MOLECULAR MARKERS OF GLIOMAS

Implications for diagnosis and new target therapies

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ACKNOWLEDGEMENTS

My Sincerest Thanks To:
Professor Marc Sanson, Director of Experimental Neuro-Oncology Lab at ICM, for the thoughtful support, constructive criticism, genuine enthusiasm and challenges we faced together over these last 3 years as well as the great opportunities he offered me for international collaborations that permitted the PhD project to advance.

I must also extend my gratitude to Professor Mauro Ceroni, my co-director and teacher, for his support and endless encouragement from the University of Pavia.

Regarding the three main projects that are presented in this thesis, a special thanks to Professor Richard Houlston, Victor Enciso Mora, Ben Kynnensley and Karim Larbreche from Institute of Cancer Research, for sharing results and advancements in GWA studies.

The project on FGFR-TACC fusions would not have been possible without Professors Antonio Iavarone and Anna Lasorella, for accepting me in their team in Columbia University and all their lab members, especially Alessandra Fucci, for her in-depth explanation of the genomic background of the spectrum of FGFR-TACC fusions and her essential support on experiments and manuscript preparation. Regarding following studies on FGFR-TACC fusions, I have to especially thank Alberto Picca for his help, Julien Savatovsky for MRI analysis and Karima Mokhtari and Frank Bielle for their essential cooperation and their original and acute observations.

I thank all our collaborators from other institutions in the setting of ANOCEF for the interest shown in the enlargement of screening and identification of newly FGFR-TACC positive patients permitting the clinical trial, which is actually going on.

Regarding the project on 2 HG non-invasive detection, I have to especially thank Francesca Branzoli, physicist at CENIR, whose earnest, successful efforts on MEGA-PRESS were crucial to the success of the study. I also thank Chris Ottololenghi for his assistance on dosages of tumor 2 HG, Stephane Lehericy, Director of Cenir and Malgorzata Marjanska for their helpful suggestion of MRS sequence and Laurent Capelle for his collaboration on patients’ enrolment.
I thank all present and former members of the Experimental Neuro-oncology Lab from ICM for their help and efforts, especially Marianne Labussiere, who supported and coached me from my very first moments in the lab, as well as Yohann Schmitt, Amithys Rahimian, Marine Giry, Catherine Carpentier and Blandine Boisselier.

I thank all my colleagues from Pitié Salpêtrière Neuro-oncology Unit for their interest and collaboration on follow-up of \textit{FGFR-TACC} patients and identification of new candidates for 2 HG detection. Your continual support during my clinical training in neuro-oncology is much appreciated.

I also owe thanks to all new colleagues from H. Foch for welcoming me: Dr. Frederic Bourdain and Dr. Stephane Gaillard enthusiastically and energetically supported my endeavours along with the whole multidisciplinary group, especially Chiara Villa, whose support for project development is crucial. To Betrand Baussart, Patrizia Farina and all members of our multidisciplinary group, you are deeply appreciated for all that you do.

I would be remiss to not mention Professor Jean Yves Delattre, who drove me and stoked my passion since I was a student and is, has been and will be my landmark in this field.

Of course I must also give a sincere thank you to Professor Hoang Xuan for giving me the opportunity to blossom as a clinician in his group, benefitting tremendously from his training and constant encouragement.

I thank Professor Stefano Bastianello and Doctor Enrico Marchioni for their precious help and the crucial discussions on neuro-oncology.

I thank also our collaborator and good friend Marica Eoli from Institute Besta, which made me grew up in neuroncology in first moments and Giulia Berzero which in turn joined us in this field for the constant help and sincere friendship.

Many thanks to the Association pour la Recherche sur les Tumeurs Cérébrales Malignes, the University of Pavia, the Department of Brain Behavioural Science, Premio Riquier, Fondazione Costa and Collegio Ghislieri for their support in developing research during these last three years.

Many thanks to Mme Isabelle Tounian and Mme Stephanie Girard who constantly assist me in daily clinical activity.
Last, but certainly not least, my sincerest thanks go to Giovanni, all my fantastic family, my nephews Anita and Ettore, friends, fellows and mates that shared this journey.

My sincerest thanks and gratitude go out to you all.
ABSTRACT

The 2016 WHO classification of gliomas integrates molecular alterations (i.e., IDH mutations, and 1p19q codeletion) to histological features, defining distinct histo-molecular entities: IDH wild-type gliomas (mostly glioblastomas), and IDH mutated gliomas, divided according to 1p19q status into astrocytomas (1p19q intact) and oligodendrogliomas (1p19q codeleted).

The first part of the manuscript is a contribution to molecular classification based on TERT promoter mutational status. We also contributed to GWAS analysis, and investigated the association between the risk loci and specific molecular entities, showing that some loci are associated with glioblastoma and IDH wild-type gliomas (rs2736100 near RTEL1, rs6010620 near TERT, rs3851634 near POLR3B) whereas others are associated to IDH mutated gliomas (rs4295627 and rs55705857 near CCDC26, rs498872 near PHLDB1, rs7572263 near IDH1, rs11196067 near VTI1A, rs648044, near ZBTB16 and rs12230172). Notably, rs4295627 and rs55705857 near CCD26 resulted strongly associated to 1p19q codeletion and to risk of oligodendrogliomas ($P=2.31 \times 10^{-94}$).

The second part of this work is devoted to the characterization of a specific oncogenic fusion between FGFR and TACC genes, which initially reported 3% of glioblastoma (GBM) and other human cancers and is proposed as a new therapeutic target. Overall, we screened 907 gliomas for FGFR3-TACC3 fusions. We found that FGFR3-TACC3 fusions exclusively affect IDH wild-type gliomas (3%), and are mutually exclusive with the EGFR amplification and the EGFR vIII variant, whereas it co-occurs with CDK4 amplification, MDM2 amplification and 10q loss. FGFR3–TACC3 fusions were associated with strong and homogeneous FGFR3 immunostaining. We show that FGFR3 immunostaining is a sensitive predictor of the presence of FGFR3-TACC3 fusions. FGFR3-TACC3 glioma patients had a longer overall survival than those patients with IDH wild-type glioma. We treated two patients with FGFR3–
TACC3 rearrangements with a specific FGFR-TK inhibitor and we observed a clinical improvement in both and a minor response in one patient. These data support the systematic screening for FGFR-TACC fusion in all IDH wild-type glioma patients who can benefit from FGFR inhibition.

In the third section, we developed a non-invasive diagnostic tool by 1H-magnetic resonance spectroscopy in IDH mutant gliomas. We optimized a uniquely different spectroscopy sequence called MEGA-PRESS for the detection of the oncometabolite 2-hydroxyglutarate (2 HG) that specifically accumulates in IDH mutant gliomas. We analysed a prospective cohort of 25 patients before surgery for suspected grade II and grade III gliomas and we assessed specificity and sensitivity, correlation with 2 HG concentrations in the tumor and associations with grade and genomic background. We found that MEGA-PRESS is highly specific (100%) and sensitive (95.2%) for the prediction of IDH mutation and correlated with 2 HG levels measured by gas chromatography-tandem mass spectrometry (GC-MS/MS) in frozen tissue. Preliminary follow-up during radio-chemotherapy regimen and anti-IDH therapy showed a decrease in 2 HG production. In conclusion, MEGA-PRESS is a reliable tool for IDH mutation prediction at pre-surgical stages and for measuring the activity of anti-cancer drugs. Long-term monitoring will help to clarify the prognostic and predictive value of 2 HG decrease during anti-cancer treatment.
RÉSUMÉ

La classification des gliomes OMS 2016 a récemment intégré aux caractéristiques histologiques deux principales altérations moléculaires (la mutation IDH et la codélétion 1p19) et a défini deux entités histo-moléculaires distincts : les gliomes IDH wild-type (principalement les glioblastomes, GBM) et les gliomes IDH mutés, séparés sur la base du statut 1p19q en astrocytomes (1p19q non codélétés) et oligodendrogliomes (1p19 codeletés).

La première partie du manuscrit est une contribution à la classification moléculaire des gliomes, basée sur la présence de la mutation du promoteur de TERT et de la mutation de IDH. Nous avons également contribué aux études de genome-wide association (GWAS), nous avons plus particulièrement exploré l’association entre les loci à risque et les sous-groupes moléculaires. Nous avons montré que certains loci sont associés aux gliomes IDH wild-type et aux glioblastomes (rs2736100 à proximité de RTEL1, rs6010620 à proximité de TERT, rs3851634 à proximité de POLR3B) et que d’autres sont associés aux gliomes IDH mutés (rs4295627 et rs55705857 à proximité de CCDC26, rs498872 à proximité de PHLDB1, rs7572263 à proximité de IDHI, rs11196067 à proximité de VTI1A, rs648044, à proximité de ZBTB16 et rs12230172). Notamment rs4295627 et rs55705857 à proximité de CCDC26 sont fortement associés à la codeletion 1p19q (P=2.31 x10-94).

La deuxième partie de mon travail est dédiée à la caractérisation de fusions spécifiques oncogéniques entre les gènes FGFR et TACC, qui avaient été initialement décrites dans 3% des GBM et dans d’autres cancers et identifiés comme une nouvelle cible thérapeutique.

Au total nous avons analysé 907 gliomes pour la présence du gène de fusion FGFR3-TACC3. Nous avons montré que les fusions FGFR3-TACC3 ne touchent que les gliomes IDH wild-type (3%), sont mutuellement exclusives avec l’amplification de EGFR et avec la forme tronquée EGFRvIII et inversement, sont associées à l’amplification de CDK4 et de MDM2 et à la délétion du 10q.
Les fusions FGFR3-TACC3 sont associées à une expression intense et diffuse de FGFR3 en immunohistochimie (IHC). Nous avons montré que l’IHC pour FGFR3 est un marqueur prédictif très sensible de la présence des fusions FGFR3-TACC3.

Les patients porteurs d’une fusion FGFR3-TACC3 ont une survie globale significativement plus longue comparés aux patients avec gliome IDH wild-type. Nous avons traité deux patients porteurs d’un gène de fusion FGFR3-TACC3 avec un inhibiteur tyrosine-kinase (TK) spécifique pour FGFR et nous avons observé une amélioration clinique avec stabilisation de maladie et une réponse mineur chez un patient.

Ces résultats justifient la réalisation d’un criblage systématique pour les gènes de fusions FGFR3-TACC3 chez tout nouveau gliome IDH wild-type nouvellement diagnostiqué qui pourrait ainsi bénéficier d’un traitement par inhibiteur TK spécifique pour FGFR.

Dans la troisième section, nous avons développé une méthode diagnostique non invasive avec la 1H spectroscopie en résonance magnétique (1H-MRS) chez les patients porteurs d’un gliome IDH muté. Nous avons optimisé une nouvelle séquence de spectroscopie différentielle-MEGA-PRESS-pour la détection de l’oncometabolite 2-hydroxyglutarate (2 HG) qui s’accumule de manière spécifique dans les gliomes IDH mutés. Nous avons analysé de façon prospective une cohorte de 25 patients avant chirurgie pour probable gliome de grade II et grade III et nous avons calculé la sensibilité, la spécificité, la valeur prédictive positive et négative de la détection du pic de 2 HG, sa corrélation avec les concentrations de 2 HG dans le tissu et l’association avec le grade et le profil génomique.

Nous avons trouvé que la MEGA-PRESS est hautement spécifique (100%) et sensible (95.2%) dans la prédiction de la présence de la mutation IDH. Son taux est corrélé aux concentrations de 2 HG mesurés sur tissu congelé par spectrométrie de masse (gas chromatography-tandem mass spectrometry GC-MS/MS).
Des résultats préliminaires de suivi de patients traités pour un gliome \textit{IDH} muté par radio-chimiothérapie ou par inhibiteur spécifique de IDH muté montrent une réduction sous traitement de la production de 2 HG. En conclusion la MEGA-PRESS est une technique fiable pour la prédiction de la mutation de \textit{IDH} en phase préopératoire et également comme mesure d’activité des thérapies anticancéreuses. Le suivi à long terme pourra préciser la valeur d’une diminution du 2 HG aussi bien pronostique que prédictive de réponse aux traitements anticancéreux.
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Anna Luisa Di Stefano
1. INTRODUCTION

1.1 Histological and Molecular Background of Gliomas

Gliomas account for 30% of all primary brain tumors and are responsible for around 13,000 cancer-related deaths in the US each year (Ostrom, 2015; Siegel, 2011). Newly diagnosed gliomas are estimated around 20,000 in the US and 2500 to 3000 in France per year (Rigau, 2011).

For the past century, the classification of brain tumors has been based largely on concepts of histogenesis that tumors can be classified according to their microscopic similarities with different putative cells of origin and their presumed levels of differentiation (Louis, 2007). However, research into glioma biology of the last two decades, has led to the discovery of molecular alterations that proved to better define biological entities and clinical aggressiveness.

As a result of the success of prognostic stratification according to genomic background of gliomas, the WHO (World Health Organization) recently updated the classification of brain tumors (Louis, 2016) and stated that two of them – the isocitrate dehydrogenase (IDH) mutations and chromosomes 1p19q codeletion – are determinant for the so-called “integrated” diagnosis, irrespective of morphological similarities of tumor cells to putative progenitors.

By now, brain tumor entities are broadly separated according to two main dichotomies:

- *IDH* mutations principally differentiate the more indolent lower-grade gliomas (grade II and grade III and progressive glioblastoma) from primary glioblastoma, the most aggressive of gliomas.
- 1p19q codeletion, which is tightly associated to *IDH* mutations, specifically tags oligodendrogliomas among lower-grades.

Isocitrate dehydrogenase is an enzyme with three isoforms, i.e., IDH1, IDH2, and IDH3 (Dang, 2010). Intra-cellularly, it catalyses the oxidative
decarboxylation of isocitrate to α-ketoglutarate (α-KG) (Arcaro, 2007; Dang, 2010). *IDH* mutations harbour in specific cancer entities: in gliomas (70–90% of low-grade gliomas and secondary glioblastoma), in haematological malignancies (~20% of acute myeloid leukaemia), in intrahepatic cholangiocarcinoma, chondrosarcoma and melanoma (Gross, 2010; Parsons, 2008; Waitkus, 2016; Yang, 2012). Glioma-specific mutations always affect the amino acid arginine in position 132 in *IDH1* and arginine at position 172 in *IDH2* (Hartmann, 2009). The *IDH1* mutation is one of the earliest known genetic events in low-grade gliomas; it is thought to be a “driver” mutation for tumorigenesis (Suzuki, 2015) probably by accumulation of the onco-metabolite 2-hydroxyglutarate (2 HG).

At a prognostic level, *IDH* mutations have revealed to have a major prognostic impact on morphological stratification based on the WHO’s 2007 glioma grades, depicting a more favourable prognosis in *IDH* mutants compared to tumors with wild-type *IDH* in all glioma grades (Sanson, 2009) and recognizing a worse outcome common to the group of *IDH* wild-type gliomas independent of their grading (Louis, 2016).

Chromosome 1p/19q codeletion is strongly associated with classical oligodendrogial features. It results from an unbalanced translocation between the entire arm of 19p and 1q (Jenkins, 2006). At the genomic level, it corresponds to a complete loss of the 1p and 19q arms, which is important to distinguish from 1p partial distal deletions (typically 1p36) that occur in astrocytic tumors and are associated with a poor prognosis (Idbaih, 2005; Idbaih, 2008; Vogazianou, 2010). 1p19q codeletion is a strong favourable prognostic factor and since 1998 it has been associated with response and benefit to adjuvant chemotherapy with PCV after radiotherapy in anaplastic oligodendrogliaoma (Cairncross, 2014).

The reasons for this better prognosis are yet to be determined. 1p19q codeleted gliomas are systematically associated with *IDH1* or *IDH2* (Labussiere, 2010) and combinations of these molecular subgroups (*IDH*mutated+1p19q codeleted/*IDH*mutated+non codeleted and *IDH* wild-type
gliomas) correlate with marked differences in survival rates (Figure 5.2, Chapter 5.1-Section 3).

The simplified algorithm of new integrated classification of gliomas from the WHO’s 2016 (Louis, 2016) basing of IDH and 1p19q status, is reported in Figure 1.1.

Figure 1.1 adapted from Louis et al. 2016. A simplified algorithm for classification of the diffuse gliomas based on histological and genetic features according to WHO 2016.
2. AIMS

The 3-year study in the present thesis is focused on implications for diagnosis and new therapies of two distinct driver molecular aberrations harbouring in the two principal entities discussed before: the *IDH* wild-type and *IDH*-mutant gliomas.

The study is articulated in three sections.

Section 1 correspond to our contribution to the molecular classification of gliomas basing on *IDH* and *TERT* mutational status and to correlations of glioma susceptibility germ line variants with tumor phenotype and molecular background.

Section 2 “Detection, Characterization, and Inhibition of *FGFR-TACC* Fusions in *IDH* Wild-type Glioma” is dedicated to a novel targetable aberration—the *FGFR-TACC* gene fusions-in *IDH* wild-type gliomas.

In this study, conducted with the collaboration of Dr. Iavarone and Dr. Lasorella’s research group at Columbia University:

- we determine distribution and frequency of *FGFR-TACC* fusions in gliomas,
- we explore *FGFR-TACC* fusions, transcripts, repertoire and genomic background of *FGFR-TACC* positive gliomas,
- we validate a screening method and correlate with *FGFR3* expression
- we characterize clinical features and evolution of patients harbouring *FGFR-TACC* fusions, and
- we explore signals of activity of specific anti-*FGFR* therapies in two patients harbouring *FGFR-TACC* fusions.
First results of this study were published in Clinical Cancer Research (Di Stefano, 2015). The expansion study, not published to date, is developed in Chapter 4.2.

In Section 3, “In vivo non-invasive detection of 2-hydroxyglutarate in IDH mutated gliomas” we focussed on the diagnostic implication of the IDH mutation in gliomas.

Aims of this study were:

- to develop and apply Magnetic Resonance Spectroscopy (MRS) protocols for non invasive detection of 2 HG accumulation in IDH mutant glioma patients,
- to explore correlations of 2 HG accumulation by MRS and 2 HG tissue dosages, and
- to follow 2 HG variations by MRS during convectional but also targeted anti-IDH therapies in IDH mutant patients.

Preliminary results correspond to the manuscript in preparation for publication.
3. SECTION 1-OUR CONTRIBUTION TO THE MOLECULAR CLASSIFICATION OF GLIOMAS

A supplementary molecular alteration that can enhance prognostic classification is the mutation in the promoter of \textit{TERT}, which encodes telomerase. The increased telomerase activity seen in cancer leads to preservation of telomeres, allowing tumors to avoid induction of senescence. Among other cancer, somatic mutations of the \textit{TERT} promoter (\textit{TERTp}-mut) have been documented in gliomas (Killela, 2013; Labussiere, 2014). The two most common mutations in \textit{TERT}, C228T and C250T, map -124 and -146 bp, respectively, upstream of the \textit{TERT} ATG site (chr5, 1,295,228 C4T and 1,295,250 C4T, respectively), creating binding sites for Ets/TCF transcription factors.

In the two following studies (Labussiere, 2014; Labussiere, 2014) we largely screened brain tumor banks from Pitié-Salpetrière (Onconeurotek) for \textit{TERTp}-mutation (807 gliomas), and we explored the prognostic value and its association with main molecular aberrations in gliomas, such as \textit{IDH} mutation, 1p19 codeletion and \textit{EGFR} amplification.

As a result, in the first article (Labussiere, 2014) we reported that:

- prevalence of \textit{TERTp}-mut is around 69\% in gliomas
- we showed that \textit{TERTp}-mut clusters specularly with \textit{IDH} mutation in 89\% of \textit{IDH}+/1p19 codeleted gliomas (corresponding to oligodendrogliomas) and also with 75\% of \textit{IDH} wild-type glioblastoma
- we showed that \textit{TERTp}-mut is a determinant prognostic factor but interestingly its prognostic impact is contextual and depends on the histologic and genomic background of the tumor
- we proposed a molecular stratification of tumors by both \textit{IDH1/2} and \textit{TERTp}-mut status in gliomas.
In a second study (Labussiere, 2014) we focussed on glioblastoma:

- showing that $TERTp$-mut is an independent factor of poor prognosis in GBM, and even stronger in $IDH$ mutated GBM
- we also proposed a refined prognostic classification of GBMs based on joint analyses of $TERT$, $EGFR$, and $IDH$.

Such molecular prognostic classification of gliomas have been reproduced by other groups (Killela, 2014) and became a paradigm to define five principal groups of gliomas with characteristic distributions of age at diagnosis, clinical behavior, acquired genetic alterations, and associated germ line variants (Eckel-Passow, 2015).

Articles we coauthored on $TERTp$-mutation in gliomas and molecular prognostic classifications (Labussiere, 2014; Labussiere, 2014) are reported in this section.
**TERT** promoter mutations in gliomas, genetic associations and clinico-pathological correlations

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**Background:** The role of telomerase reverse transcriptase (TERT) in gliomagenesis has been recently further strengthened by the frequent occurrence of TERT promoter mutations (TERTp-mut) in gliomas and evidence that the TERT SNP genetic rs2736100 influences glioma risk. TERTp-mut creates a binding site for Ets/TOF transcription factors, whereas the common rs2853669 polymorphism disrupts another Ets/TOF site on TERT promoter.

**Methods:** We sequenced for TERTp-mut in 807 glioma DNAs and in 235 blood DNAs and analysed TERT expression by RT-PCR in 151 samples. TERTp-mut status and TERTp-polymorphism rs2853669 were correlated with histology, genomic profile, TERT mRNA expression, clinical outcome and rs2853601 genotype.

**Results:** TERTp-mut identified in 40.8% of gliomas (491 out of 807) was globally associated with poorer outcome (Hazard ratio (HR) = 1.50). We defined, based on TERTp-mut and IDH mutation status, four prognostic groups: (1) TERTp-mut and IDH-mut associated with TpTPq codetiation, overall survival (OS) > 17 years; (2) TERTp-wt and IDH-mut, associated with TP53 mutation, OS = 97.5 months; (3) TERTp-mut and IDH-wt, with no specific association, OS = 31.6 months; (4) TERTp-mut and IDH-wt, associated with EGFR amplification, OS = 15.4 months. TERTp-mut was associated with higher TERT mRNA expression, whereas the rs2853669 variant was associated with lower TERT mRNA expression. The mutation of CCA (a repressor of ETV1-3 belonging to the Ets/TOF family) was also associated with TERT mRNA upregulation.

**Conclusions:** In addition to IDH mutation status, defining the TERTp-mut status of glioblastomas should allow further prognostic stratification of patients with gliomas. We also show that TERTp-mut, rs2853669 variant and IDH mutation influence Tert expression. This effect could be mediated by Ets/TOF transcription factors.

The telomerase reverse transcriptase (TERT) gene encodes a highly specialised reverse transcriptase, which adds hexamer repeats to the 3’ end of chromosomes (Aubert and Lansdorp, 2008; Cesar and Roeder, 2010). The increased telomerase activity seen in cancer leads to preservation of telomeres, allowing tumours to avoid induction of...
Prognostic impact of TERT promoter mutation in gliomas.

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Somatic and constitutional TERTp-mut status. Tumours from 807 patients (451 male; median age at diagnosis 51.0 years, range, 17.3-89.1; 206 grade II, 206 grade III and 395 grade IV) were screened for TERTp-mut. Complete patient characteristics are shown in Supplementary Table 2. Tumours from 491 of the 807 patients (60.8%) were TERTp-mut-355 C228T (72.3%) and 136 C228T (27.7%). One GBM and two grade II oligodendrogliomas carried both C228T and C282T. These three cases were considered as TERTp C228T mutant in all subsequent analyses. To confirm the mutations were somatic, we screened germline DNA of 91 of the cases. No mutation was detectable in germline DNA. We also investigated for the presence of TERTp-mut, in 89 familial glioma patients and 64 glioma patients with a second cancer – 14 with endometra (Supplementary Table 2). In none of the cases was a −149, −124 or −57 mutation identified.

28385609 genotypes were available for 389 of the tumours. The distribution of genotypes showed no significant departure from HW (39 CC, 161 CT, 185 TT; P = 0.67). There was no difference in the distribution of genotypes between the TERTp-mut and the TERTp wt cases (TT 45.8% vs 53.3%, CT 44.3% vs 36.0% and CC 10.0% vs 50.0%, respectively).

We then investigated a purported association between somatic TERTp-mut and rs2736100 genotype in 518 glioma patients, finding no association in the whole group (odds ratio 1.8, 95% confidence interval 0.8 to 3.67, P = 0.18). The results were similar when stratifying by IDH1 status and tumour class (Supplementary Table 3).

Case-control comparison showed a stronger association with rs2736100 with IDH1-wt gliomas but not with TERTp-mut gliomas (Supplementary Table 4). Collectively, these data imply there is no association between TERTp-mut and rs2736100 genotype. In
addition, we did not find any significant association between TERT promoter mutation and the other gliomas susceptibility SNPs rs1979158, rs2325386, rs4395627, rs907756, rs4980773 and rs6160620 (data not shown).

TERT-pmt is associated with GBM and EGRF amplification, and with oligodendroglioma, 1p/19q codeletion and CIC mutation. TERT-pmt was associated with the age at diagnosis in all gliomas (median age 56.1 years for TERT-mutated patients vs 40.0 years; t-test P=0.0001) and when stratified by grade (median age at diagnosis 46.4 years vs 36.1 for grade II; P=0.008; 53.3 vs 37.8 for grade III; P=0.0001 and 39.6 vs 35.6 years for grade IV, P=0.0001).

TERT-pmt was more frequent in GBM than in grade II or III tumours (296 out of 395 vs 75.8% vs 189 out of 412 = 45.9% \( P < 10^{-5} \)), more frequent in oligodendroglioma than in astrocytomas/oligodysplasias for grade III (52 out of 81 = 64.2% vs 46 out of 125 = 36.8%; \( \chi^2 \) test \( P = 0.0001 \)) and for grade II (70 out of 119 = 58.8% vs 21 out of 87 = 24.1%; \( \chi^2 \) test \( P < 10^{-5} \)). Additionally, there was no difference in the ratio of C228T/C250T mutations among the different grades (Table 1). TERT-pmt was identifiable in 87.9% (44 out of 100) of gliomas with 1p/19q codeletion 09 oligodendrogliomas, 17 oligodysplasias; 26 (24.3%) on C228T and 68 (63.6%) on C250T in contrast with 38.8% of non-codeleted gliomas (341 out of 880, \( \chi^2 \) test \( P = 0.0001 \)). EGRF amplification was present in 383 tumours (142 GBM) and was mutually exclusive with 1p/19q codeletion: 163 (40.1%) having TERT-pmt (124, C228T), as compared with 51.8% (323 out of 624) of EGRF non-amplified tumours (\( \chi^2 \) test \( P = 0.0001 \)). The association of TERT promoter mutations with other molecular alterations commonly seen in gliomas is detailed in Supplementary Table 3. We investigated whether there was a relationship between CIC inactivating mutations and TERT-pmt in grades II and III. CIC mutation was associated with TERT-pmt in 85% of the cases (28 out of 33), compared with 61% (25 out of 41) in CIC-wt tumours (\( \chi^2 \) test \( P = 0.04 \)).

TERT-pmt is associated with increased TERT mRNA expression. We investigated the transcriptional consequences of TERT-pmt in 155 tumours for which mRNA was available. We found a three-fold increase in mRNA expression between TERT-pmt and non-mutated groups (mean ± s.e.m. 1.03 ± 0.37 vs 0.34 ± 0.08 AU; Mann–Whitney test \( P = 0.0001 \), Figure 1A).

Since the presence of the rs2833669 C allele disrupts an Ets binding site (Rachakonda et al., 2013), we investigated the effect of rs2833669 genotype on TERT mRNA expression. Tumours harbouring the variant allele (CC + CT) showed a two-fold reduction in TERT expression, as compared with TT homozygotes (mean ± s.e.m. 0.50 ± 0.06 vs 2.97 ± 0.67 AU; Mann–Whitney test \( P = 0.0001 \)). This relationship was also seen in the TERT-pmt mutant cohort, however, we did not evidence any significant association in TERT-wt tumours (Figure 1B and C).

Figure 1. Expression of TERT mRNA in gliomas. The Mann–Whitney test was used to compare the expression of the different groups. (A) Expression of TERT mRNA according to TERT promoter mutation status. TERT-pmt mutation (C228T n = 88 or C250T n = 30) is associated with higher TERT mRNA expression compared with TERT-wt group (n = 33) (\( P < 0.0001 \) in both cases). (B) Expression of TERT mRNA according to rs2833669 status. Variant allele carriers (n = 70) present a lower TERT expression than TT homozygotes (n = 66) (\( P < 0.0001 \)). (C) Expression of TERT mRNA according to TERT-pmt and rs2833669 status. TERT mRNA expression is lower for the variant allele carrier (n = 62) compared with TT (n = 54) in TERT-pmt subgroup (\( P = 0.0079 \)). For TERT-pmt group, only seven CC + CT samples and eight TT samples were available. (D) Expression of TERT mRNA according to CIC mutation status. TERT mRNA expression is increased in CIC mutant tumours (n = 18) compared with CIC wild type (n = 11) (\( P = 0.0494 \)). (E) Impact of rs2833669 and CIC mutational status on TERT expression. In the CIC-wt cohort, TERT expression was lower in CC + CT subgroup, as compared with TT subgroup (\( P < 0.0159 \)). For the variant allele carriers (CC + CT), expression of TERT was increased in the CIC mutant group (n = 8), as compared with CIC wt (n = 5) (\( P = 0.0016 \); **P < 0.05; ***P < 0.01; ****P < 0.0001).
Prognostic impact of TERT promoter mutation in gliomas. BRITISH JOURNAL OF CANCER

Since ETS/TCF transcription factors, including ETV1/4 transcription factors are controlled by CIC (Dennizn et al., 2011), we also investigated a specific relationship with CIC mutation. We found TERT mRNA expression was two-fold higher in CIC mutant tumours, compared with CIC wild-type gliomas (Mann-Whitney test, P = 0.003) for the whole group (figure 1D), and for the carriers of the variant allele (figure 1E). The variant allele C was also associated with a decrease in TERT mRNA expression in the CIC wt group.

Prognostic impact of TERTp-mut is dependent on tumour grade. For patients with grade III and IV gliomas, TERTp-mut was associated with a significantly shorter PFS and OS (figure 2; Supplementary Table 6). For example in grade III gliomas, medium OS of TERT promoter mutant normal patients was twice longer (62.6 vs 29.4 months) than OS of TERT promoter mutated (log-rank test P = 0.013). This was in sharp contrast with low-grade gliomas, where OS was better for patients with TERT-p-mut (>16 years vs 97.5 months, P = 0.0031). There was no difference in outcome between controls and C228T and C250T TERT-p-mut in any of the analyses.

In a multivariate Cox model analysis incorporating IDH mutation, age at diagnosis, 1p/19q codeletion, MGMT promoter methylation, Karnofsky performance status, WHO grade and extension of surgery (Table 2), TERT-p-mut was seen to be an independent negative prognostic factor for OS (Hazard ratio (HR) = 1.50; 95% CI: 1.07–2.09, P = 0.018).

TERTp-mut is associated with specific prognostic and molecular subgroups. Given TERTp-mut is associated with both 1p/19q codeletion and EGFR amplification, which are mutually exclusive alterations with opposite prognostic effects and TERT-p-mut had a different effect in low- and high-grade gliomas, prompted us to refine our survival analysis (Figure 3). Gliomas can be stratified into four distinct prognostic groups according to IDH and TERTp-mut status: (1) TERTp-mut and IDH-wt, highly associated with 1p/19q codeletion (83.3%, 94 out of 111); OS > 17 years; (2) TERTp-wt and IDH-mut, associated with TP53 mutation (67.7%, 67 out of 99; OS = 97.5 months); (3) TERTp-wt and IDH-wt, with no specific association (all negative); OS = 31.6 months; (4) TERTp-mut and IDH-wt, highly associated with EGFR amplification (44.1%, 361 out of 363; OS = 15.0 months) (Figure 4).

TERTp-mut confers a poor prognosis except if associated with 1p/19q codeletion. We considered the prognostic impact of the above classification in grades II, III and IV (Figure 5; Supplementary Table 7). In grades II and III, TERTp-mut was predictive of a longer survival in the IDH mutated group, but shorter survival in the IDH wt group. This finding can be explained by the fact that 94 out of 114 of TERTp-mut-IDH wt are 1p/19q codeleted. Indeed in the GBM group that do not include any 1p/19q codeleted, TERTp-mut is associated with a particularly poor prognosis in IDH-wt tumours (OS = 13.8 vs 6.5 months, P = 0.006) but surprisingly also in IDH-mut (OS = 13.8 vs 29.1

![Figure 2](https://example.com/fig2.png)

Figure 2. Prognostic impact of TERT promoter mutation status on overall survival and PFS, according to grade. Results were compared using the log-rank test (Mantel Cox). In grade II gliomas (n = 206), TERTp mutation is associated with better survival (median > 16 years vs 97.5 months, P = 0.013). There is also a trend for better PFS (median 41.3 vs 33.3 months, P = 0.069) whereas in grade III (n = 206) and grade IV gliomas (n = 396), TERTp mutation is associated with poorer survival (median 29.4 vs 62.6 months P = 0.013 and 13.8 vs 18.4 months P = 0.001) and PFS (median 15.1 vs 22.4 months P = 0.006 and 8.3 vs 10.4 months P = 0.0001).

![Figure 3](https://example.com/fig3.png)

Figure 3. Prognostic impact of TERT promoter mutation status on overall survival and PFS, according to grade.

www.bjocancer.com | DOI:10.1038/bjc.2014.538 2027
Table 2. Cox model for overall survival and progression-free survival

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Alphabets: CI = confidence interval; HR = hazard ratio; IDH = isocitrate dehydrogenase; KPS = Karnofsky Performance Status; TERT = telomerase reverse transcriptase. The analysis was conducted on 80 tumours with all parameters available.

Figure 3. Association of TERT promoter mutations with the major genetic alterations in gliomas (n= 806). Each tumour is represented by a column. A yellow box indicates the presence of the genetic alteration, the absence in blue, and the cases not assessed are indicated in grey. The stratification has been done using four groups: IDH-mut, TERTp-mut, IDH-mut + TERTp-mut, IDH-wt + TERTp-mut and IDH-wt + TERTp-mut. TERTp mutation is associated with two mutually exclusive alterations: tp16q co-deletion and EGFR amplification.

Figure 4. Prognostic stratification of gliomas according to IDH and TERT promoter mutation status (n= 806). (A) Overall survival; (B) Progression-free survival. We identified four prognostic subgroups, (1) TERTp-mut and IDH-mut (OS > 17 years, PFS 66.9 months), (2) TERTp-wt and IDH-mut (OS > 97.5 months, PFS 28.6 months), (3) TERTp-wt and IDH-wt (OS > 31.6 months, PFS 14.1 months) and (4) TERTp-mut and IDH-wt (OS > 15.0 months, PFS 8.5 months).

months, P = 0.022) (Figure 6). In contrast TERTp-mut was associated with a poorer outcome in IDH-wt gliomas irrespective of grade (OS: 76.2 vs 94.8 months in grade II, P = 0.18 vs 36.3 months in grade III, P = 0.007; 13.7 vs 17.5 months in grade IV, P = 0.066).

DISCUSSION

Given that 40% of tumours being TERTp-mut, TERT is the most frequently mutated gene in gliomas thus far identified (Ariga et al., 2013; Killefa et al., 2013; Liu et al., 2013). We found TERTp-mut glioma patients were older, consistent with previous reports of other malignancies (Griewank et al., 2013; Killefa et al., 2013).

Unlike melanomas, in which germline TERTp mutations have been reported to cause familial melanoma (Horn et al., 2013), we found no evidence that TERTp-mut contributes substantially to predisposition to gliomas or the glioma/melanoma syndrome.

Our data showed that TERTp-mut is generally associated with poorer outcome in high-grade gliomas, consistently with previous data, on glioma (Killefa et al., 2013, 2014), and other tumours (Rachakonda et al., 2013). In contrast, however, we observed a trend for better outcome in low-grade gliomas. Stratifying tumours by IDH1/2 and TERTp-mut status provides insight into this apparent paradox, identified four molecular subtypes of gliomas with distinct prognosis. In IDH mutated tumours, TERTp-mut is largely confined to tp16q co-deleted oligodendrogial tumours that have the best outcome (Kakish et al., 2007; van den Bent et al., 2013). Mutation of CIC, recently identified (Bettegowda et al., 2013).
Yip et al, 2012 is also primarily a feature of this group (Figure 3). In contrast, if TERTp-mut is associated with IDH1/2 wild-type tumours, than it is mainly seen in the context of GBM (27% out of 340) with almost half of them (124 out of 276) having an EGFR amplification which is associated with poor outcome. In our study, this subgroup also included 54 grade III gliomas that had a particularly poor OS of 20.1 months. Taken together, our data show that the prognostic impact of TERTp-mut is highly contextual and depends on the histologic and genomic background of the tumour.

From a mechanistic point of view, TERTp mutation leads to the creation of a putative binding site for Ezh2/TCF transcription factors (Huang et al, 2013), leading to a two- to four-fold higher expression of telomerase (Agata et al, 2013; Huang et al, 2013; Naft et al, 2013; Rachakonda et al, 2013). The activity of telomerase reverse transcriptase is closely correlated with TERT mRNA level. The expression of TERT is regulated by many transcription factors binding motifs located in its promoter and by epigenetic and chromatin remodelling mechanisms (Kyo et al, 2008; Zhu et al, 2010). Among the complex regulation of telomerase expression, rs2835669 has been shown to modulate both TERT expression and impact on prognosis in bladder cancer (Rachakonda et al, 2013). Indeed, the presence of the variant allele disrupts a pre-existing Ezh2 binding site and results in the decrease of TERT expression in our series. However, unlike bladder cancer, rs2835669 variant does not modify the prognostic impact of TERTp mutation in our glioma series (data not shown). Our data also suggest a link between CIC mutation and TERT expression in the context of glioblastomas. Indeed, the presence of the variant allele of rs2835669 did not result in a reduction of TERT expression in the CIC mutant subgroup.

Among the 40% gliomas lacking TERTp mutation, ~50% harbour an IDH mutation (mostly astrocytomas (43 out of 180) and oligoastrocytomas (91 out of 180), which are frequently TPS3 mutated. In this group, mutations in the ATRX gene (alpha thalassaemia/mental retardation syndrome X-linked), or in its
Partner death domain-associated protein (DDAX), which are involved in alternative lengthening telomere (ALT) phenotype, have been frequently documented (Jiao et al., 2012; Kamata et al., 2012; Lin et al., 2012; Kildla et al., 2013) and are mutually exclusive with telomerase reactivation. The IDH-wt and TERTp-wt group includes mostly GBM tumours (SOH, 66 out of 114). The 'triple negative' low-grade gliomas, characterised by a poorer outcome also conform to these categories (Mebius et al., 2010). Telomere maintenance mechanism has not been investigated yet in this subgroup.

A more detailed analysis shows the four group classification, recently reported (Kildla et al., 2014) is an oversimplification (see Supplementary Figure 2 and Figure 3a; for example, the TERTp-wt-IDH-mut is indicative of better outcome for grades II and III with an OS > 17 years, but is associated with a poorer outcome in GBM (OS=13.8 months), whereas in GBM the best group
Prognostic impact of TERT promoter mutations in gliomas.

Anna Luisa Di Stefano

This study is supported by grants from the Institute Nationale du Cancer (INCA), the Association pour la Recherche contre le Cancer (ARC), the Ligue Nationale contre le Cancer (LNC), and the Association pour la Recherche sur les Tumeurs Cérébrales (ARTC). ALD is supported by an investigator fellowship from Collegio Ghislieri, Pavia, Italy. VQ and SM are supported by a grant from the Association pour la Recherche contre le Cancer (ARC). SM is supported by a grant from the Ligue Nationale contre le Cancer (LNC).

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Combined analysis of TERT, EGFR, and IDH status defines distinct prognostic glioblastoma classes

ABSTRACT

Objective: To identify the prognostic significance of TERT promoter (TERTp-mut) and their associations with common molecular alterations in glioblastomas (GBMs).

Methods: We sequenced the TERTp-mut in DNA from 395 GBMs and analyzed the results with their respective histology, genetic profile (IDH mutation, EGFR amplification, CDKN2A homozygous deletion, loss of chromosome 10, TP53 mutation), and overall survival (OS).

Results: TERTp-mut were found in 299 of 395 GBMs (77.9%) and were associated with an older age (median 59.6 years for TERTp-mut vs 53.6 years for TERTp-wild type (TERTp-wld), p < 0.0001). TERTp-mut was an independent factor of poor prognosis (OS = 13.8 vs 18.4 months, in both IDH-mutated (OS = 13.6 vs 17.5 months, p = 0.006) and IDH-wt GBMs (OS = 13.7 vs 17.5 months, p = 0.006). TERTp-mut was associated with IDH-wt, EGFR amplification, CDKN2A deletion, and chromosome 10a loss, but not with MGMT promoter methylation. In the TERTp-mut group, OS was twice longer in EGFR-wt than in EGFR amplification GBMs (OS = 26.6 vs 13.3 months, p = 0.0025). In the EGFR-wt group, patients with TERTp-mut had a significantly better outcome (OS = 26.3 vs 12.5 months, p = 0.00001), whereas in the EGFR amplification group, patients with TERTp-mut survived longer (OS = 15.8 vs 13.3 months, p = 0.05). Taken together, the absence of both EGFR amplification and TERTp-mut is associated with longer survival in patients with GBM (26.5 months for patients with IDH-wt, 36.7 months for patients with IDH mutation).

Conclusions: The analysis of TERTp-mut, in combination with EGFR amplification and IDH mutation status, refine the prognostic classification of GBMs. Neurology® 2014;83:1200-1206

GLOSSARY

CDKN2A = cyclin-dependent kinase inhibitor 2A; EGFR = epidermal growth factor receptor; GBM = glioblastoma; IDH = isocitrate dehydrogenase; MGMT = methylguanine methyltransferase; OS = overall survival; PFS = progression-free survival; TERT = telomerase reverse transcriptase; TERTp-mut = TERT promoter mutation; TERTp-wld = TERT promoter wild type; TP53 = tumor suppressor p53; wt = wild type.

Recently, mutations affecting the promoter region of the telomerase reverse transcriptase (TERT) gene have been reported in numerous cancers.1,2 Gliomas and especially glioblastomas (GBMs) were among the most frequently affected tumors.3,4 These mutations occurred in 2 homozygous positions (chr8, 1,295,228 C>T and chr8, 1,295,259 C>T), located ~124 and ~146 base pairs upstream from the ATG start site (~124 G>A and ~146 G>A).5 Both mutations conferred enhanced TERT promoter activity, possibly by generating a consensus binding site (CCCTCAGGCCCGAAG) for E-twenty-six transcription factors.6 The TERT gene codes for a highly specialized reverse transcriptase catalyzing, with other members of the telomerase complex, the 3′ extension of chromosome ends by adding hexamers repeats.6 TERT expression and telomerase activity are usually low in normal tissues, and the constant shortening of telomeres finally leads to cell senescence. In contrast, most human...
cancers are characterized by an increased activity of telomerase allowing the maintenance of telomere lengths, thus avoiding induction of senesence and conferring unrestricted growth properties to cancer cells.\(^{(6,11)}\)

A number of genetic and genomic alterations have already been described in GBM, including epidermal growth factor receptor (EGFR) amplification, cyclin-dependent kinase inhibitor 2A (CDKN2A) homozygous deletion, methylguanine methyltransferase (MGMT) promoter methylation, and isocitrate dehydrogenase (IDH) mutation. To date, only MGMT promoter methylation and IDH mutation have been proven to be prognostic in GBM.\(^{(6,11)}\)

In this study, we investigated the prevalence and the prognostic impact of TERT promoter mutations (TERTp-mut), in a series of 395 patients with GBM treated in our department. We then correlated the TERTp-mut status with the other genetic alterations.

**METHODS**

**Patients and tissue samples.** Selection of patients was based on the following criteria: histologic diagnosis of primary GBM according to the World Health Organization classification, and clinical data and follow-up available in the neuro-oncology database (Oncovisual, Paris). We considered primary GBM when the first symptoms appeared less than 3 months before the patient was referred to the clinic. We included patients known to have a history of silicon or known low-grade gliomas.

The Qiagen DNA Mini Kit was used to extract tumor DNA from frozen tumors, as described by the manufacturer (Qiagen, Courtabœuf, France). DNA was extracted from blood samples using a conventional saline method.

For the determination of EGFR amplification, CDKN2A homozygous deletion, and loss of heterozygosity 9p and 10, genomic profiling was performed by comparative genomic hybridization array analysis or single nucleotide polymorphism array as previously described.\(^{(6,11)}\) MGMT promoter methylation status was determined by bisulfite sequencing using specific PCR amplification and restriction digestion.

**Standard protocol approvals, registrations, and patient consent.** Collection of tumor and blood samples and clinicopathologic information was undertaken with informed consent and written approval of a committee in accordance with the terms of the Declaration of Helsinki.

**Determination of TERTp-mut status.** The promoter region of the TERT gene was amplified as follows: TERT-F (5'-TGGGGCGGATCTGACCTCCT-3') and TERT-R (5'-AGAAGCTGACGGCCTGG-3') primers at 94°C for 3 minutes or 90°C for 35 cycles at 94°C (15 seconds), 60°C (15 seconds), and 72°C (2 minutes), with a final step at 72°C for 6 minutes. PCR products were then purified with the Agencourt AMPure XP PCR purification protocol (Agencourt, Beverly, France). Purified PCR products were then sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, United Kingdom). Sequences were paired with the Agilent Human CancerGenome panel according to the manufacturer’s instructions (50 ng of genomic DNA; Applied Biosystems, Saint Aubin, France).

**Statistical analysis.** The p-value test was used to compare the gene expression distribution. The association with continuous variables was calculated with a Mann-Whitney test.

**Overall survival (OS)** was defined as the time between the diagnosis and death or last follow-up. Patients who were still alive at the last follow-up were considered as censored events in the analysis. Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Patients who were recurrence-free at the last follow-up were considered as censored events in the analysis. To find clinical and/or genomic factors related to OS (or PFS), survival curves were calculated according to the Kaplan-Meier method, and differences between curves were assessed using the log-rank test. Variables with a significant p value were used to build a multivariate Cox model. Two-sided p values <0.05 were considered significant.

**RESULTS**

**Somatic and constitutional TERTp-mut status.** A population of 395 primary GBMs was screened for the presence of TERTp-mut. Median age at diagnosis was 58.5 years (range 18.2–94.1). Median Karnofsky Performance Score was 80 (range 0–100). At diagnosis, 281 patients (71.8%) underwent partial or total surgical resection and 214 (54.3%) were biopsy. One hundred and forty-four patients were treated upfront with radiotherapy alone, 25 (6.6%) were treated adjuvantly, 28 (7.1%) were not treated at all, and 11 (2.8%) were treated with external beam radiotherapy. The remaining 110 patients were treated with chemotherapy. Fourteen patients did not receive any specific oncologic treatment. The information was missing for 36 patients, who have been excluded from all PFS analyses. Median OS was 14.8 months and median PFS was 8.6 months.

We found 219 (71.5%) TERTp-mut, including 222 C228T (74.2%) and 77 C250T (25.8%) mutations. One tumor had both C228T and C250T mutations. This patient was considered as C228T TERTp-mut for all subsequent analyses. Patients with TERTp-mut were older than patients with TERTp-wild-type (TERTp-wt) GBM: median age at diagnosis was 59.6 vs 53.9 years, respectively (p < 0.0001). There was no difference of age at diagnosis between patients harboring C228T and C250T TERTp-mutations (data not shown).

To confirm that such mutations were all somatic events, we investigated the presence of the TERTp-mut in blood DNA corresponding to 56 TERTp-mut GBMs. No mutation was found in blood DNA (data not shown).

TERTp-mut is an independent factor of poor prognosis in GBM. In patients with GBM, patients with TERTp-mut had significantly shorter OS and PFS than patients with TERTp-wt. Median OS was 13.8
months in patients with TERTp-mut compared to 18.4 months in patients with TERTp-wt (p < 0.0001) (figure 1). Accordingly, PFS was 8.3 and 16.4 months, respectively (p < 0.0001). We did not find any difference in outcome between the C228T and C228T TERTp-mut (figure e-1 on the Neurology® Web site at Neurology.org). We then input the following factors as candidate variables in the multivariate Cox proportional hazards regression model analysis: age at diagnosis, IDH mutation, extent of surgery, concurrent and adjuvant chemotherapy, Karnofsky Performance Score, MGMT promoter methylation status, and TERTp-mut. TERTp-mut appeared as an independent

We then input the following factors as candidate variables in the multivariate Cox proportional hazards regression model analysis: age at diagnosis, IDH mutation, extent of surgery, concurrent and adjuvant chemotherapy, Karnofsky Performance Score, MGMT promoter methylation status, and TERTp-mut. TERTp-mut appeared as an independent

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Abbreviations: CI = confidence interval; HR = hazard ratio; IDH = isocitrate dehydrogenase; KPS = Karnofsky Performance Score; MGMT = methylguanine methyltransferase; TERT = telomerase reverse transcriptase; TMZ = temozolomide.
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<td>7.8</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis</td>
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<td></td>
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<tr>
<td>≥50 y</td>
<td>7.5</td>
<td>-0.0001</td>
</tr>
<tr>
<td>≤50 y</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>Treatments</td>
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<td></td>
</tr>
<tr>
<td>Concurrent and adjacent TMZ</td>
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<td>-0.0001</td>
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<tr>
<td>Other</td>
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<tr>
<td>MGMT promoter</td>
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<tr>
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<tr>
<td>Nonmethylated</td>
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<tr>
<td>TERT promoter</td>
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</tr>
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<td>-0.0001</td>
</tr>
<tr>
<td>Nonmethylated</td>
<td>10.4</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CI = confidence interval, HR = hazard ratio, IDH = isocitrate dehydrogenase, KPS = Karnofsky Performance Score, MGMT = methylguanine methyltransferase, TERT = telomerase reverse transcriptase, TMZ = temozolomide.

prognostic factor for both OS and PFS in GBM (tables 1 and 2).

TERT mutations are associated with specific prognostic and molecular subgroups. The association of TERTp-mut with the other molecular alterations frequently found in GBMs is presented in table 3.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TERTp-mut</th>
<th>TERTp-wt</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR amplification</td>
<td>12/13549 (92.8)</td>
<td>13/563 (23.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Or 1p/19q loss</td>
<td>23/11969 (99.4)</td>
<td>41/465 (8.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td>CDKN2A deletion</td>
<td>14/11969 (47.2)</td>
<td>3/565 (5.31)</td>
<td>0.0013</td>
</tr>
<tr>
<td>IDH-mutation</td>
<td>8/190 (4.2)</td>
<td>22/291 (7.5)</td>
<td>0.0001</td>
</tr>
<tr>
<td>TP53 mutation</td>
<td>395/144 (27.3)</td>
<td>16/414 (3.17)</td>
<td>0.0001</td>
</tr>
<tr>
<td>MGMT promoter-methylated</td>
<td>15/7844 (19.37)</td>
<td>4/650 (0.64)</td>
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<tr>
<td>TERTp-mut = TERT/promoter wild type</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Abbreviations: CDKN2A = cyclin-dependent kinase inhibitor 2A; Or = chromosome; EGFR = epidermal growth factor receptor; IDH = isocitrate dehydrogenase; MGMT = methylguanine methyltransferase; TP53 = tumor protein p53.

TERTp-mut = TERT promoter mutation, TERTp-wt = TERT promoter wild type, TP53 = tumor protein p53.

IDH mutation was associated with TERTp-wt GBM (22/38 [57.9%] vs 8/284 [2.8%] in TERTp-mut GBM). We therefore required whether the higher incidence of IDH mutation could explain the better outcome of TERTp-wt patients compared with TERTp-mut GBM. However, stratifying our population according to the TERTp status, we found that TERTp-mut was prognostic in both IDH-wt GBM (OS = 13.7 vs 17.5 months, p = 0.0006) and IDH-mutation GBM (OS = 13.8 vs 17.6 months, p = 0.062). Moreover, it is particularly striking to note that IDH mutation was associated with a better outcome in TERTp-wt GBM (OS = 37.6 vs 17.5 months, p = 0.04) but not TERTp-mut GBM (OS = 13.8 vs 17.5 months, p = 0.04). Therefore, comparing according to TERTp status, we found that IDHp-mut = IDH promoter mutation was associated with a better outcome in TERTp-wt GBM (OS = 37.6 vs 17.5 months, p = 0.04) but not TERTp-mut GBM (OS = 13.8 vs 17.5 months, p = 0.04).

In contrast to IDH mutation, EGFR amplification, present in 144 GBMs, was associated with TERTp-wt GBM (9/1299 [8.8%] vs 13/796 [1.6%] in TERTp-mut GBM). We found that EGFR amplification had no prognostic impact per se (figure 3). However, when stratifying according to TERTp status, we found that EGFR amplification was associated with shorter OS and PFS in the TERTp-wt group (OS = 12.3 vs
26.6 months, \( p = 0.005 \)) but not in the TERTp-mut group (OS = 13.8 vs 12.3 months, \( p = \text{not significant} \)) (Figure 3). It is striking to note that in the EGFR wt group, patients with TERTp-mut had a poorer outcome compared to patients with TERTp-wt (OS = 12.5 vs 26.6 months, \( p < 0.0001 \)), whereas in the EGFR amplified group, patients with TERTp-mut had a better outcome than patients with TERTp-wt (OS = 15.8 vs 13.8 months, \( p = 0.05 \)). To better understand this paradox, we compared the age of these different populations: there was no significant difference of age in the EGFR amplified group between TERTp-mut patients (59.1 years) and TERTp-wt patients (55.7 years, \( p = 0.5 \)), whereas TERTp-mut patients were significantly older than TERTp-wt patients (66 vs 69 years, \( p = 0.0001 \)) in the EGFR nonamplified group. We therefore analyzed the impact of the TERTp-mut according to age in the EGFR-wt group. The results, reported in figure 3, show that in each age category (<50 years; 50-65 years; >65 years), patients with TERTp-wt had a longer survival, but this was particularly relevant in the group of patients younger than 50 years.

We also found that TERTp-mut were associated with chromosomal 1q loss and CDKN2A homozygous deletion, but these associations did not result in a prognostic stratification of our cohort.

**Prognostic classification of GBMs based on TERTp, EGFR, and IDH status.** Building on these results, we propose a 4-group molecular classification of GBMs: (1) GBMs with TERTp-mut constituting a homogeneous group (OS = 13.8 months); (2) GBMs with EGFR amplification and TERTp-wt (OS = 13.3 months), all of which are IDH-wt; (3) IDH mutation having no prognostic impact in this group; and (4) EGFR wt and TERTp-wt, characterized by a much better prognosis particularly in the presence of the IDH mutation (OS = 37.6 months), but even in the absence of the IDH mutation (26.5 months) (figure 4).

![Figure 2 Prognostic impact of TERT promoter mutation in glioblastomas stratified according to IDH mutation](image)

![Figure 3 Prognostic stratification of glioblastomas according to TERT promoter mutation and EGFR amplification status](image)
**Figure 4** Survival curves corresponding to the 4 prognostic groups

TERP-mut | IDH = 13.8 months; EGFR mut with IDH-mut (OS = 13.3 months); IDH-mut with IDH-mut and IDH-wt (OS = 26.5 months); IDH-mut with TERP-mut; IDH-wt mutation (OS = 37.6 months; amp = amplification; EGFR = epidermal growth factor receptor; IDH = isocitrate dehydrogenase; mut = mutation; OS = overall survival; TERP = telomerase reverse transcriptase; TERP-mut = TERP promoter mutation; TERP-wt = TERP promoter wild type; wt = wild type.

**DISCUSSION**

**TEPT** is the most frequently mutated gene in GBMs, suggesting that it may be an early event in the development of these tumors. These mutations create a pronecophen site for the C-mos-serotonin complex factor transcription factors and increase 2- to 4-fold transcriptional activity of the promoter. Increasing telomerase activity could be a selective advantage and promote immortalization of cells by preventing the senescence induced by telomere shortening.

In our series, **TERP-mut** were associated with an older age at diagnosis, as previously reported in medulloblastomas,15 conjunctival melanomas,16 and recently in gliomas.17 Telomeres are shorter in the GBMs of older patients, and shortening of telomere shortening may therefore be more critical in older patients. Accordingly, the polymorphism rs1736100, which maps to the **TEPT** locus, has also been associated with the **IDH-wt** and older age gliomas.19

In this work, we show that **TERP-mut** is an independent factor of poor outcome in GBMs. Indeed, we clearly show here that this effect is not due to the association of **IDH** mutation with **TERP-mut** status as previously believed.20 Moreover, the impact of **TERP-mut** is even stronger in patients with **IDH** mutation than in patients with **IDH-wt**, with a median OS decreasing from 37.6 months in **TERP-wt** to 13.8 months in **TERP-mut**. In other words, our data suggest that the favorable prognostic impact of the **IDH** mutation in GBMs is lost in the presence of an associated **TERP** mutation, because the survival in **IDH** mutation **TERP-mut** GBMs is identical to standard (i.e., **IDH-wt**) GBMs.

We further dissected the prognostic impact of the **TERP-mut** in the context of different genetic backgrounds. **EGFR** amplification is a hallmark of GBMs. It affects 40% of GBMs and is mutually exclusive with **IDH** mutation. In line with previous reports, we show here that **EGFR** amplification has no prognostic impact in the whole GBM population.21,22 However, determination of the **TERP-mut** status revealed that in the **TERP-mut** group, patients with **EGFR-wt** had a median survival twice superior to that of patients with **EGFR** amplification. Consequently, we show a very sharp and opposite effect of the **TERP-mut** on survival according to **EGFR** status. Similar results have been obtained in medulloblastomas, also showing an opposite prognostic effect of **TERP-mut** according to different genetic subtypes.20 Our study also makes it clear that the difference of age—a well-known prognostic factor in GBM—is not a valid explanation, because in the **EGFR** amplified subgroup, patients with **TERP-mut** were older but still had a better outcome (OS = 15.8 vs 13.3 months, p = 0.05).

Finally, we propose a 4-group molecular classification that summarizes our results: when either **EGFR** amplification or **TERP-mut** is present, the prognosis is poor with median survival ranging from 12 to 16 months. Median survival is much better when none of these 2 aberrations is present, ranging from more than 2 years for patients with **IDH-wt**, to more than 3 years for patients with **IDH** mutation.

In this study, we found that the **TERP-mut** is a strong and independent prognostic marker in GBM, and is not related to **IDH** status. We also show an opposite prognostic effect of **TERP-mut** in **EGFR-wt** GBMs. Finally, we propose a refined prognostic classification of GBMs based on the joint analysis of **TERP**, **EGFR**, and **IDH**.

**AUTHOR CONTRIBUTIONS**

M.L. and M.S. designed the study and wrote the manuscript; M.L., B.R., A.K., K.L., M.D., A.S., P.P., and G.F. performed data analysis. M.L. performed the statistical analysis. Statistical data were collected and analyzed by V.A. M.L. and M.S. are responsible for the data analysis and interpretation. All authors approved the manuscript.

**ACKNOWLEDGMENT**

The authors are indebted to Marcello Scala for editing the manuscript.

**STUDY FUNDING**

Supported by the Ugo Nobile National center for Cancer (ENCCO) and the Italian National Cancer (RNC). Anna Luisa Di Stefano is supported by an investigator fellowship from Collegio Cistercense, Pisa, Italy.

**DISCLOSURE**

The authors report no declared conflict of interest in the manuscript. Go to Neurology.org for full disclosure.

Received December 29, 2013; accepted in final form on July 2, 2014.

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3.1 Glioma Susceptibility Loci Reflect a “IDH-Based Watershed” in Gliomagenesis

Understanding of the genetic susceptibility of glioma has been transformed by recent genome-wide association studies (GWAS), which recently disclosed a dichotomy between IDH mutated and IDH wild-type gliomas in heritability of gliomas. Starting from 2009, GWAS have progressively identified single nucleotide polymorphisms (SNPs) at 26 loci influencing glioma risk and have provided evidence for a polygenic basis of genetic susceptibility of gliomas (Shete, 2009; Sanson, 2011; Wrensch, 2009; Kinnersley, 2015; Walsh, 2014; Enciso-Mora, 2013; Jenkins, 2012).

Inherited predisposition to glioma is also suggested by a number of rare inherited cancer syndromes, such as Turcot’s and Li–Fraumeni syndromes, and neurofibromatosis, which however, even collectively, account for little of the two-fold familial risk of glioma and for <5% of glioma cases (Hemminki, 2009).

Giving heterogeneity of gliomas, in 2012 we started exploring associations between the first known 7 glioma-risk SNPs at that time, and tumor histological and molecular genetic profile (Di Stefano, 2013). Interestingly, in this study we found that certain SNPs -rs2736100 (annotating TERT) and rs6010620 (RTEL1) are associated with high-grade phenotype and molecular aberrations and that rs4295627 (CCDC26) and rs498872 (PHLDB1) are associated with low-grade disease, IDH mutation, and 1p-19q codeletion.

We then proposed for the first time a model of case-control analyses based on four tumor molecular classes by IDH status, 1p19q codeletion and EGFR amplification [Figure 3.1 adapted from (Di Stefano, 2013)].

In this study, we found that susceptibility alleles on TERT, RTEL1, CCDC26, PHLDB1 predispose to different molecular subgroups of gliomas and that IDH somatic mutation is the most robust watershed for these distinct etiologic pathways [Figure 3.1; (Di Stefano, 2013)].
Figure 3.1 adapted from (Di Stefano, 2013). Glioma susceptibility loci predispose to specific molecular classes of gliomas. In A) Kaplan–Meier curves in 1372 glioma patients show significant overall survival difference between tumor class 1-IDH mut/1p-19q codel/EGFR normal-(median OS 211.2 months), tumor class 2-IDH mut/1p-19q normal/EGFR normal- (median OS 103.9 months), tumor class 3-IDH wt/1p-19q normal/EGFR normal- (median OS 26.5 months), and tumor class 4-IDH wt/1p-19q normal/EGFR amplification-(median OS 16.6 months). In B) Glioma risk, stratified by specific tumor class, is represented (OR and 95% CI) for each single SNP. IDH mutation/1p-19q codeletion/EGFR normal status; IDH mutation/1p-19q normal status/EGFR normal status; IDH wild-type/1p-19q normal status/EGFR normal status; IDH wild-type/1p-19q normal status/EGFR amplification.

More recently, others and we have reported supplementary susceptibility variants for gliomas using an imputation based approach and genotyping confirmation.

We reported a rare variant rs78378222 (minor allele frequency 0.013) annotating TP53, which is strongly associated to glioma risk ($P=6.86 \times 10^{-24}$) (Enciso-Mora, 2013). This locus does not show a differential association between rs78378222 histological phenotype (GBM versus non-GBM tumors) and is not associated with TP53 somatic mutation.

In a following study, thanks to advances in high-density genotyping and imputation reference panels we were able (Enciso-Mora, 2013) to deeply examine 8q24.21, the region encompassing the rs4295627 (CCDC26), which correspond to the region with the highest signal of association with IDH mutation and 1p19 codeleted gliomas (OR 2.44 $P=10^{-9}$). Analysis revealed an imputed low-frequency SNP rs55705857 ($P=2.24 \times 10^{-38}$) and stratifying by glioma subtype, we showed that the association with rs55705857 is confined to non-glioblastoma (non-GBM) tumors with the tightest association ever found in GWAS ($P=1.07\times 10^{-67}; P=2.31 \times 10^{-94}$in the pooled analysis, of three additional datasets).
rs55705857 maps to a highly evolutionarily conserved sequence within the long non-coding RNA CCDC26, raising the possibility that this region (8q24.21) contains a germ line alteration that facilitates the initiation or progression of gliomas with IDH mutations (Enciso-Mora, 2013).

More recently, to expand the repertoire of glioma susceptibility loci, others and we recently performed the following:

- a first meta-analysis of four GWAS (totalling 4,147 cases and 7,435 controls), reporting new risk loci for glioblastoma (GBM) at 12q23.33 (rs3851634, near POLR3B, \(P=3.02\times10^{-9}\)) and non-GBM at 10q25.2 (rs11196067, near VTI1A, \(P=4.32\times10^{-8}\)), 11q23.2 (rs648044, near ZBTB16, \(P=6.26\times10^{-11}\)), 12q21.2 (rs12230172, \(P=7.53\times10^{-11}\)) and 15q24.2 (rs1801591, near ETFA, \(P=5.71\times10^{-9}\)) (Kinnersley, 2015).

- a second larger meta-analysis, in the setting of the Glioma International Case Control Consortium, including past published GWAS and a new GWAS with replication comprising 12,496 cases and 18,190 controls (Melin et al. Nat Genet, in press)

This study identified 13 new risk loci, reaching a total of 26 glioma risk loci identified so far.

Among them, rs7572263, mapping to 2q33.3, is associated with non-GBM \((P=2.18\times10^{-10}\), Odds Ratio=1.20) and interestingly localizes ~50 kb telomeric to the gene encoding IDH1. Since IDH mutation is predominate, as mentioned before, in non-GBM glioma, the association at 2q33.3 could potentially disclose additional insights into the aetiological basis of IDH mutant gliomagenesesis. Reported articles are included in this section.
Genome-wide association study identifies multiple susceptibility loci for glioma

Ben Kinnersley¹, Marianne Labussière², Amy Holroyd¹, Anna-Luisa Di Stefano²,³,⁴, Peter Broderick¹, Jayaram Vijayakrishnan¹, Karima Mokhtari²,³,⁵, Jean-Yves Delattre²,³,⁴, Konstantinos Gousias⁶, Johannes Schramm⁶, Minouj K. Schoenmaker¹, Sarah J. Flemming¹, Stefan Herrmann⁸,⁹, Stefanie Heilmann⁸, Stefan Schreiber¹⁰,¹¹, Heinz-Erich Wichmann¹²,¹³, Markus M. Nöthen⁹, Anthony Swerdlow¹⁴, Mark Lathrop⁵,¹⁵,¹⁶, Matthias Simon⁶, Melissa Bondy⁷, Marc Sanson²,³,⁴ & Richard S. Houlston¹

Previous genome-wide association studies (GWAS) have shown that common genetic variation contributes to the heritable risk of glioma. To identify new glioma susceptibility loci, we conducted a meta-analysis of four GWAS (totaling 4,147 cases and 7,435 controls), with imputation using 10,000 Genomes and UK10K Project data as a reference. After genotyping, an additional 1,490 cases and 1,723 controls we identified new risk loci for glioblastoma (GBM) at 1q22.33 (rs3856364, near PCORB2, \(P = 3.02 \times 10^{-29}\)) and non-GBM at 10q25.2 (rs1799607, near VTTA, \(P = 4.32 \times 10^{-18}\)), 11q23.2 (rs6848044, near ZBTB16, \(P = 6.26 \times 10^{-5}\)), 12q12 (rs12220172, \(P = 7.53 \times 10^{-10}\)) and 15q24.2 (rs1880199, near EPHA, \(P = 5.71 \times 10^{-8}\)). Our findings provide further insights into the genetic basis of the different glioma subtypes.
Glialomas account for ~40% of all primary brain tumours and cause around 13,000 deaths in the United States of America each year.1 Glialomas are heterogeneous and different tumour subtypes, defined in part by malignancy grade for example, pilocytic astrocytoma WHO grade I, diffuse low-grade glioma WHO grade II, anaplastic glioma WHO grade III and glioblastoma (GBM) WHO grade IV, can be distinguished.2 Glialomas are typically associated with a poor prognosis irrespective of clinical care, with the most common type, GBM, having a median overall survival of only 15 months.3

While the glioma subtypes have distinct molecular profiles resulting from different ontological pathways,6 no environmental exposure has however, consistently been linked to risk except for ionizing radiation, which only accounts for a very small number of cases7. Direct evidence for inherited predisposition to glioma is provided by a number of rare inherited cancer syndromes such as Turcot’s and Li-Fraumeni syndromes, and neurofibromatosis8. Even collectively, these diseases however account for little of the twofold increased risk of glioma seen in first-degree relatives of glioma patients9. Support for polygenic susceptibility to gliomas has come from genome-wide association studies (GWASs) that have identified single-nucleotide polymorphisms (SNPs) at eight loci influencing glioma risk: 4q21.2 (near TERC), 5p15.33 (near TERT), 7p13.2 (near EGFR), 8q24.21 (near CCDC26), 9p21.3 (near CDKN2A/CDKN2B), 11q23.2 (near PHD1), 17p13.1 (17p32) and 20q13.33 (near KYSL).6 Perhaps not surprisingly there is variability in genetic effects on glioma by histology with subtype-specific associations at 5p15.33, 20q13.33 and 9p21.1 for GBM and at 11q23.3 and 8q24 for non-GBM gliomas.6

Recovery of untyped genotypes via imputation has enabled fine mapping and refinement of association signals, for example, in identification of rs570857 in the 4q24 association signal in glioma.10 Recently, the use of the 1000 Genomes Project and the UK10K project as a combined reference panel has been shown to improve accuracy compared with using the 1000 Genomes Project alone, allowing imputation of alleles with frequencies <0.5% to be viable.12 Here we report a meta-analysis of four GWASs totalling 4,473 glioma cases and 5,435 controls to identify new glioma susceptibility loci, after imputation using the 1000 Genomes and the UK10K Project data as reference. After genotyping an additional series of 1,490 cases and 4,055 controls, we identified new risk loci for GBM at 12p23.33 and non-GBM at 10q25.2, 11q33.2, 12q12.2 and 15q24.2. Our findings provide further insights into the genetic basis of the different glioma subtypes.

Results

Association analysis. To identify additional glioma susceptibility loci we conducted a pooled meta-analysis of four GWASs in populations of European ancestry, the UK-GWAS, the French-GWAS, the German-GWAS and the US-GWAS, that were genotyped using either Illumina HumanHap 317, 317-I + 24B, 370Duo, 550, 610 or 1M arrays (Supplementary Table 1). After filtering, the studies provided genotypes on 4,473 cases and 7,431 controls of European ancestry (Supplementary Table 1, Supplementary Fig. 1). Consistent with our previous analysis,5, quintile–quintile (Q–Q) plots for the German and the US series showed some evidence of inflation (inflation factor based on the 90% least-significant SNPs, λQ5 = 1.15 and 1.11, respectively). However after correcting for population substructure using principal component analyses as implemented in Eigenstrat,13 λQ5 for all studies was ≤1.05 (Supplementary Fig. 2). To achieve consistent and dense genome-wide coverage, we imputed unobserved genotypes at >10 million SNPs using a combined reference panel comprising 1,292 individuals from the 1,000 Genomes Project and 3,291 individuals from the UK10K project. Q–Q plots for all SNPs (minor allele frequency (MAF) >0.5%) post-imputation did not show evidence of substantive over-dispersion introduced by imputation after Eigenstrat adjustment (combined λQ5 = 1.07, λQ5 for individual studies: 1.04–1.06; Supplementary Fig. 2).

Pooling data from each GWAS into a joint discovery data set, we derived joint odds ratios (ORs) and 95% confidence intervals (CIs) under a fixed-effects model for each SNP with MAF >0.01. Meta-analysis across the four GWASs for each SNP showed ORs ranging from 0.85 to 1.66 with CIs for 95% confidence intervals. A total of 14 SNPs for follow-up, mapping to distinct loci not previously associated with glioma risk (Fig. 1 and Supplementary Table 2). In contrast we found no significant support for the association between rs1980116 near TERC (3q28) and risk of high-grade glioma recently reported by Walsh et al.19 (combined P value for GBM = 0.17; Supplementary Table 2 and Supplementary Fig. 3). While the UK-GWAS and the study of Walsh et al. share use of the UK1958 Birth Cohort controls, the other three GWASs we analysed are fully independent.

After filtering at P < 5.0 × 10⁻⁶ in each all glioma, GBM or non-GBM, we selected 14 SNPs for follow-up, mapping to distinct loci not previously associated with glioma risk (Fig. 1 and Supplementary Table 2). In the joint analysis five SNPs showed an association with tumour risk, which was genome-wide significant (Table 1)—rs3851634 (12q23.3, PGBM = 3.02 × 10⁻²), rs1980116 (3q28, 4.32 × 10⁻⁶), rs6864044 (11q23.2, PGBM = 6.26 × 10⁻¹¹), rs12292172 (12q12.2, PGBMGR = 7.53 × 10⁻⁸), and rs189359 (15q24.2, PGBM = 5.71 × 10⁻⁸). In the joint analysis the established locus rs1229217 (12q12.2) maps to ZBTB16 (zinc finger and BTB domain-containing protein 16, alias PLZF) (Fig. 2c), rs1196067, rs6864044 (11q23.2) is also located on 1q24.3. The association signal at 12q23.3 defined by rs3851634 was specific for GBM. The rs3851634 maps to intron 12 of the gene encoding polymeric III, RNA, subunit b (POLR3B, Fig. 2a) within a ~30-kb block of linkage disequilibrium (LD) at 12q23.3, which also contains the genes CHAF1B and TBP.22 The other four SNP associations defined by rs1196067, rs6864044, rs12292172 and rs189359 were specific to non-GBM glioma. rs1196067 (12q23.2) is located in intron 7 of ZBTB16 (zinc finger and BTB domain-containing protein 16, alias PLZF), rs6864044 (11q23.2) is also located on chromosome 1q24.3.
In the case of non-GBM SNPs rs11196067 showed the strongest association with grade II glioma \( (P = 5.2 \times 10^{-8}) \) and 7p33 non-mutated glioma \( (P = 5.82 \times 10^{-8}) \); rs68044 with grade II oligodendroglioma \( (P = 0.026) \) and 10q non-deleted glioma \( (P = 0.006) \); rs1801591 with grade II astrocytoma \( (P = 0.001) \) and IDH1/IDH2 mutated \( (P = 0.003) \) and 10q non-deleted glioma \( (P = 0.003) \).

Functional annotation of risk variants. For each of the sentinel risk SNPs at the five risk loci (as well as correlated variants, \( r^2 > 0.8 \)) we examined published data \(^{4,35}\) and made use of the online resources HaploReg v3, RegulomeDB and SearSeq for evidence of functionality and regulatory motifs at genomic regions (Supplementary Table S5), rs1801591, which is responsible for the EIF5A p.Thr171Le substitution, resides within a highly conserved region of the genome (genomic evolutionary rate profiling \( \text{GERP} = 5.65 \)) and the amino-acid change is predicted to be damaging (PolyPhen-2 = 1). Although rs648044 exhibits low evolutionary conservation \( \text{GERP} = 9.32 \) it maps within a strong DNase hypersensitivity site and predicted enhancer/super-enhancer element for multiple tissues including the brain. The region surrounding rs648044 is also predicted to interact with the ZBTB36 promoter, which combined with alteration of a PAX-5 motif is suggestive of direct functional impact; rs12230172 localizes within a moderately conserved region \( \text{GERP} = 3.41 \) and occupies promoter histone marks in the brain as well as enhancers predicted to associate with transcriptional start sites for PHLD1 and GLIPR1; rs11196067 in VEGFA, while having a low conservation score \( \text{GERP} = 0.719 \), occupies enhancer histone marks in embryonic stem cells although not in brain cells. Similarly, rs3851634 maps to a moderately conserved region \( \text{GERP} = 2.37 \) and occupies enhancer histone marks in 18 organs including the brain.

eQTL analysis of the five new glioma SNPs. To gain further insight into the functional basis of rs11196067, rs648044, rs12230172, rs1801591 and rs3851634 associations we performed an expression quantitative trait loci (eQTL) analysis using RNA-Seq expression data on 389 low-grade gliomas (LGGs) and 138 GBMs from The Cancer Genome Atlas (TCGA), together with lymphoblastoid cell line RNA-Seq data on 562 samples from GLUVAHNS \(^{39} \). We examined for an association between SNP genotype and expression of genes mapping within 1Mb of the sentinel SNP (Supplementary Data 2). After adjusting for multiple testing within each region no statistically significant eQTL was seen for rs11196067, rs12230172, rs1801591 or rs3851634. The strongest association between rs648044 genotype and gene expression was with ZW10 in LGG \( (P = 5.7 \times 10^{-7}) \), with the risk allele (T) associated with lower expression, remaining significant after adjustment for multiple testing. To explore the possibility that rs648044 is correlated with a SNP exhibiting a stronger association with ZW10; we examined associations with ZW10 expression in LGG tumours in all SNPs in LD \( (r^2 > 0.4) \) with rs648044. All of the proxy SNPs examined were more weakly associated with ZW10 than rs648044 (Supplementary Table 6). Following on from these analyses we made use of publically available eQTL mRNA expression array data on adipose tissue, lymphoblastoid cell lines and skin from 856 twins (MzTHER) \(^{22} \) and 5,311 non-transformed peripheral blood samples using the blood eQTL browser \(^{39} \). The risk allele (C) of rs3851634 was associated with significantly lower levels of POLR3B \( (P = 7.49 \times 10^{-4}) \) in peripheral blood analysis with a nominally significant association in skin \( (P = 0.0052) \). The risk allele (T) of rs1801591, was associated with significantly lower
ETFA levels in peripheral blood (P = 7.90 × 10⁻²); there was a nominally significant association in McTHER lymphoblastoid cell lines (P = 0.037).

Somatic mutation of newly implicated risk genes in glioma. We examined mutation data from TCGA for evidence of recurrent mutation in genes annotated by the new GWAS signals. Collectively, POLR3E, ETFA, VTI1A, ZTB3, and PHLDA4 are altered in 8% (22/286) of LGG as compared with 3% (8/275) of GBM (P = 0.014, Supplementary Table 7) providing support for these genes having a role in glioma tumorigenesis.

Individual variance in risk associated with glioma SNPs. To explore the relative contributions of previously reported and newly described loci to glioma risk, we applied the method of Pharoah et al. to eight previously reported SNPs as well as the five new risk SNPs (Supplementary Table 8). The variance in risk attributable to all 12 SNPs is 26%, 27% and 43% for all gliomas, GBM and non-GBM, respectively.

Pathway enrichment of glioma GWAS SNPs. To gain further insights into the biological basis of associations we performed a pathway analysis on GWAS associations in all gliomas, GBM and non-GBM. Applying a false discovery rate (FDR) threshold of <0.1 revealed enrichment for 14 pathways in all gliomas, 8 in GBM and 9 in non-GBM tumours (Supplementary Table 9).

Table 1 | Association between SNP and glioma risk in discovery and replication data sets for rs17196067, rs482044, rs12230172, rs3851634 and rs18805919.

<table>
<thead>
<tr>
<th>SNP</th>
<th>MAF</th>
<th>P (95% CI)</th>
<th>OR (95% CI)</th>
<th>OR (95% CI)</th>
<th>OR (95% CI)</th>
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<tr>
<td>rs17196067</td>
<td>0.38</td>
<td>0.41</td>
<td>FTE</td>
<td>5.09 × 10⁻¹⁵</td>
<td>0.79 (0.71-0.89)</td>
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<tr>
<td>(VTI1A)</td>
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<td></td>
<td>GER</td>
<td>0.44</td>
<td>0.26 (0.12-0.69)</td>
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<td>rs482044</td>
<td>0.40</td>
<td>0.38</td>
<td>USA</td>
<td>0.016</td>
<td>0.98 (0.89-1.08)</td>
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<tr>
<td>(ZTB3)</td>
<td></td>
<td></td>
<td>Replication</td>
<td>0.56</td>
<td>0.97 (0.88-1.07)</td>
</tr>
<tr>
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<td>0.46</td>
<td>Combined</td>
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<td>0.89 (0.85-0.93)</td>
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<td>(Igfaspauc)</td>
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<td></td>
<td>G/T</td>
<td>0.018</td>
<td>0.98 (0.92-1.07)</td>
</tr>
<tr>
<td>rs3851634</td>
<td>0.27</td>
<td>0.30</td>
<td>Combined</td>
<td>5.29 × 10⁻³</td>
<td>0.97 (0.93-1.01)</td>
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<tr>
<td>(PORD)</td>
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<td></td>
<td>Replication</td>
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<tr>
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<td>0.10</td>
<td>0.09</td>
<td>Combined</td>
<td>1.07</td>
<td>0.25 (0.04-1.65)</td>
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<tr>
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<td></td>
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<td>0.44 (0.05-3.91)</td>
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<td>0.25 (0.04-1.65)</td>
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<tr>
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<td>0.09</td>
<td>Combined</td>
<td>1.07</td>
<td>0.25 (0.04-1.65)</td>
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<tr>
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<td>Replication</td>
<td>0.52</td>
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<td>(PORD)</td>
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<td>Replication</td>
<td>0.52</td>
<td>0.25 (0.04-1.65)</td>
</tr>
</tbody>
</table>

Pathways implicated in GBM tumours primarily include DNA repair and Notch-signalling, whereas for non-GBM tumours pathways were primarily associated with cell-cycle progression and energy metabolism (Supplementary Table 9).

Discussion

To our knowledge, we have performed the largest GWAS of glioma to date, identifying five novel glioma susceptibility loci at 11q23, 11q25, 11q25.1, 11q23.3, 12q21.2, and 15q12 and taking the total count of risk loci to 12. Through making use of a combined reference panel from the UK1000 and the 1000 Genomes Projects we were able to recover genomically from ~8 million SNPs for association analysis, a significant increase from using array SNPs alone. In addition, we have provided further evidence that genetic susceptibility to glioma can be subtype specific, emphasising the importance of searching for histology-specific risk variants.

While desperting the functional impact of these SNP associations on glioma development requires additional analyses, a number of the genes implicated have relevance to the biology of this cancer a priori. As well as participating in regulating unstimulated trafficking of secretory vesicles, VTI1A plays a key role in neuronal development and in selectively maintaining spontaneous neurotransmitter release22. More recently, recent GWAS have identified associations between the VTI1A SNPs rs7086803 and lung cancer23. rs7086803 and rs1224908 are not
correlated with each other ($r^2 = 0.22, \beta = 0.72$) and are also not correlated with rs11980607 ($r^2 = 0.03, \beta = 1.01(0.22$, respectively), suggesting the existence of multiple risk loci within the region with different tumour specificities.

ZBTB16 is highly expressed in undifferentiated, multipotential progenitor cells and its expression has been shown to influence resistance to retinoid-mediated re-differentiation in t(11;17)(q23;q21) acute promyelocytic leukaemia. The BTB domain of ZBTB16 has transcriptional repressive activity and interacts with components of the histone deacetylase complex thereby linking the transcription factor with regulation of chromatin condensation. Although rs6480441 lies within an enhancer active in brain and is predicted to interact with the ZBTB16 promoter, providing an attractive functional basis for the 11q23.2 association through differential ZBTB16 expression, we found a strong association between rs6480441 and ZBT10 expression in LGG ($P = 7.5 \times 10^{-7}$). Since ZBT10 plays a role in chromosome segregation, it also represents a plausible candidate for the 11q23.2 association.

We also observed a strong association between EFTA expression and rs1801591 in peripheral blood ($P = 7.9 \times 10^{-15}$). EFTA participates in mitochondrial fatty acid beta oxidation, shuttling electrons between flavoprotein dehydrogenases and the membrane-bound electron transfer flavoprotein ubiquinone oxireductase. Mutations of EFTA have been reported to be a cause of recessive glutaric aciduria IIb (RGLB20, 29), which...
features gliosis. While the pThreon171His change is reported to decrease thermal stability of eIF4A2 thereby providing evidence for a functional difference the strong eGOL data is consistent with the functional basis for the E1942A association being mediated through differential expression.

RNA polymerase II (POLR2A) is involved in the transcription of small noncoding RNAs and short interspersed nucleic elements, as well as all transfer RNAs. Although mutations in POLR2A have been shown to cause recessive hypomyelinating leukoencephalopaty12 thus for there is no evidence implicating the involvement of thrombopoetin. Albeit in whole blood there was a strong association between POLR2A expression and rs3858164 (P = 7.49 × 10^-6), providing a possible functional basis for the 7.8% association.

At 12q27.2 rs13220172 maps within RPP11-RH213, a lincRNA of currently unknown function. Although only being adjacent to PHLD1, the known 11q23.3 association map to the related gene PHLD1, which is also specific to non-GBM tumours. Although a role for PHLD1 in glioma has yet to be established downregulation of PHLD1 in neuronal cells has shown to enhance cell death without Fas induction25, additionally PHLD1 expression may be involved in regulation of anti-apoptotic effects of IGF1 (ref. 34).

Intriguingly across all of the four GWAS data sets we analysed we did not replicate the association between rs130116 (near TERT) at 5q26.2 and risk of high-grade glioma recently reported by Walsh et al.28 (P = 8.3 × 10^-3), OR = 1.20 versus P = 0.18, OR = 1.06 relative to the G-allele in our GWAS data set), despite our study having a similar power to demonstrate a relationship (1,783 GBM cases, 7,435 controls in our study as compared with 1,644 cases, 7,736 controls). It is, however, noteworthy that the Walsh et al.28 analysed both anaplastic astrocytoma and GMB. While we cannot demonstrate a significant association (rs10190P16 and TPT3 mutated glioma (P = 0.06), Supplementary Table 1) suggesting the role of the SNP in glioma risk is even more limited.

Our findings provide further evidence for an inherited genetic susceptibility to glioma. Future investigation of the genes targeted by the risk SNPs we have identified is likely to yield additional insights into this malignancy. We estimate that the risk loci we have identified for glioma account for 27 and 43% of the familial risk of GBM and non-GBM tumours, respectively, of which 11% can be explained by the loci rarely reported in this study (Supplementary Table 8). Although the power of our study to detect major common loci (MAF > 0.2) conferring risk ≥ 2 was high (> 89%), we had low power to detect alleles with smaller effects and/or MAF < 0.1. By implication, variants with smaller effects may only be detected by sequencing studies.

In addition, with which we have recently shown, stratified analysis of glioma by molecular profile may lead to the discovery of additional subtype-specific risk variants. However, such subtype analyses can increase the statistical burden of adjusting for multiple testing. For example, if we apply the Bonferroni correction for GMB and non-GBM subtypes, the rs1196522 (VHLA) association at P = 8.44 × 10^-3 would not be declared genome-wide significant. An issue in future subtype analyses of glioma will therefore be to have sufficient study power to mitigate type II error given the additional constraints of multiple testing. Further efforts to expand the scale of GWAS meta-analyses through international consortia and increasing the number of samples taken forward to large-scale replication will be required to address this challenge.

Methods

Ethics. Collection of blood samples and clinicopathological information from patients and controls was in accordance with informed consent and relevant ethical review board approval in accordance with the tenets of the Declaration of Helsinki. Ethical committee approval for this study was obtained from relevant centres (UK: South East Multicentre Research Ethics Committee (MREC) and the Scottish multicentre Research Ethics Committee (SREC); France: APHP Ethical Committee-CPB (centres de Protection du Personne); Germany: Ethics Commission of the Medical Faculty of the University of Bonn, and DSM, University of Texas MD Anderson Cancer Institutional Review Board).

Genomic-wide association studies. We used GWAS data previously generated on four non-overlapping case–control series of Northern European ancestry, which have been the subject of previous studies27,30,34 and are listed in Supplementary Table 1. Briefly, the UK GWAS was based on 698 cases (481 male mean age 46 years) ascertained through the INTERPHONE study27. Individuals from the UK: Birth Cohort (n = 2,000) served as a source of controls. The US GWAS was based on 1,283 cases (786 males, mean age 47 years) ascertained through the MD Anderson Cancer Center, Texas, between 1990 and 2008. Individuals from the Cancer Genetic Markers of Susceptibility (CGEMS), n = 2,240, were ascertained as controls27. The French GWAS study ascertained 1,409 patients with glioma ascertained through the Service de Neurologie Miseante, Centre Hospitalier Paul-Laprade, Paris. The controls (n = 1,213) were ascertained from the SUMMIT (Study Utilizing Multimodal Imaging of Tumors) study of 1,273 healthy subjects (mean aged 55-90 years; mean age 56-66 years)29. The German-GWAS ascertained 990 patients who underwent surgery for a glioma at the Department of Neurosurgery, University of Bonn Medical Center, between 1996 and 2009. Controls subjects were taken from three population studies: KORA (Cooperative Health Research in the Region of Augsburg; n = 488; ref. 30); PONSIG (Population Genic: Cohort n = 670; ref. 48) and from the HUte Haidler Kiel study (n = 384; ref. 48).

Replication genotyping. For replication we made use of DNA from 1,689 glioma cases recruited as an ongoing UK study of primary brain tumours (National Brain Tumour Trust). Controls were healthy individuals that had been recruited to the National Study of Childhood Cancer Genes79 and the European Lung Cancer Prognostication Study80. All cases and controls were UK residents and had self-reported European ancestry. Controls reported no personal history of cancer at the time of ascertainment. Genotyping of the GWAS data was performed using Illumina Human550v1.1 BeadChip (rs278994, n = 119,407; ref. 81) and rs2237302 (rs1013564; n = 82,053) and rs1985162 were performed using competitive allele-specific PCR (CAAS) chemistry (UCG, Herlev, Denmark). UK primer sequences detailed in Supplementary Table S1). Conditions used are available on request. Glioma risk for this SNP was 3% in 95%. To ensure quality of genotyping in all arrays, at least two negative controls and at least two duplicates (showing a concordance > 99%) were genotyped. For SNPs with MAF < 3%, at least two known heterozygotes were included per genotyping plate, to aid genotyping.

Statistical and bioinformatic analysis. Data were imputed for all samples for over 10 million SNPs using MACH21 v.1.06 (ref. 40) and software in the 1000 Genomes Project (Phase 1 integrated release, March 2012 v.433) and the UK Biobank data (ALL/AF/ACG, KAG Yakutia, CoEase, and Turkmen). DGA1/GA5MQ0G/EAG/DGA5QMQ0G/EAG500MR0G000A9, studies only) as reference panels (Supplementary Table S1). Genotypes were aligned to the positive strand of both, imputation and genotyping. Imputation was conducted separately for each scan in which neither imputation or GWAS data sets were used in a common set of 423,149 SNPs. Poorly imputed SNPs defined by an imputation score (R2) < 0.70 and Hardy-Weinberg equilibrium (p < 0.05) were excluded from the analysis. Tests of association between imputed SNPs and glioma were performed under a polygenic dosage model in SUMMIT v.2.3 (ref. 40).

Coevolution for the GWAS data sets were inferred using Network (part of the EGenoNetworks.org suite of APIs) using —network —node 100000 —api 100000 —input —output —network —node 100000 —api 100000 —input —output. No significant coexpression was found using the default settings.

The significance of differential genotyping of cases and controls was evaluated using Q-Q plots of test statistics. The inflation factor was bias in the 90% non-significant SNPs as previously advocated28. Testing for secondary signals was carried out in SUMMIT, adjusting for the sentinel SNP using the ‘Bonferroni’ option. Visualisation of population ancestry was carried out in enea by monitoring query samples onto eigenvectors inferred from the 1000 Genomes Project populations (Supplementary Fig. 1). Meta-analysis of GWAS data sets under a fixed-effects model was undertaken in META v.1.8 (ref. 89) using the inverse-variance approach. Cochran’s Q statistic to test for heterogeneity and the P statistic to quantify the proportion of the total variation due to heterogeneity was calculated28. P-values < 0.05 are considered characteristic of large heterogeneity28. In addition, analyses stratified by glioma tumour histology and molecular characteristics were performed. All statistical P values were two sided.

Further analysis of individual variants in risk associated with glioma-risk SNPs were carried out using the method described in Plaschos et al.34 assessing the familial
risk of glioma to be 1.77 (ref. 51). Briefly, for a single allele (i) of frequency $p_i$, relative risk $R_i$ and log of the variance $V_{i}$ the risk distribution due to that allele is given by:

$$V_{i} = (1 - p_i^2)(1 - 2p_i) - (p_i^2 - 1) + E_i^2 = E_i^2$$

Where $E_i$ is the expected value of $i$.

For multiple risk alleles the distribution of risk in the population tends to the normal with variance:

$$\sum_i V_i$$

The total genetic variance ($V$) for all risk alleles that have been estimated to be

$$V = \sum_i V_i$$

LD matrixes were calculated in schools of 1,000 B (ref. 52) using UKGAS data and plotted using PLINK (ref. 13). LD bands were defined on the basis of haplotype recombination rate (HRR) as defined using the Oxford recombination hotspots and the lack of distribution of correlation intervals defined by Gueydon et al. 119. SNPs were annotated for functional effect using REGULOMDB.118 Haplotype v.2.3 (refs 53 and 119) and SNAP (v.3.15.0) the three servers make use of data from ENCODE.117 GERP conservation metrics, combined annotation-dependent depletion (CADDP).119保守度和PhyloP (v.2.0) scores. We searched for evidence of association with SNPs obtained through the L1000 panel and with associated COAD/PRAD risk in p-values and $\beta$-values with a correction for multiple testing. In this context, we found no strong evidence for association or for a significant risk. However, our findings highlight the importance of further investigation in this area.

References:


Acknowledgements
In the US, funding was provided to Cancer Research UK D020205, supported by the Bobby Moore Fund, the Wellcome Trust and the IE Fouding Medical Research Trust. K.B.S. is supported by a BBSRC studentship funded by the Sir John Rose Foundation. The National Forum from Cancer Research and the Cancer Research Network and we acknowledge the contribution of all clinicians and health-care professionals to the patients. The UK INTERPHONE study was supported by the European Union Fifth Framework Program Quality of Life and Management of Living Resources (QLK4-CT-1999-01758). The National Forum from Cancer Research and the Cancer Research Network and we acknowledge the contribution of all clinicians and health-care professionals to the patients. The UK INTERPHONE study was supported by the European Union Fifth Framework Program Quality of Life and Management of Living Resources (QLK4-CT-1999-01758). The National Forum from Cancer Research and the Cancer Research Network and we acknowledge the contribution of all clinicians and health-care professionals to the patients.
A.M. Lakhmir and M. Brandel for help in collecting data, and Y. Han for database support. For the Surname study, we are indebted to B. Haefliger (Basel), S. Ott and Dr. A. Miller-Fahs (Basel) for help with the acquisition of clinical data, and R. Mühlbrier (Basel) who provided technical support. The UK study utilized data and control genotyping data generated by the Wellcome Trust Case-Control Consortium. A full list of the investigators who contributed to the generation of the data is available from www.wtccc.org.uk. The UK-GWA study made use of control genotypes from the Cancer Genetic Markers of Susceptibility (CGEMS) prostate and breast cancer studies. A full list of the investigators who contributed to the generation of the data is available from http://cgems.nimh.nih.gov. French cohorts were taken from the SU.VI.MAX study. The German GWA study made use of genotyping data from three population control sources: KORA-gus, The Heinz Nixdorf RECALL study and IONPEN. The HSNP cohort was established with the support of the Heinz Nixdorf Foundation. Francois de Grouchy received support from the IONPEN Programme of the University of Bonn, Germany. We are grateful to all investigators who contributed to the generation of this data set. UK Biobank data generation and access were organized by the UK Biobank consortium and funded by the Wellcome Trust. The results here are in part based on data generated by the TCGA Research Network, http://tcga.cancer.gov.

Author contributions
S.H.L. and B.R. designed the study. D.J.L. drafted the manuscript with contributions from all other authors. B.E. performed statistical and bioinformatics analysis. P.B. performed laboratory management and reverse replication genotyping and sequencing of UK cases and controls by S.H.L. A.M. and T.R. in the UK, A.S. M.M. S.P. and R.B. developed patient recruitment, sample acquisition and performed sample collections of cases in the UK, M.B. developed protocols and patient recruitment in Germany, M.S. H.E.W., S.S. and J.S. developed patient recruitment and blood sample collection, M.S. assessed DNA isolates and arranged and performed case and control ascertainment and supervision of DNA extractions. S. Heimes, S. Heilner and K.G. performed experimental works in France, S.S. and J.S.S. developed patient recruitment, J.S.S. and A.S. performed reverse genotyping and K.G. performed the immunohistochemical analysis. M. Lafarge performed laboratory management and reverse genotyping of the French samples. All authors contributed to the final paper.

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How to cite this article: Kramer, S. et al. Genome-wide association study identifies multiple susceptibility loci for glioma. Nat. Genet. 46, 637–643, doi: 10.1038/ng.2730 (2014).

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Genome-wide association study reveals specific differences in genetic susceptibility to glioblastoma and non-glioblastoma

Beatrice Melin*, Jill Barnholtz-Sloan†, Margaret R. Wrensch*, Christoffer Johansen‡, Dora Il'yasova§, Ben Almersdottir, Quinn Ostrom, Karim Labroche, Yanwen Chen, Georgina Armstrong, Yanhong Liu, Jeanette E Eckel-Passow, Paul A Drcker, Marianne Ledousèche, Anste Di Stefano, Peter Broderick, Pilar Galan, Karina Mohkan, Jean-Yves Delattre, Konstantinos Goulias, Johannes Schramm, Minouk I. Schaefer, Sarah J Fleming, Stefan Herms, Stefanie Heilmann, Markus M Nothen, Heinz-Erich Wichmann, Stefan Schreiber, Anthony Swerdlow, Mark Latroop, Matthias Simon, Marc Sanson, Ulrike Andersson, Preethe Rejrojanun, Stephen Chenock, Marthe Livet, Zhuoming Wang, Meredith Yeager, (on behalf of the glioma scan group, John Winc,
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<th>Altitude</th>
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<th>MRE</th>
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Table 1: Association statistics for top SNP at each of the newly reported glioma risk loci. Associations at P=5x10^-8 are highlighted in bold. Odds ratios were derived with respect to the risk allele and highlighted in bold. Risk allele frequency (RAF) is according to European sample from 1000 genomes project. The RPD column indicates the average imputation info scores across the studies in the discovery phase, with a score of 2 indicating the SNP is directly genotyped in all studies.

Figure 2: Forest plots of effect size and direction for the new SNPs associated with glioma risk.

rs12752552 (1p31.3)
Figure 3: Regional plots of discovery-phase association results, recombination rates and chromatin state segmentation tracks for the new glioma risk loci. 1p31.3 (GBM)

![Regional plots of discovery-phase association results, recombination rates and chromatin state segmentation tracks for the new glioma risk loci. 1p31.3 (GBM).]

Figure 4: Relative impact of SNP associations at known and newly identified risk loci for GBM and non-GBM tumours.

![Relative impact of SNP associations at known and newly identified risk loci for GBM and non-GBM tumours.]

Anna Luisa Di Stefano
Supplementary Figure 1: Q-Q plots of association statistics for the GWAS datasets pre- and post-imputation.

A. GiCC (Genotyped (unadjusted)), B. GiCC Genotyped (adjusted for first 2 PCs), C. GiCC Genotyped and Imputed (unadjusted), D. GiCC Genotyped and Imputed (adjusted for first 2 PCs), E. UCSC (genotyped (unadjusted)), F. UCSC Genotyped (adjusted for first 4 PCs), G. UCSC Genotyped and Imputed (unadjusted), H. UCSC Genotyped and Imputed (adjusted for first 4 PCs), I. MDA Genotyped and Imputed (unadjusted), J. MDA Genotyped and Imputed (adjusted for first 4 PCs), K. NHGRI genotyped and imputed (unadjusted), L. NHGRI genotyped and imputed (adjusted for first 4 PCs).

<table>
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Supplementary Table 1: Summary characteristics of the GiCC substudies

<Table content...>
Supplementary Figure 1: PCA plots of the GWAS datasets

(a) GICC study

Supplementary Figure 3: Forest plots of effect size and direction for the SNPs from previously reported loci associated with glioma risk.

rs1920116 (3q26.2)
### Supplementary Table 2: Best association signals from previously published risk loci discovered in European populations. Shown for each region are the GWAS tagSNP as well as the most associated variant within a 500kb window in the imputation and the associated odds ratio and p-value associated with each odds ratio derived with respect to the allele underlined and highlighted in bold. Shown are the association statistics for previously published SNPs in each locus, as well as the most associated SNP in this study if differing from the published SNPs. Risk allele frequency (AF) is according to European samples.

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**Supplementary Table 4: Individual variance in risk associated with glioma SNPs.** For each glioma risk locus, the relative risk per risk allele of the highest associated SNP is given. Risk allele frequency (RAF) is according to European samples from 1000 genomes project.
Figure 1: Genome-wide meta-analysis $P$-values ($-\log_{10}P$, y axis) plotted against their chromosomal positions (x axis).

(a) All glioma
4. SECTION 2-THERANOSTIC MARKERS

4.1 Detection, Characterization, and Inhibition of FGFR-TACC Fusions in IDH Wild-type Glioma

Glioblastoma multiforme (GBM) is among the most lethal and frequent primary brain tumors. Targeted therapies against common genetic alterations in GBM have not changed the dismal outcome of the disease (Weathers, 2014; Omuro, 2013).

The genetic background of glioblastoma is commonly characterized by the absence of the IDH mutation (94%), recurrent chromosomal abnormalities (7p gain, 10q loss, 13q loss), oncogenes amplifications (EGFR, CDK4) and oncosuppressors deletions (Brennan, 2013).

This same genetic background, notably the absence of the IDH mutation, may be recapitulated in a small percentage of lower grade gliomas (grade III and grade II), showing an aggressive clinical behaviour (Sanson, 2009).

As discussed in the introduction section, the recent update of the WHO classification of brain tumors dichotomized the classification and prognostication of gliomas according to the IDH status (Louis, 2016) and recognized a worse outcome common to the group of IDH wild-type gliomas independent of their grading.

Because of the failure of conventional therapies in the control of IDH wild-type gliomas, there is a major need for new druggable targets in this subgroup of patients.

In 2012, Singh et al. reported that a small subset of GBMs (3.1%; 3 of 97 tumors examined) harbours oncogenic chromosomal translocations that fuse in-frame the tyrosine kinase coding domains of fibroblast growth factor receptor (FGFR) genes (FGFR1 or FGFR3) to the transforming acidic coiled-coil (TACC) coding domains of TACC1 or TACC3, respectively (Figure 4.1) (Singh, 2012).
The FGFR-TACC fusion protein displays oncogenic activity when introduced into astrocytes or stereotactically transduced in the mouse brain. The fusion protein, which localizes to mitotic spindle poles, has constitutive kinase activity and induces mitotic and chromosomal segregation defects and triggers aneuploidy (Figure 4.1) (Singh, 2012).

Inhibition of FGFR kinase corrects the aneuploidy, and oral administration of an FGFR inhibitor prolongs survival of mice harbouring intracranial FGFR3-TACC3–initiated glioma (Figure 4.1.1) (Singh, 2012).

However, the full repertoire of the structural variants of FGFR-TACC fusions is incomplete as well as the genetic and phenotypic signature of FGFR-TACC positive gliomas. Clinical activity of specific inhibition with anti-FGFR therapies in patients harbouring this oncogenic alteration is also incomplete.
Figure 4.1 Adapted from Singh et al. 2012. FGFR3-TACC3 gene fusion.
A) Genomic fusion of FGFR3 exon 17 with intron 7 of TACC3. In the fused mRNA, exon 16 of FGFR3 is spliced 5' to exon 8 of TACC3. Solid black arrows indicate the position of the fusion-genome primers. B) Sanger sequencing chromatogram showing the reading frame at the breakpoint and putative translation of the fusion protein. T, threonine; S, serine; D, aspartic acid; F, phenylalanine; E, glutamic acid. C) Schematics of the FGFR3-TACC3 protein. Regions corresponding to FGFR3 or TACC3 are shown in red or blue, respectively. The fusion protein joins the tyrosine kinase domain of FGFR3 to the TACC domain of TACC3. D) Survival of glioma-bearing mice was tracked after intracranial implantation of Ink4A; Arf−/− astrocytes transduced with FGFR3-TACC3. After tumor engraftment, mice were treated with vehicle or AZD4547 (50 mg/kg) for 20 days (vehicle, n = 7 animals; AZD4547, n = 6; P = 0.001). E) FGFR3-TACC3 localizes to spindle poles, delays mitotic progression, and induces chromosome segregation defects and aneuploidy. (A) Confocal microscopy analysis of FGFR3-TACC3 (red) covering the spindle poles of a representative mitotic cell. α-tubulin, green; DNA [stained with 4',6-diamidino-2-phenylindole (DAPI)], blue.
Since 2013, in the collaborative setting with research group led by Dr. Lasorella and Dr. Iavarone, we have been able to perform the largest screening for this therapeutic target in up to 907 patients to date.

Results from first analysis in a dataset of 584 GBM and 211 grade II and grade III gliomas were published in Clinical Cancer Research in 2015 (Di Stefano, 2015). In this article:

- we confirmed that RT-PCR sequencing is a sensitive and specific method to identify \(FGFR-TACC\)-positive patients,
- we detected for the first time \(FGFR3-TACC3\) fusions in about 3% of \(IDH\) wild-type non-GBM (grade II and grade III) and we confirmed the frequency of 3% of \(IDH\) wild-type glioblastoma,
- we found that \(FGFR3-TACC3\) fusions are associated with uniform intratumor expression of the fusion protein
- we found that the presence of \(FGFR-TACC\) fusions are mutually exclusive of the \(IDH\) mutation, \(EGFR\) amplification, \(EGFR\) vIII variant and that it is associated with higher frequency of \(MDM2\) and \(CDK4\) amplifications, and
- we observed a clinical benefit in two \(FGFR3-TACC3\)-positive patients treated with a FGFR inhibitor.

The corresponding article is included in this section.

Next, we started three supplementary studies, based on the prospectical screening for \(FGFR-TACC\) fusions at Pitié-Salpetrière Hospital of new diagnosed \(IDH\)-wild type glioma cases.

This screening accounts for 907 analysed cases, of which 40 \(FGFR3-TACC3\) positive patients have been identified so far, corresponding to the largest series ever identified.

Expansion studies are focused on:
- an extensive characterisation of new FGFR3-TACC3 transcripts and molecular features, together with clinical and histological phenotypes in a larger repertoire of 40 FGFR3-TACC3 positive gliomas patients (Section 4.2)

- an exploratory study on the presence of new acquired mutations associated with resistance in one FGFR3-TACC3 positive patient recurring after specific anti-FGFR therapy (Section 4.3)

- a “phase Ib/phase II clinical trial testing efficacy and tolerability of an anti-FGFR therapy-AZD4547-in glioma patients harbouring FGFR-TACC fusions at recurrence” (NCT02824133, TARGET trial, PI Prof Marc Sanson) which is the first world-wide phase II trial, testing the efficacy of the anti-FGFR therapy AZD4547 in this selected subgroup of patients. The TARGET trial started in September 2015, which included 12 patients, and is now on the expansion phase. Preliminary results of this trial are not shown in this thesis.
Detection, Characterization, and Inhibition of FGFR-TACC Fusions in IDH Wild-type Glioma

Anna Luisa Di Stefano 1,2,3, Alessandra Fucci 1, Veronique Frattini 4, Marianne Labussiere 5, Karina Mokhtarian 1,2, Pietro Zoppoli 1, Yannick Marie 2, Aurelie Bruno 1, Blandine Bossiesser 5, Marine Girv 1, Julián Savatovsky 4, Mehdi Touati 1, Hayat Belal 1, Aurelie Kammoun 1, Ahmed Idbaih 1, Caroline Houllier 4, Ferg R. Lucy 4, Jean-Charles Soria 1, Josef Tabernero 4, Marics Esil 1, Rosina Pieterra 1, Stephen Yip 1, Kevin Petrecca 1, Jennifer A. Chan 1, Gaetano Finocchiaro 1, Anna Lasorella 1, Marc Samson 2,3, and Antonio Iavarone 1,6

Abstract

Purpose: Oncogenic fusions consisting of fibriloadulterin growth factor receptor (FGFR) and TACC are present in a subgroup of glioblastoma (GBM) and other human cancers and have been proposed as new therapeutic targets. We analyzed frequency and molecular features of FGFR-TACC fusions and explored the therapeutic efficacy of inhibiting FGFR kinase in GBM and grade II and III glioma.

Experimental Design: Overall, 795 gliomas (544 GBM, 85 grade II and III with wild-type and 126 with IDH1/2 mutation) were screened for FGFR-TACC breakpoints and associated molecular profile. We also analyzed expression of the FGFR3 and TACC3 components of the fusions. The effects of the specific FGFR inhibitor IN-412754493 for FGFR-TACC3-positive glioma were determined in preclinical experiments. Two patients with advanced FGFR3-TACC3-positive GBM received IN-412754493 and were assessed for therapeutic response.

Results: Three of 85 IDH1/2 wild-type (3.6%) but none of 126 IDH1/2-mutant grade II and III glioma harbored FGFR3-TACC3 fusions. FGFR-TACC3 rearrangements were present in 17 of 544 GBM (3.1%). FGFR3-TACC3 fusions were associated with strong and homogeneous FGFR3 immunostaining. They are mutually exclusive with IDH1/2 mutations and FGFR amplification, when they co-occur with IDH4 amplification. IN-412754493 inhibited growth of glioma cells harboring FGFR3-TACC3 in vitro and in vivo. The two patients with FGFR3-TACC3 rearrangements who received IN-412754493 manifested clinical improvement with stable disease and minor response, respectively.

Conclusions: FGFR sequencing is a sensitive and specific method to identify FGFR3-TACC3-positive patients. FGFR-TACC3 fusions are associated with uniform immunohistochemical expression of the fusion protein. The clinical response observed in the FGFR3-TACC3-positive patients treated with an FGFR inhibitor supports clinical studies of FGFR inhibition in FGFR3-TACC3-positive patterns. Clin Cancer Res 21(19): 4907-18; 2015 AOCR.

© 2015 American Association for Cancer Research. See related commentary by Almena and Buss, p. 3105.
Translational Relevance

This article reports an unbiased screening assay for FGFR1-TACC3 fusion genes in gilbertoma that reveals the high prevalence of variants that are generated by FGFR1-TACC3 chromosomal translocation in human cancer. FGFR1-TACC3 fusions occur in grade II and III gliomas harboring wild-type IDH1 with frequency similar to glioblastoma (GBM), thereby providing a clue to the aggressive clinical behavior of this glioma subtype. The molecular characterization of fusion-positive gliomas revealed that FGFR1-TACC3 is mutually exclusive with IDH1 amplification but co-occurs with CDK1 amplification. FGFR1-TACC3-positive glioma displays strikingly uniform and strong expression of the fusion protein at the single-cell level. Preclinical experiments with FGFR3-TACC3-positive glioma cells treated with the thiol-activated growth factor receptor (FGFR) inhibitor IN-42754493 showed strong antitumor effects, and treatment of two patients with recurrent GBM harboring FGFR1-TACC3 resulted in clinical improvement and radiologic tumor reduction. These findings validate the treatment with FGFR inhibitors of patients with gliomas harboring FGFR1-TACC3 chromosomal translocations.

(3, 4). Underlying biologic features, including infiltrative growth behavior, intrinsic tumor heterogeneity, and adaptive resistance mechanisms, coupled with the unique challenges of interventional locoregional treatment, present significant problems in effective management. Despite surgery and chemoradiotherapy, most patients rapidly recur and no effective treatment options are available at that stage. Besides GBM, which features the highest grade of malignancy among gliomas (grade IV), lower grade gliomas, which include grades II and III, are a heterogeneous group of tumors in which specific molecular features are associated with divergent clinical outcomes. The majority of grade II and III gliomas (but only a small subgroup of GBMs) harbor mutations in IDH1 genes (IDH1 or IDH2), which confer a more favorable clinical outcome. Conversely, the absence of IDH mutations is associated with the worst prognosis (5).

We have recently identified FGFR1-TACC3 gene fusions (mostly FGFR1-TACC3 and rarely FGFR1-TACC3) as an example of highly oncogenic and recurrent gene fusions in GBM. The FGFR1-TACC3 fusions that have been identified so far include the tyrosine kinase (TK) domain of FGFR and the coiled-coil domain of TACC proteins, both necessary for the oncogenic function of FGFR1-TACC3 fusions. We also treated tumor dependency on FGFR1-TACC3 fusions in preclinical mouse models of FGFR1-TACC3 glioma and observed marked antitumor effects by FGFR inhibition (6). After our report, FGFR1-TACC3 fusions have been identified in pediatric and adult gliomas, bladder carcinoma, squamous lung cancer, and head and neck carcinoma, thus establishing FGFR1-TACC3 fusions as one of the chromosomal translocations most frequently found across multiple types of human cancer (6-15).

From a mechanistic standpoint, we discovered the unexpected capacity of FGFR1-TACC3 fusions to trigger aberrant chromosome segregation during mitosis, thus initiating chromosome instability (CIN) and aneuploidy, 2 hallmarks of cancer. However, we still have an incomplete understanding of the full repertoire of the structural variants of FGFR1-TACC3 fusions occurring in GBM and lower grade gliomas. Furthermore, it remains unknown whether FGFR1-TACC3 fusions mark distinct grades of gliomas and GBM subtypes.

To date, 8 variants of the FGFR1-TACC3 fusion have been reported that mostly differ for the breakpoint in the TACC3 gene (6-15). Because of the close proximity of FGFR1 and TACC3 (the 2 genes map at a distance of 70 kb on chromosome 4p16.3), detection of FGFR1-TACC3 rearrangements by FISH is not a feasible option with the currently available methods. Here, we report a novel screening method for FGFR1-TACC3 fusions that include a RT-PCR assay designed to identify the known and novel FGFR1-TACC3 fusion transcripts, followed by confirmation of the in-frame breakpoint by Sanger sequencing. Using this assay, we have analyzed a dataset of 368 GBMs and 211 grade II and III gliomas. A clonal question with fundamental clinical relevance for any novel candidate target mutation is the frequency of the alteration in the cancer cell population, thus discriminating between a clonal or subclonal origin of the mutation. In fact, GBM is characterized by a formidable degree of subclonal heterogeneity, whereby neighboring cells display amplification and expression of different receptor tyrosine kinase (RTK) coding genes (16-19). This notion poses major therapeutic challenges for targeting any individual RTK will result, at best, in the eradication of a limited tumor subclone. In this study, we determine that brain tumors harboring FGFR1-TACC3 fusions manifest strong and homogeneous intratumoral expression of the FGFR1 and TACC3 component intercalated in the fusion points, when matched by immunostaining. We also report a significant clinical benefit following treatment with a specific inhibitor of FGFR1-2 in 2 patients with GBM who harbored FGFR1-TACC3 rearrangement.

Materials and Methods

Patients and tissue samples

This study includes a cohort of 749 consecutive patients with histological diagnosis of gliomas from 5 institutions. Forty-nine recurrent gliomas from Mie Sapporo hospital and one recurrent glioma from the University of Calgary (Calgary, Canada) were also included. A summary of the patient cohort is provided in Table 1. Tumor specimens, blood samples, and clinicopathologic information were collected with informed consent and relevant ethical board approval in accordance with the tenets of the Declaration of Helsinki. For the samples from the Paris-Saint-Lazare Hospital, clinical data and follow-up are available in the neur-oncology database [Cerebrotech, C.H. Pitie-Salpetriere, Paris].

Two recurrent patients with GBM harboring FGFR1-TACC3 were enrolled in the dose-escalation part of NCT04154609 trial (NCT01962532) at the Gustave Roussy Institute (Paris, France).

Identification of fusion transcripts and analysis of genomic breakpoints

Total RNA was extracted from frozen tissues using TRIzol (Invitrogen) according to manufacturer’s instructions. Two to three hundred nanograms of total RNA was transfected with the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) or SuperScript II (Invitrogen). RT-PCR was performed using Assay-Primed Tag DNA Polymerase (Invitrogen). Primer pairs used for the FGFR3-TACC3 fusion screening were: FGFR3/12S-FW: 5'-CGGCTGAGGCTGAGAACGCGG-3' and FGFR3/12S-BV: 5'-AACGCTGCGGAGGCTGAGA-3', amplification conditions were 94°C 3 minutes (94°C 30 seconds/66°C 30 seconds/66°C 1
minute 60 seconds) for 35 cycles, 60°C for 2 minutes. FGFRI
-TACC1 fosmons were amplified with FGG1ex 6-F2, 5/-ECC
TGGCCAGAGCGATTCG-3’ and TACC3 5/-B3, 5/-CAGACT
CAACGCAGCGAG-3’ primers (54°C 30 seconds/95°C 20 se-
conds/65°C 1 minute 40 seconds for 35 cycles). PCR products
were subjected to sanger sequencing.

FGFR3-TACC1 transgenic background were analyzed in 6
FGFR3-TACC1-positive samples, 5 of which from the Pitt-Sal-
pitak Hospital and I from Montreal Neurological Institute
(Montreal, Canada). Three additional samples (5/22, TCGA
27-1833, and TCGA 06-4950) available from our previous study (25) were also included in the analysis. Fifty nanograms of gDNA
was used in the PCR reaction, performed with Accuprime
Ex Polymerase (Invitrogen) and PCR products were longer
sequenced. Primers used in genotyping were designed according to the breakpoint sequence in the mRNAs, the list of primers used are:
FGGR3ex 6-F2, 5/-ECCAGAGCGATTCG-3’ (PCR ampli-
samples 3046, 4373, 4872, 4875, 4458, 5682, OPK-14, 06-4950, 27-1833
and sequencing samples 4048, 4137, 4326, 4894, 5682, OPK-14,
06-4950, 27-1833). FGGR3ex 6-F2, 5/-GACCCTCGCGCCTCG-
CAGG-3’ (PCR and sequencing sample 3046). TACC3ex 9N
5/-AAGGGCACCCAGACAGAA-3’ (PCR and sequencing sam-
ple 4051 and 4204). TACC3ex 9N, 5/-TCCAGAG
GGATCCAGATCCAGTGC-3’ (sequencing samples 4373 and 4458).
TACC3ex CAGAGCACCAAGGCAGCCCTCAGC-3’ (PCR
samples 4373, 4872, 4894, 4875). TACC3ex CAGAGCAC
CGAGCCCTCAGC-3’ (PCR and sequencing samples 27-1833
and 2037). PCR conditions were: 94°C 30 seconds/60°C 30 seconds/68°C 2 minutes 30 seconds for 40 cycles. For amplifications performed with the primer TACC
ex CAGAGCACCAAGGCAGCCCTCAGC-3’, the program was
94°C 30 seconds/60°C 15 seconds/68°C 2 minutes 30 seconds
for 40 cycles.

Quantification of FGFRI and TACC3 transcripts by GEM

The relative expression of FGG1 and TACC3 transcripts were
analyzed by qRT-PCR. Primer pairs with comparable efficiency of amplification
were identified, and efficiency was assessed using serial dilutions of cDNAs (25) prepared from DAPI-stained 46;X/45;X/46,XX/45,XX/46.XX
in one experiment. The effects of FGFRI and TACC3 expression on cell growth were
assessed by qRT-PCR.

Table 1. Frequency of FGFR3-TACC1 fusions in GBM and grade II-III glioma.

<table>
<thead>
<tr>
<th>Tumor sample source</th>
<th>Cases (GBM)</th>
<th>Detected fusions</th>
<th>Immunostaining FGFR3 positive/sample analyzed</th>
<th>Immunostaining TACC1 positive/sample analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitt-Salpitak Hospital</td>
<td>380</td>
<td>9</td>
<td>1/30</td>
<td>1/30</td>
</tr>
<tr>
<td>British Neurological Institute</td>
<td>85</td>
<td>5</td>
<td>1/15</td>
<td>1/15</td>
</tr>
<tr>
<td>University of Calgary</td>
<td>60 - 185</td>
<td>2 + 185</td>
<td>1/10 + 1/185</td>
<td>1/30 + 1/185</td>
</tr>
<tr>
<td>Montreal Neurological Institute</td>
<td>9</td>
<td>0</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>University of British Columbia</td>
<td>3</td>
<td>0</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Total</td>
<td>584 (100%)</td>
<td>17 (29%)</td>
<td>17 (29%)</td>
<td>17 (29%)</td>
</tr>
</tbody>
</table>

Note: Distribution of the FGFR3-TACC1 fusions in GBM (red) and lower grade gliomas (blue) samples were according to the institution of origin. The table reports a number of cases analyzed, number of tumors harboring FGFR3-TACC1 fusions (blue), and results of FGFR3 immunostaining. Lower grade glioma samples are further stratified according to IDH status and WHO grade. The respective frequency of FGFR3-TACC1 in GBM, grade II-III glioma (red) and (blue) and IDH mutant (red) gliomas is reported in parentheses.

*5 cases, GBM.

1Recent case from the University of Calgary.

2Total number of cases in the current study is 584.

3Mutations in both cases were observed in GBM (red) and lower grade glioma (blue).

4Information on FGFR3 and TACC1 expression was not available for all cases.

5Information on FGFR3 and TACC1 expression was not available for all cases.

6Information on FGFR3 and TACC1 expression was not available for all cases.
defined according to the absence of IDH1-R132H immunohistochemical and/or mutations in IDH1 and IDH2 genes. IDH1 promoter status was determined by the same technique in 277 samples (23). Hypomethylation of the IDH1 promoter was tested in 242 samples by bisulfite pyrosequencing (26). The presence of FGF23 was evaluated by RT-PCR in 118 samples using FGF23-FW 5′-CTCGAGGCAGGGACGGATACACG-S′ and FGF23-RW 5′-CTCGACCATGCGAGTCTGTTGTTT-S′ primers (25).

Copy number variations and miRs have been performed on 192 tissue samples using CGH arrays using BAC arrays (n = 187), Agilent 4 × 180 K (n = 2), NimbleGen 4 × 720 K (n = 2), and Agilent 8 × 60 K (n = 1). Results were normalized using control DNA from matched blood samples as previously described (28). Additional analysis of 193 tumor specimens were performed by SNP array, using Illumina Omni (n = 110), Illumina HumanCore (n = 32), Illumina T06 (n = 27) or Illumina 610 K (n = 24), as previously described (27). Array processing was outsourced to Integrative. Raw copy number numbers were estimated at each of the SNP and copy number markers. Bioconductor software packages were then used to segment copy number data. Segments were mapped to high general assembly (28). Copy number alterations magnitudes called log R ratio (LRR) were classified using simple thresholds: deletion (LRR ≤ −1), loss (−1 < LRR ≤ −0.2), gain (0.2 ≤ LRR ≤ 1), or amplification (LRR ≥ 1) according to default Nexus 7.5 software. For additional 56 gliomas, 10x low was assessed on tumor and blood DNA for microsatellite analysis, whereas amplification of FGF23, IDH2, and DCC and deletion of CDKN2A gene were determined by qPCR, as previously reported (29, 30).

The molecular profiles obtained in The Sapienza2012 dataset were combined with those available in The Cancer Genome Atlas (TCGA) data portal. TCGA GBM segment copy number variation profile was downloaded from the UCSC Cancer Genomics Browser (11). Copy number variations (CNV) were measured using the MyPhenome Genome-Wide Human SNP Array 6.0 platform from the Broad TCGA genome characterization center (32). Raw copy number numbers were estimated at each of the SNP and copy number markers. Circular binary segmentation was then used to segment the copy number data (28). Segments were mapped to high general assembly at Broad.

For CNV analysis of the regions across FGF23 and TACC3 genes, we considered samples for which RNAseq and CNV data were available. For RNAseq performing of FGF23 and TACC3 fusion had been performed. Overall, USCI GBM (all with wild-type IDH1 genotype) satisfied these criteria. Among them, 230 were FGF23-TACC3 fusion, whereas 33 were FGF23-TACC3-negative. The CNV magnitudes, called LRR, were classified using the following thresholds: deletion (LRR ≤ −1), loss (−1 < LRR ≤ −0.2), gain (0.2 ≤ LRR ≤ 1), or amplification (LRR ≥ 1), according to the MyPhenome software (35). The analysis of the genomic regions encompassing FGF23, IDH1, IDH2, CDKN2A, and TACC3 genes, was performed to evaluate their CNV. Gene expression status was inferred according to Ibrumman and colleagues (14). The frequency of the aberrations of these genes in FGF23-TACC3-positive and -negative samples were calculated and the obtained data were then combined with the Osaka-Sapienza Hospital database.

Statistical analysis

Differences in the distribution of continuous variables were assessed using the Fisher exact test. The P-values were adjusted for multiple testing according to Benjamin and Hochberg false discovery rate (FDR). A P-value ≤ 0.05 (2-sided) was considered to be statistically significant.

Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Patients who were still alive at the last follow-up were considered as censored events in the analysis. Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Patients who were recurrence-free at the last follow-up were considered as censored events in the analysis. Survival curves were calculated by the Kaplan-Meier method and differences between curves assessed using the log-rank test. A log-rank test P ≤ 0.05 (2-sided) was considered to be statistically significant.

Cell culture and cell growth assay

GIC-1123 gliomaspheres were cultured in murine neural medium (Fremington) supplemented with B27, N2 (Fremington), LIF, and EGF (20 ng/mL, PeproTech). Mitotic arrestin M6A-Akt/7 were cultured in DMEM supplemented with 2% FBS. Cells were seeded at 1000 cells per well in a 96-well plate and to 1 × 10^6 cells were plated and treated with IDX-47754893 (Active Biochem, PA-1278) or dimethyl sulfoxide (DMSO) in 1% FBS. Data were recorded after 72 hours, cell viability was assessed using the MIT assay. Data are mean ± SEM of 6 replicates. Experiments were performed 3 times.

Subcutaneous xenografts and drug treatment

GIC-1123 cells (5 × 10^3) were injected subcutaneously in the flank of athymic nude (nude) mice (Charles River Laboratory). Mice carrying about 200 mm3 subcutaneous tumors were randomized to receive 12 mg/kg IDX-47754893 (Active Biochem, PA-1278) or dimethyl sulfoxide (DMSO) in 1% FBS. Tumor diameters were measured with calipers and tumor volumes estimated using the formula: 0.5 × length × width^2. Data are mean ± SD of 5 mice in each group. Mice were sacrificed when tumors in the control group reached the maximum size allowed by the IACUC Committee at Columbia University (New York, NY).

MRI imaging and evaluation of clinical response to IDX-47754893

Baseline and follow-up imaging measurements were performed on 1.5 T Siemens MRI scanner using T1-weighted images before gadolinium injection. Axial or 3D FLAIR (fluid-attenuated inversion recovery), dynamic susceptibility contrast perfusion (0.1 mmol/L of gadobutrol), and 3D T1-weighted images after gadolinium injection. Tumor response was assessed according to the RECIST criteria (33). Contrast-enhancing lesion volume was assessed with the help of a semiautomated volumetry tool (Segment3D), based on shape detection and thresholding, with control and manual correction of edges when necessary. Gross or partial necrosis (34) or residual enhancer portion of the lesion may be affected by operator subjectivity; we included them both for volumetric and axial measurements.

DSC (dynamic susceptibility contrast) perfusion datasets were processed with vendor’s software suite (Neuroperfusion, Philips), including co-registration and rCBV (relative cerebral blood volume) parametric maps generation with 3 different algorithms (Geometric, Variogram, Binning, Arterial Input Function-based deconvolution and model-free).
Results
Detection of FGFR1–TACC3 and FGFR3–TACC3 fusions in GBM and grade II–III glioma

To determine the frequency and molecular features of FGFR1–TACC3 fusions in human patients with glioma, we screened a cohort of 584 GBM and 231 grade II–III glioma tumors at St. Vincent’s Medical Center (Table 1). One-hundred eight were grade III (49 IDH1 wild-type, 52 IDH2 mutant, and 7 IDH2 mutant) and 103 were grade II (46 IDH1 wild-type, 63 IDH2 mutant, and 4 IDH2 mutant). We also established the IDH mutation status of 333 GBM and determined that 301 had wild-type IDH1 (2 and 30 were mutated at codon 132 of IDH1). We designed a RT–PCR assay for the detection of all known and possibly new variants of FGFR1–TACC3 and FGFR3–TACC3 fusions that include the mRNA sequence coding for the key FGFR-TEK and TACC domains required for the oncogenic activity of the fusion protein (Figs. 1 and 2A–D). Overall, we found 20 tumors with an FGFR1–TACC3 fusion, of which 17 were GBM (70% positive) and 3 lower grade gliomas harboring wild-type IDH2 genes (3.5% positive). The size of the FGFR1–TACC3 RT–PCR amplicons ranged from 928 bp (for FGFR1ex10–TACC3ex3) to 1,706 bp (for FGFR1ex8–TACC3ex1). The FGFR1–TACC3 fusion was detected in one grade II IDH1 wild-type glioma (Fig. 1). Conversely, we did not find any IDH1/2-mutant gliomas harboring FGFR1–TACC3 fusions (P = 0.02). Sanger sequencing of the fusion amplicons revealed that each FGFR1–TACC3 DNA joined in frame the sequence coding for the entire TK domain upstream of TACC–coding sequences that invariably include the coiled-coil TACC domain (Fig. 1). However, we detected a notable variability among FGFR1–TACC3 fusion isoforms, whereby 3 of the identified variants occurred only in individual cases (Fig. 1). Furthermore, 5 fusion transcripts emerged as new variants that have not been reported before in human cancer (marked in red in Fig. 1). Next, we designed a RT–PCR primer to map the genomic breakpoints coordinates for 9 FGFR1–TACC3–positive samples for which gDNA was available (Supplementary Figs. S1 and S2). We successfully reconstructed the genomic breakpoints by Sanger sequencing and found that they differ for each of the 9 positive cases. Interestingly, even cases harboring the same (FGFR1–TACC3 transcript fusions (19/45) and 7/47) joining exons 17 of FGFR1 to exon 6 of TACC3 to 54/65 and 43/73 joining exon 17 of FGFR3 to exon 6 of TACC3 to 54/65 and 47/1855 joining exon 17 of FGFR1 to exon 11 of TACC3 had different genomic breakpoints (Supplementary Fig. S2). Taken together, the above findings indicate that the noticeable variability among FGFR1–TACC3 fusion transcripts and genomic breakpoints is efficiently resolved by the RT–PCR screening assay.

Immunohistochemical analysis of FGFR3–TACC3-positive tumors
We analyzed the expression of the FGFR3 fusion protein by IHC or IF using an antibody that recognizes the N-terminal region of FGFR3 (FGFR3-N) in 12 GBM and 3 lower grade gliomas harboring FGFR3–TACC3 fusions for which sufficient tissue was available. Interestingly, each of the 15 positive tumors but none of those that had scored negative in the RT–PCR assay displayed strong positivity for FGFR3 in the vast majority of tumor cells but...
not endothelial cells throughout the analyzed tumor section (Fig. 2A–I). Notably, IF using an antibody that recognizes an epitope at the C-terminus of TACC3, which is inevitably retained within FGFR3–TACC3 variants (TACC3-C), reproduced the staining pattern of the FGFR3-V antibody in FGFR3–TACC3-positive tumors. Conversely, negative or very weak staining was obtained in FGFR3–TACC3-positive tumors with antibodies recognizing the regions of FGFR3 (FGFR3 C-terminal region, FGFR3-C) and TACC3 (TACC3 N-terminal region, TACC3-N). Consequently, FGFR3–TACC3 fusion proteins (Supplementary Fig. S3A) consistently quantifiable IF-PCR of GBM harboring FGFR3–TACC3 fusions showed that the expression of the N-terminal coding region of FGFR3 and the C-terminal coding region of TACC3 (which are included in the fusion gene) is markedly higher than the expression of the C-terminal coding region of FGFR3 and the N-terminal coding region of TACC3, which are excluded from the fusion transcripts (Supplementary Fig. S3B). We also analyzed one recurrent GBM from a patient whose tumor had been found positive for FGFR3–TACC3 at the initial diagnosis and who had received after concurrent radiotherapy and temozolomide treatment. The recurrent tumor retained the same FGFR3–TACC3 fusion gene and protein that was present in the untreated GBM as determined by RNA sequencing and FGFR3 IF, respectively (Supplementary Fig. S3D). Although this requires additional validation, the retained uniform positivity for FGFR3 in this recurrent GBM suggests that targeting the FGFR3–TACC3 fusion protein at eclipse is a valid therapeutic strategy.

Clinical and molecular characteristics of glioma patients with FGFR3–TACC3 fusions

Clinical and molecular profiling data were available for 591 patients, including 380 GBM (9 with FGFR3–TACC3 fusions) and 211 lower grade gliomas (3 with FGFR3–TACC3 fusions). Of these 12 patients, 5 are males and 7 females, aged 48 to 92 years (median = 61 years). We sought to determine the molecular
prolonged FGFR3-TACC3-positive gliomas. To do so, we combined the analysis of CNVs and somatic mutations of key GBM genes in one dataset with the SNP66 high density genomic array analysis of 138 TCGA-derived GBM samples fully associated for FGFR3-TACC3 fusion genes (for RNA-seq) and single-cell PCR analysis of these samples had revealed that 3% of them harbor FGFR3-TACC3 fusions (ref. 6). Patients with FGFR3-TACC3 fusions displayed unique characteristics (Table 2). FGFR3-TACC3 fusions were mutually exclusive with EGFR amplification (9 of 16 vs. 166 of 411, P = 0.0064, TDR; a value corrected for multiple comparisons = 0.0031) and showed a clear trend against the presence of the PCD17 transcript variant (9 of 16 vs. 37 of 213; P = 0.031). Conversely, FGFR3 amplification was significantly more frequent in FGFR3-TACC3-positive tumors (7 of 16 vs. 41 of 408, P = 0.006; TDR; a value corrected for multiple comparisons = 0.0019). A less significant association of FGFR3-TACC3 fusions was also seen with amplification of MYC (1 of 16 vs. 24 of 408, P = 0.0164, TDR; a value corrected for multiple comparisons = 0.0024) and with gain of chromosome 10q, loss of chromosome 10p, and mutation of the MGMT promoter (Table 2). When compared with the SW wild-type patient population of grade II and III gliomas (Table 2) and glioblastoma, there was no significant difference in PFS or OS between patients positive or negative for FGFR3-TACC3 (Supplementary Fig. S4A and S5B).

Finally, we sought to establish whether the CNV analysis of the FGFR3 and TACC3 genomic loci could be used to predict positivity for FGFR3-TACC3 fusions. The analysis of high-density SNP66 arrays of the 135 GBM samples from TCGA revealed that 13 samples displayed different degree of copy number gains encompassing the FGFR3 and TACC3 loci (Supplementary Fig. S6). However, none of them harbored FGFR3-TACC3 fusions. Conversely, the 3 FGFR3-TACC3-positive samples in the dataset harbored microsatellite events involving only the exons of the FGFR3 gene that are included in the fusion breakpoint. This finding suggests that any CNV analysis that is less accurate than high-density SNP arrays could fail to identify the genomic marks associated with the rare FGFR3-TACC3-positive cases.

Preclinical and clinical relevance of targeting FGFR3-TACC3 fusions

JN-42754693 is a potent, oral pan-FGFR tyrosine kinase inhibitor with IC50 values in the low nanomolar range for all members of the FGFR family. It has demonstrated potent anti-tumor activities in mouse models with FGFR alterations, including squamous non-small cell lung cancer, gastric cancer, breast cancer, hepatocellular cancer, endometrial and bladder cancers (34–35). To ask whether JN-42754693 is effective in targeting specifically FGFR3-TACC3-positive cells, we treated with JN-42754693 mouse astrocytes expressing FGFR3-TACC3, FGFR3-TACC3 containing a mutation that inactivates the kinase activity of FGFR3 (FGFR3-TACC3(1203D)) or the empty vector. We also studied the effect of JN-42754693 on human glioma stem cells GIC-1123 that harbor the FGFR3-TACC3 gene fusion (6). These experiments revealed that both mouse astrocytes and GIC-1123 that express FGFR3-TACC3 but not cells expressing the knockin fusion or the empty vector are highly sensitive to JN-42754693 inhibition by JN-42754693 with an IC50 of 3.05 and 1.35 nM/ml/1, respectively (Fig. 3A and B). Next, we tested whether oral treatment with JN-42754693 of orthotopic xenografts of human GIC-1123 affects tumor growth. Mice were randomized to receive vehicle or JN-42754693 (1 mg/kg), monitoring the in vivo results. JN-42754693 elicited a potent growth inhibition of GIC-1123 tumor xenografts (Fig. 3C and D) with a statistically significant tumor regression after 2 weeks (P value of the slope calculated from the trend starting point = 0.04). The above findings provide a strong foundation for further studies of patients with GBM harboring FGFR3–TACC3 rearrangements with JN-42754693.

Two patients with recurrent GBMs harboring FGFR3-TACC3 fusions were treated with JN-42754693 in a less-invasive phase I trial (36). Patients 1, 2, aged 52 years, underwent partial surgical resection of a right parietal GBM, followed by fractionated radiotherapy and concurrent temozolomide as first-line treatment (36). The RT-PCR sequencing analysis of the GBM specimen revealed positivity for the FGFR3-TACC3 fusion (FGFR3 exon7/TACC3 exon5, single copy). Supplementary Figs. S5 and S2) and the immunohistological findings using FGFR3 antibody on paraffin-embedded sections showed strong positivity in a large fraction of tumor cells (not shown). After 5 cycles of temozolomide, the patient presented with diseases and received a local treatment with JN-42754693. After 2 cycles of temozolomide, the patient presented with diseases and received a local treatment with JN-42754693. After 2 cycles of temozolomide, the patient presented with diseases (Fig. 4A) and was withdrawn from the study because of disease progression (Fig. 4A). At this time, the patient was enrolled in the JN-42754693 trial and received JN-42754693 (12 mg/kg administered in cycles of 7 days on and 7 days off treatment). After 3 weeks, the patient reported a marked clinical improvement (complete regression of lesions and headache). On MRI, the sum of product diameters (RANO criteria; Fig. 4B) and voluntary (Fig. 4C) measured without excluding sync and anatomic components showed disease stabilization. However, the tumor mass underwent significant decrease of the enhancing
parachyme (−44%) with formation of a cystic portion in the centrum sem. The objective response was further corroborated by the marked reduction of the extent of tumor vascularity estimated by quantitative analysis of DSCV (relative cerebral blood volume) from dynamic susceptibility MR perfusion maps (37) (Fig. 4D). Stabilization lasted for 115 days. During INI-42756493 treatment, mild and manageable toxicity was observed (grade 1 hyperglycemia, asthenia, dysguesia, dry mouth, keratitis, and grade II nail changes). After 4 months, tumor progressed on MRI locally as well as in the contrast-enhanced area and T2/FLAIR hyperintensity. The patient was reoperated and subsequently treated with CCNU. He is still alive, but in progression after 21 months from diagnosis and 287 days from the start of the anti-FGFR therapy.

Patient 2 is a 64-year-old woman, affected by left parietal GBM, diagnosed by stereotactic biopsy. The tumor was positive for FGFR3-TACC3 gene fusion by RT-PCR sequencing and showed diffuse FGFR3 expression in most tumor cells (Fig. 2A, C and F, sample 43). The patient received as first-line treatment fractionated radiotherapy and temozolomide according to the Stupp protocol (36), but after 2 cycles of monthly temozolomide, she presented with clinical deterioration including progressive headache, right homonymous hemianopsia and memory impairment. Brain MRI performed after 3 and 4 months after the completion of concurrent chemoradiotherapy revealed tumor progression with increase of the left parietal mass and the appearance of a small contrast-enhanced lesion (Fig. 4E). The patient was thus enrolled in the INI-42756493 trial (12 mg/kg administered in cycles of 7 days followed by 7 days off-treatment), and showed clinical improvement after 4 weeks (progression of headaches, visual field defect, and memory impairment). Best response was observed after 34 days of treatment with a 22% reduction of tumor size according to the RANO criteria (Fig. 4F) and 28% according to homogeneity (Fig. 4G). Grade 1 hyperglycemia, nail changes, and mucositis were observed. Clinical status remained stable until disease progression occurring 135 days after the start of the anti-FGFR therapy. The patient is still alive and is receiving a third-line chemotherapy with nitrosourea and bevacizumab.

Discussion

FGFR3-TACC3 fusions are potent oncogenic events that when present in brain tumor cells confer sensitivity to FGFR inhibitors (6). Since our original identification of recurrent FGFR3-TACC3 fusions in GBM, small subgroups of patients harboring FGFR3-TACC3 translocations have been identified in several other tumor types (7–15). Here, we report an unbiased RT-PCR-screening analysis for the identification of all possible functional FGFR3-TACC3 fusion transcripts. The screening of a large glioma dataset from multiple institutions not only confirmed that FGFR3-TACC3...
Figure 4. Biologic and posttreatment MRI of patients treated with IPI-491766 (Patient 1A-1D). A, post-contrast T1-weighted images show the tumor mass on the left parietal lobe. The interval (days) from the beginning of follow-up is indicated above each MRI. B, analysis of tumor volume (cm$^3$) before and during the anti-FGFR treatment (mean area). C, analysis of tumor volume (cm$^3$) before and during the anti-FGFR treatment (mean area). D, two different MRI slice levels of superior and middle part of the lesion are presented. E, analysis of T1-weighted images with gadolinium in 20 days after anti-FGFR treatment. F, two different MRI slice levels of superior and middle part of the lesion are presented. G, analysis of T1-weighted images with gadolinium in 20 days after anti-FGFR treatment.
Disclosure of Potential Conflicts of Interest

J. S. Jansen reports receiving honoraria and travel reimbursement from Bayer and Philips Healthcare. I. C. Soria and T. Tabernero are consultants/advisory board members for Bayer; no potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Writing, review, and/or revision of the manuscript (A. Di Stefano, T. Giordano, F. Fanti, P. Zappoli, N. Teodori, F. Lee, I. C. Soria, I. Tabernero, C. Hamburger, A. Lasera, M. Sesion, A. Lasera).
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Di Stefano, M. Labianca, M. Gay, R. Fiumara, A. Lasera.
Other (e.g., clinical data validation, literature search, clinical trial coordination, etc.): A. Zaffaroni, M. Sesion, A. Tenenbaum.

Grant Support

This work was supported by National Cancer Institute grants R01CA161444 and R01CA103126 (to A. Lasera) and R01CA77560 (to I. C. Soria). National Institute of Neurological Disorders and Stroke (R01NS041775) (to A. Lasera), and a grant from The Cristiano Foundation (to I. C. Soria). The Italian National Research Council (CNR) and the Italian Ministry of Health awarded I. C. Soria (to the National Institute of Neurological Sciences) and A. Lasera (to the National Institute of Neurological Sciences) with grants for the Clinical Research and Development Foundation (FIRB). I. C. Soria was supported by a fellowship from the Italian Ministry of Health (Ricerca Finalizzata). F. Lee is supported by a fellowship from the American Brain Tumor Association (ABTA). A. Di Stefano was supported by the Foundation IOM and PRIN 2011-2013, 20132050599_000. S. Yip is supported by the research scientist award from the Vancouver General Health Research Institute (VGHRI). Funding and distribution of brain tumor samples from EKB is made possible by generous financial support of BrainTumorBC.

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Received, August 22, 2014; revised December 18, 2014; accepted January 4, 2015; published OnlineFirst January 21, 2015.
Anna Luisa Di Stefano

Published Online First January 21, 2015. DOI: 10.1158/1078-0432.CCR-14-2199

FGFR1-TACC Identification and Inhibition in Glioma Patients

Clinical Cancer Research

Detection, Characterization, and Inhibition of FGFR–TACC Fusions in IDH Wild-type Glioma

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4.1.1 SUPPLEMENTARY MATERIALS

Supplementary Figures and Tables

Supplementary Figure 1 from article (Di Stefano, 2015).
Genomic PCR images and Sanger sequences of FGFR3-TACC3 genomic breakpoints. Fusion specific PCR products and Sanger sequencing chromatograms showing the FGFR3-TACC3 genomic breakpoints. The genomic sequences corresponding to FGFR3 and TACC3 are indicated in red or blue, respectively. M, DNA adder; C-, Negative Control.
Supplementary Figure 2 from article (Di Stefano, 2015).
Schematics of FGFR3-TACC3 genomic break points. Schematic representation of the genomic fusions between FGFR3 and TACC3 compared to the corresponding mRNA in red and blue report the regions belonging to FGFR3 and TACC3, respectively. The genomic breakpoint coordinates, according to the genome build GRCh37/hg19, are indicated above each fusion gene.
Supplementary Figure 3 from article (Di Stefano, 2015).
**Evaluation of the expression of FGFR3-TACC3 fusion elements.** (A) Microphotographs of immunofluorescence staining of a representative GBM harboring FGFR3-TACC3 fusion using antibodies that recognize the N- and C- termini of FGFR3 (FGFR3-N, FGFR3-C) and TACC3 (TACC3-N, TACC3-C), are in red. Nuclei are counterstained with DAPI, shown in blue. (B) Quantitative RT-PCR of four representative GBM carrying FGFR3-TACC3 fusion and three negative controls using primer pairs that amplify FGFR3 and TACC3 regions included in or excluded from the fusion transcripts, as indicated in the diagram. OAW28: ovarian cystoadenocarcinoma cell line harboring wild-type FGFR3 and TACC3 genes; GBM55 and GBM0822: GBM harboring wild-type FGFR3 and TACC3 genes; GBM3808; GBM1133; GBM0826; GBM3048: GBM harboring FGFR3-TACC3 (F3-T3) fusion. Error bars are SD of triplicate samples.
Supplementary Figure 4 from article (Di Stefano, 2015). The FGFR3-TACC3 fusion gene and protein are retained in recurrent GBM. (A) FGFR3-TACC3 fusion specific RT-PCR product from untreated and recurrent GBM from patient #3124. (B) Sanger sequencing chromatogram showing the identical reading frame at the breakpoint and the putative translation of the fusion protein in the untreated and recurrent tumor from the same patient. The fused exons at mRNA level are shown. Regions corresponding to FGFR3 and TACC3 are indicated in red and blue, respectively. T = threonine; S = serine; D = aspartic acid; V = valine; K = lysine; and A = alanine. (C) Representative microphotographs of FGFR3 immunofluorescence (IF) staining in both untreated and recurrent GBM. Blue staining indicates DAPI; Red staining indicates FGFR3. Magnification is 10x.
Supplementary Figure 5 from article (Di Stefano, 2015).

PFS and OS of FGFR3-TACC3-positive glioma patients. (A) Kaplan-Meier curves in IDH wild-type glioma patients don’t show significant differences in Progression Free Survival (PFS) between FGFR3-TACC3 positive (N=12, median PFS=11.20 months) and FGFR3-TACC3 negative (N=274, Median PFS=12.27 months) (P=0.85). (B) Kaplan-Meier curves in IDH wild-type glioma patients don’t show significant differences in Overall Survival (OS) between FGFR3-TACC3 positive (N=12, Median OS=32.80 months) and FGFR3-TACC3 negative (N=326, Median OS=18.60 months) (P=0.6). Red indicates FGFR3-TACC3 positive patients and green indicates FGFR3-TACC3 negative patients. Open circles represent censored patients.
Supplementary Figure 6 from article (Di Stefano, 2015). Analysis of SNP6.0 arrays of GBM harboring CNVs of FGFR3 and TACC3 genomic loci. CNVs of the FGFR3/TACC3 genomic loci in “gain labeled” (LRR > 0.2) TCGA samples. The CNA magnitudes (expressed as log2 ratio) were classified using simple thresholds: deletion (x < -1), loss (-1 < x ≤ -0.2), gain (0.2 ≤ x <) or amplification (x >). Gains are in gradients of red, losses in gradients of blue. Samples with uniform gains/amplification of FGFR3 and TACC3 lack FGFR3-TACC3 fusions. Samples harboring FGFR3-TACC3 fusions (F3-T3) show microamplifications involving the first FGFR3 exons, which are spliced in the fusion gene.
Supplementary Table 1 from article (Di Stefano, 2015). Summary of FGFR-TACC fusion transcripts identified in all cancer types. FGFR3-TACC3 fusion variants are ranked according to their prevalence across any cancer type. The number of FGFR-TACC fusions identified in each tumor type, including those identified in the present study, is also indicated.
4.2 Clinical Phenotype, Genetic Background and Correlations with FGFR3 Expression of FGFR-TACC Positive Gliomas

4.2.1 BACKGROUND

FGFR-TACC fusions are potent oncogenic events that when present in brain tumor cells confer sensitivity to FGFR inhibitors (Singh, 2012) that had been detected as a recurrent event in about 3% of IDH-wild type gliomas. We have recently reported an unbiased screening assay for FGFR–TACC fusions by RT-PCR in glioma that overcomes the great variability of variants that are generated by FGFR–TACC chromosomal translocation and we described the spectrum of transcripts, genomic breakpoints and molecular features of the 15 gliomas (12 GBMs and 3 lower grade gliomas) identified at that time (Di Stefano, 2015). However, exhaustivity was affected by the rarity of this aberration and the small sample size collected.

In the same study, we observed that all the 15 FGFR–TACC–positive glioma displayed strikingly uniform and strong expression of the FGFR3-N terminus as a result of accumulation of the fusion protein, suggesting that FGFR-TACC fusions are early events compatible with the glioma-initiating functions (Di Stefano, 2015).

Based on this observation we hypothesized that immunostaining using an antibody that recognized the N-terminus of FGFR3 might be useful as a prescreening method on paraffin embedded samples, but no data on sensitivity and specificity were available from a prospective cohort.

In this study we perform an institutional prospective binary screening for FGFR-TACC fusions by IHC and by RT-PCR in all new diagnosed IDH wild-type gliomas in Pitié-Salpêtrière Hospital. We calculate predictive values of IHC regarding the presence of the FGFR-TACC fusion transcripts, and we depict clinical features of a multicentric case series of 40 FGFR3-TACC3 positive glioma patients, the largest series identified to date.
4.2.2 METHODS

Patient and Tissue Samples

This study includes a cohort of patients with histologic diagnosis of glioma from 9 institutions in the setting of ANOCEF (Association des Neuro-Oncologue d'Expression Française) and POLA French Networks (Hopital Pitié-Salpêtrière, Onconeurotek, Paris; Hopitaux Civils de Lyon; Hopital Foch Suresnes; Institut du Cancers d’Angers; Hopital la Timone, Marseille; CHU Bordeaux, Hôpital Roger Salengro, Lille; CHU de Toulouse).

This cohort included a retrospective series of glioma patients from Pitié-Salpêtrière Hospital, being the object of a previous publication (Di Stefano, 2015), a new prospective series of newly diagnosed glioma patients starting from January 2014 from Pitié-Salpêtrière Hospital, and cases from other institutions.

In the prospective cohort, all cases underwent parallel-blinded analysis by FGFR3 IHC and RT-PCR while cases from other institutions were addressed at our centre in order to perform RT-PCR, screening for the fusion transcripts after local detection of FGFR3 expression by IHC.

Tumor specimens, blood samples and clinico-pathological information were collected with informed consent and relevant ethical board approval in accordance with the tenets of the Declaration of Helsinki. For the samples from the Pitié-Salpêtrière Hospital, clinical data and follow-up are available in the neuro-oncology database (Onconeurotek, GH Pitié-Salpêtrière, Paris).

Identification of Fusion Transcripts and analysis of Genomic Breakpoints

Total RNA was extracted from frozen tissues using Trizol (Invitrogen) according to manufacturer instructions. Two to three hundred nanograms of total RNA were
retro-transcribed with the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) or SuperScript II (Invitrogen). RT-PCR was performed using AccuPrime Taq DNA Polymerase (Invitrogen). Primer pairs used for the FGFR3-TACC3 fusions screening were: FGFR3ex12-FW: 5’-CGTGAAGATGCTGAAAGACGATG-3’ and TACC3ex14-RV: 5’-AAACGCTTTGAAGAGGTCGGAG-3’; amplification conditions were 94°C-3min, (94°C-30sec/61°C-30sec/68°C-1min40sec) for 35 cycles, 68°C-7min. FGFR1-TACC1 fusions were amplified with FGFR1ex16-FW: 5’TGCCTGTGGAGGAACTTTTCA-3’ and TACC1ex13-RV: 5’-CCCAAACCTCAGCAGCCTAAG-3’ primers (94°C-30sec/60°C-30sec/68°C-1min40sec for 35 cycles). PCR products were subjected to Sanger sequencing.

Immunohistochemistry and Histological Diagnosis

For the immunohistochemical analysis (IHC) of FGFR3 expressions, deparaffinization and immunolabeling of the sections were performed by a fully automated immunohistochemistry system Ventana benchmark XT system® (Roche, Basel, Switzerland) using as primary antibody the mouse monoclonal anti-FGFR-3 diluted 1:500 (clone B9, Santa Cruz Biotechnology), and using as chromogen: streptavidin–peroxidase complex with diaminobenzidin. The percentage of immunopositive tumor cells among the total number of tumor cells together with the maximal intensity of the immunolabelling were evaluated by visual semi-quantitative examination and tumor samples were then classified according to “weak”, “moderate” and “intense” staining for FGFR3. Integrated diagnosis was reviewed according to WHO 2016 (Louis, 2016) by two independent expert pathologists (KM and FB).
Molecular Characterization of Tumor Samples

Mutational status of *IDH1, IDH2, TERT* promoter, Histones *H3B* and *H3F3A, PTEN, BRAF* V600, was analysed as well as the methylation status of the *MGMT* promoter.

Expression of *IDH1-R132H* mutation was analyzed by IHC as previously described (Reyes-Botero, 2014) and *IDH1* and *IDH2* gene mutations were identified by Sanger sequencing (Sanson, 2009).

*IDH* wild-type tumors are defined according to the absence of *IDH1-R132H* immunopositivity and/or mutations in *IDH1* and *IDH2* genes. *TERT* promoter status was determined by the Sanger sequencing (Labussiere, 2014; Labussiere, 2014).

Hyper-methylation of the *MGMT* promoter was tested by bisulphite pyro-sequencing (Quillien, 2012). The presence of *EGFRvIII* was evaluated by RT-PCR using *EGFR- FW5’- TTCGGGAGCAGCGATGCGAC-3’* and *EGFR-RV ‘CTGTCCATCCAGGG AGGAGTA-3’* primers (Idbaih, 2009).

Copy number variations analyses have been performed using CGH arrays using BAC arrays (N=235). Results were normalized using control DNA from matched blood samples as previously described (Idbaih, 2008). Additional analyses of 154 tumor specimens were performed by SNP array, using Illumina Omni (Gonzalez-Aguilar, 2012). Array processing was outsourced to Integragen. Raw copy numbers were estimated at each of the SNP and copy-number markers. The biodiscovery property SNP-FASST2 algorithm was then used to segment copy number data. Segments were mapped to hg18 genome assembly (Olshen, 2004).

Copy number alterations (CAN) magnitudes called log-R ratio (LRR) were classified using simple thresholds: deletion (x ≤ -1), loss (-1 < x ≤ -0.2), gain (0.2 ≤ x <) or amplification (x ≥) according to default Nexus 7.5 software.

Targeted gene capture followed by sequencing with parallel next-generation sequencing (NGS) for *IDH1, IDH2, TERT* promoter, *H3B* and *H3F3A, BRAF*
V600 mutations, *EGFR, CDK4, MDM2* amplifications, p16 deletions and chromosomal gain and losses was performed in 29 glioma cases.

**Statistical Analysis**

Differences in the distribution on categorical variables were analyzed using Fisher Exact test. The *P* values were adjusted for multiple testing according to the Benjamini and Hochberg false discovery rate (FDR). A q-value of 0.05 (2-sided) was considered to be statistically significant.

Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Patients who were still alive at the last follow-up were considered as censored events in the analysis. Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Patients who were recurrence-free at the last follow-up were considered as censored events in the analysis. Survival curves were calculated by the Kaplan-Meier method and differences between curves assessed using the Log-Rank test. A Log-Rank test *p*-value ≤ 0.05 (two-sided) was considered to be statistically significant. Chi-square tested sensitivity, specificity, and positive and negative predictive values of FGFR3 staining to detect the presence of *FGFR3-TACC3* fusions.

**4.2.3 RESULTS**

To determine the frequency and features of *FGFR–TACC* fusions in human patients with glioma, we screened a cohort of 907 gliomas (655 grade IV, 144 grade III and 108 grade II).

This cohort included a retrospective series of 591 glioma patients from Pitié Salpêtriere, being object of a previous publication (Di Stefano et al. 2015), a new prospective series of 236 newly diagnosed glioma patients starting from January 2014 from *Pitié Salpêtriere Hospital* and 80 supplementary cases from other institutions.
176 cases had IDH mutations (36 grade IV, 72 grade III and 68 grade IV).
The RT-PCR assay was used for screening, as previously reported (Di Stefano, 2015), which allows for the detection of possible variants of FGFR3–TACC3 fusions that retain the mRNA sequences coding for the key FGFR-TK and TACC domains required for the oncogenic activity of the fusion protein.
Overall, we found 40 tumors harbouring FGFR3–TACC3 fusions. According to the 2016 WHO classification, 34 were glioblastoma IDH wild-type, 3 anaplastic astrocytoma IDH wild-type and 3 diffuse astrocytoma grade II IDH wild-type (see Table 4.2.1).
In this cohort, results were consistent with what we previously discovered in that all glioma harbouring FGFR3-TACC3 fusions are IDH wild-type. Conversely, all 176 IDH mutated gliomas of the cohort were negative for FGFR3-TACC3 fusions.
Sanger sequencing of the fusion amplicons revealed that each FGFR–TACC cDNA joined in-frame the sequence coding for the entire TK domain upstream of TACC coding sequences that invariably include the coiled-coil TACC.
We confirmed, in these larger series’, that FGFR3–TACC3 fusion isoforms are notably variable, even though isoforms FGFR3-exon 17-TACC3-exon 11 (17/40, 42%), FGFR3-exon 17-TACC3-exon 10 (12/40, 30%) and FGFR3-exon 17-TACC3-exon 8 (4/40, 10%) occurred more frequently. Seven supplementary variants occurred only in individual cases (Table. 4.2.1). Among them, we identified two new fusion transcripts (FGFR3-exon 17-TACC3-exon 13 and FGFR3-exon 17-TACC3-exon 4) as new variant currently unreported (underlined in Table 4.2.1).
Range of size of the FGFR3–TACC3 RT-PCR amplicons was comprised between 805 bp (for FGFR3ex18-TACC3ex13) and 1706 bp (for FGFR3ex18-TACC3ex4), comparing to our previous findings (Di Stefano, 2015).
Clinical and histo-molecular features (according to WHO 2016) of patients harbouring $FGFR3$-$TACC3$ fusions in this series are detailed in Table 4.2.1. Sex ratio was 0.9 (21 females and 19 males); median age at diagnosis was 61 years old (range 35-87).

Information on tumor location was available for all patients harbouring $FGFR3$-$TACC3$ fusions. In all 40 patients, $FGFR3$-$TACC3$ gliomas are supra-tentorial and located in cerebral lobes. No patients present tumors in the deep structures of the cerebrum, ventricles, cerebellum or in the brainstem. Gliomas in the frontal lobe accounted for 40% (16/40), temporal lobe for 27% (11/40), parietal/parieto-occipital lobe for 30% (12/40) and occipital lobe for 1 out of 40.

Gliomas were located more frequently in the right hemisphere (58%; 23/40) than in the left (42%; 17/40).
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<td>49</td>
<td>Right Frontal lobe</td>
<td>anaplastic astrocytoma IDH wild-type</td>
<td>Intense</td>
<td>FGFR3 EX1 TACC3 EX11</td>
<td>Alive</td>
<td>54.1</td>
<td>*recent study</td>
</tr>
<tr>
<td>36</td>
<td>F</td>
<td>74</td>
<td>Left Frontal lobe</td>
<td>glioblastoma IDH wild-type</td>
<td>Intense</td>
<td>FGFR3 EX1 TACC3 EX11</td>
<td>Alive</td>
<td>46.7</td>
<td>Di Stefano et al. 2015</td>
</tr>
<tr>
<td>37</td>
<td>F</td>
<td>77</td>
<td>Right Temporal lobe</td>
<td>diffuse astrocytoma IDH wild-type</td>
<td>Intense</td>
<td>FGFR3 EX1 TACC3 EX11</td>
<td>Alive</td>
<td>73.4</td>
<td>Di Stefano et al. 2015</td>
</tr>
<tr>
<td>38</td>
<td>F</td>
<td>59</td>
<td>Left Temporal lobe</td>
<td>glioblastoma IDH wild-type</td>
<td>Intense</td>
<td>FGFR3 EX1 TACC3 EX11</td>
<td>Alive</td>
<td>40.6</td>
<td>Di Stefano et al. 2015</td>
</tr>
<tr>
<td>39</td>
<td>F</td>
<td>53</td>
<td>Right Temporal lobe</td>
<td>glioblastoma IDH wild-type</td>
<td>Intense</td>
<td>FGFR3 EX1 TACC3 EX11</td>
<td>Alive</td>
<td>40.6</td>
<td>*Di Stefano et al. 2015</td>
</tr>
<tr>
<td>40</td>
<td>F</td>
<td>62</td>
<td>Left Temporal lobe</td>
<td>glioblastoma IDH wild-type</td>
<td>Intense</td>
<td>FGFR3 INT1 TACC3 EX7</td>
<td>Dead</td>
<td>0.9</td>
<td>*Di Stefano et al. 2015</td>
</tr>
</tbody>
</table>

Table 4.2.1 Clinical and histological diagnosis of patients harboring FGF3-TACC3 fusions. Details about FGFR3-TACC3 variant and FGFR3 staining are reported. Underlined FGF3-TACC3 isoforms emerged as new variants in the present study. Abbreviations: M=male; F=female; EX=exon; INT=intron; OS=overall survival from diagnosis; na=not available
Next, we explored if FGFR3-TACC3 positive patients had a different clinical course and outcome. We compared overall survival from time of diagnosis of FGFR3-TACC3 positive patients with the IDH wild-type glioma of this cohort (including grade II, grade III and grade IV). Remarkably, we observed that survival is significantly longer in FGFR3-TACC3 patients than FGFR3-TACC3 negatives (median OS 40.1 and 20.0 months respectively) ($P=0.03$) and this difference is confirmed, even more pronouncedly, in the grade IV glioma subgroup (median OS FGFR3-TACC3 positives 40.1 months versus FGFR3-TACC3 negatives OS 19.0; $P=0.006$), as showed in Figure 4.2.1. FGFR3-TACC3 isoforms did not correlate with different survival ranges.

![Figure 4.2.1 OS of FGFR3-TACC3-positive glioma patients.](image)

**Figure 4.2.1 OS of FGFR3-TACC3-positive glioma patients.** (A) Kaplan-Meier curves in IDH wild-type glioma patients show significant differences in between FGFR3-TACC3 positive (N=40, median OS=40.1 months) and FGFR3-TACC3 negative (N=469, Median OS= 20.0 months) ($P=0.03$). (B) Kaplan-Meier curves in IDH wild-type glioma grade IV subgroup show significant differences in Overall Survival (OS) between FGFR3-TACC3 positive (N=34, Median OS=40.1 months) and FGFR3-TACC3 negative (N=400, Median OS=19.0 months) ($P=0.006$). Red indicates FGFR3-TACC3 positive patients, green indicates FGFR3-TACC3 negative patients and open circles represent censored patients.
**FGFR3 Immunostaining is Highly Specific of the Presence of FGFR3-TACC3 Fusions**

In the previous study, we observed that all gliomas harbouring FGFR3-TACC3 fusions strong and homogeneous FGFR3 immunostaining, with an antibody that recognized the N-terminus of FGFR3 which is retained and overexpressed in the fusion protein. To determine sensitivity and specificity of FGFR3 immunostaining to predict the presence of FGFR3-TACC3 fusions we analysed a prospective cohort of 236 subjects using parallel-blinded immunostaining and RT-PCR assay. Results are detailed in Table 4.2.2. FGFR3 immunostaining was positive in all patients harbouring the FGFR3-TACC3 fusions and, conversely, any FGFR3-TACC3 positive glioma scored negative at immunostaining. Then, sensitivity of FGFR3 immunostaining to predict the FGFR3-TACC3 fusions was 100%, specificity was 81.8%, positive predictive value (VPP) was 19.6%, and negative predictive value (VPN) 100% (chi2 37.9).

80 cases from other institutions were addressed at our centre in order to perform RT-PCR screening for the fusion transcripts after local detection of FGFR3 expression by IHC. Of them, RT-PCR analysis was contributive in 77 patients while in the remaining 3, RNA concentration was too low and no supplementary frozen tumor was available. We added these supplementary 77 cases from other institutions to the prospective institutional cohort in order to calculate the positive predictive value in a larger sample. Adding these 77 supplementary patients, scoring positive for FGFR3 staining (59 FGFR3-TACC3 negative and 18 FGFR3-TACC3 positive by RT-PCR), the positive predictive value increased to 21.8%.
Table 4.2.2 Parallel-blinded analysis by FGFR3 immunostaining (IHC) and RT-PCR assay in an institutional prospective cohort of 236 patients. FGFR3 immunostaining proved highly sensitive 100%. Specificity was 81.8%.

<table>
<thead>
<tr>
<th>FGFR3-TACC3 negative</th>
<th>FGFR3 IHC +</th>
<th>FGFR3 IHC -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR3-TACC3 positive</td>
<td>41</td>
<td>185</td>
<td>226</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>185</td>
<td>236</td>
</tr>
</tbody>
</table>

Regarding the intensity of FGFR3-N immunostaining 30 out of the 40 gliomas harbouring FGFR3-TACC3 fusions scored “intense” expression of FGFR3, 5 “moderate” (Patients 1, 18, 28, 32 and 38 in Table 4.2.1) and 1 “weak” (Patient 11 in Table 4.2.1). No information of FGFR3 IHC was available for four of the patients (Patients 15, 26, 35 and 36).

While in our previous study we observed that all FGFR3-TACC3 positive gliomas shared a diffuse an intense pattern of FGFR3 expression, in this larger series we observe that rarely FGFR3-TACC3 fusions may present a weaker expression of FGFR3, suggesting that weak FGFR3 staining does not rule out the presence of FGFR3-TACC3 fusion transcript. Transcript sequencing and FGFR3 immunostaining of the only patient showing a “weak” expression of FGFR3 in this series (Patient 11) are shown in Figure 4.2.2.
**Figure 4.2.2 Representation of FGFR3 expression in Patient 11.** (A) FGFR3-TACC3 fusion specific RT-PCR product from untreated GBM from Patient 11, glioma sample ID 5862; 5008 Positive control. (B) Sanger sequencing chromatogram for FGFR3-TACC3 transcript showing a breakpoint at FGFR3 Exon 17 and TACC3 Exon 11. Regions corresponding to FGFR3 and TACC3 are indicated in red and blue, respectively. (C) Is a representative microphotograph of FGFR3 immunostaining scoring 20% of labeled tumor cells. Magnification is 200x.

**Molecular Alterations in IDH Wild-Type Gliomas Harbouring FGFR3-TACC3 Fusions**

We sought to update and extend to a larger series and a larger panel of chromosomal aberration than what was previously reported on the molecular profile of FGFR3-TACC3-positive glioma and to compare to IDH wild-type FGFR3-TACC3 negative gliomas.

Genomic data were available for up to 595 IDH wild-type gliomas, and 40 FGFR3-TACC3 positives are presented in **Table 4.2.3**.

According to our previous analysis, we confirmed in a larger series the mutual exclusivity of EGFR amplifications and FGFR3-TACC3 fusions (0/34 cases with EGFR amplification in FGFR3-TACC3 positives versus 139/358 (62%) in IDH wild-type FGFR3-TACC3 negatives; \( P=0.0001 \)), and the higher frequency of CDK4 amplification in FGFR3-TACC3 positives gliomas (5/29, 17% vs. 24/337, 7% in FGFR3-TACC3 negatives; \( P=0.06 \)).
Moreover, we confirm that MDM2 amplifications are more frequent in FGFR3-TACC3 fusions (5/30, 16% in F3-T3 positives versus 17/380, 4% in F3-T3 negatives; \( P = 0.01 \)) and that EGFRvIII is not represented in FGFR3-TACC3 fusions (\( P = 0.01 \)).

Interestingly, as a new result we found a significantly higher frequency of 10q loss in FGFR3-TACC3 gliomas compared to FGFR3-TACC3 negative gliomas (23/25, 92% versus 225/346, 65%; \( P = 0.004 \)).

In these series’ we could extend analysis to other rare molecular aberrations such as PTEN mutation, MET amplification, histones H3F3A and H3B mutations and BRAF V600E mutations, and we did not find any FGFR3-TACC3 positive gliomas harbouring these alterations.

We found no statistical association between FGFR3-TACC3 fusions and other genetic and epigenetic alterations that commonly occur in gliomas harbouring wild-type IDH genes (TERT promoter mutations, gain of chromosome 7p, loss of chromosomes 13q and 14q and methylation of the MGMT promoter, Table 4.2.3).
**Table 4.2.3** Molecular alterations in *IDH* wild type glioma harboring *FGFR3-TACC3* fusions (635 *IDH* wild-type gliomas; 40 *FGFR3-TACC3* positive vs. 595 *FGFR3-TACC3* negative). The table reports the absolute number and frequency (percentage) of individual glioma-specific molecular alterations in tumors scoring positive or negative for *FGFR3-TACC3* fusions. Statistically significant associations are indicated in red (Fisher Exact test). Comparing to our previous study, we found a significant higher frequency of 10q loss in *FGFR3-TACC3* positive and lower of *EGFRvIII* variant (underlined). q-values adjusted with FDR are reported.

<table>
<thead>
<tr>
<th>Alteration</th>
<th>N° of <em>FGFR3-TACC3</em> positive</th>
<th>% of <em>FGFR3-TACC3</em> positive</th>
<th>N° of <em>FGFR3-TACC3</em> negative</th>
<th>% of <em>FGFR3-TACC3</em> negative</th>
<th>P (Fisher test)</th>
<th>q (FDR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>EGFRvIII</em></td>
<td>0/14</td>
<td>0%</td>
<td>139/358</td>
<td>38%</td>
<td>0.0001</td>
<td>0.01</td>
</tr>
<tr>
<td><em>CDH4</em> amplification</td>
<td>5/29</td>
<td>17%</td>
<td>24/337</td>
<td>7%</td>
<td>0.06</td>
<td>0.14</td>
</tr>
<tr>
<td><em>MDM2</em> amplification</td>
<td>5/30</td>
<td>16%</td>
<td>17/380</td>
<td>4%</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>p16 deletion</td>
<td>9/28</td>
<td>32%</td>
<td>169/358</td>
<td>45%</td>
<td>0.23</td>
<td>0.43</td>
</tr>
<tr>
<td>10q loss</td>
<td>23/23</td>
<td>92%</td>
<td>225/346</td>
<td>63%</td>
<td>0.004</td>
<td>0.03</td>
</tr>
<tr>
<td>7p gain</td>
<td>22/23</td>
<td>83%</td>
<td>212/310</td>
<td>69%</td>
<td>0.23</td>
<td>0.43</td>
</tr>
<tr>
<td>13q loss</td>
<td>3/22</td>
<td>13%</td>
<td>53/318</td>
<td>17%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>14q loss</td>
<td>3/21</td>
<td>14%</td>
<td>45/297</td>
<td>15%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TERT promoter mutation</td>
<td>19/25</td>
<td>76%</td>
<td>234/301</td>
<td>78%</td>
<td>0.80</td>
<td>0.98</td>
</tr>
<tr>
<td>MGMT promoter hypermethylation</td>
<td>8/15</td>
<td>40%</td>
<td>82/180</td>
<td>45%</td>
<td>0.79</td>
<td>0.97</td>
</tr>
<tr>
<td><em>H3B</em> and <em>H3F3A</em> mutations</td>
<td>0/8</td>
<td>0%</td>
<td>7/99</td>
<td>7%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>BRAF</em>V600E mutation</td>
<td>0/12</td>
<td>0%</td>
<td>3/224</td>
<td>1%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>MET</em> amplification</td>
<td>0/9</td>
<td>0%</td>
<td>0/31</td>
<td>0%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>PTEN</em> mutation</td>
<td>3/9</td>
<td>33%</td>
<td>21/108</td>
<td>19%</td>
<td>0.38</td>
<td>0.53</td>
</tr>
</tbody>
</table>

**4.2.4 DISCUSSION**

*FGFR-TACC* fusions are potent oncogenic events that specifically recur in the setting of *IDH* wild-type gliomas.

Knowledge of specific histological and clinical characteristics of gliomas harbouring *FGFR3-TACC3* fusions is affected by the rarity of this alteration and the small sample size of positive cases of *FGFR3-TACC3*.

Beginning with the RT-PCR-sequencing assay, we have previously validated all possible functional *FGFR3-TACC3* fusion transcripts; in this study we have extended the screening up to 907 cases and increased the number of identified *FGFR3-TACC3* positive cases to 40.
The first relevance of our findings is that, among them, 24 patients are alive at the end of this follow-up and could potentially benefit from anti-FGFR therapies, concurrent with the example we have previously reported of the two patients treated with anti-FGFR therapy at recurrence (Di Stefano, 2015).

According to the remarkable variability of \textit{FGFR3}-\textit{TACC3} fusion events we have previously reported (Di Stefano, 2015), in this larger series we were able to detect two new isoforms of \textit{FGFR3}-\textit{TACC3} fusions (\textit{FGFR3 EX17-TACC3 EX13} and \textit{FGFR3 EX17-TACC3 EX4}), occurring in individual cases. Adding these novel identified variants, the numbers of isoforms that others and we have reported so far, increase to 14 (Supplementary Table 1 from Di Stefano, 2015). However, by increasing the repertoire of variants in this study, we observe that two variants \textit{FGFR3 EX17-TACC3 EX11} and \textit{FGFR3 EX17-TACC3 EX10} are highly recurrent and cover 42% and 30% of the series, respectively.

From our previous study we know that structural heterogeneity of \textit{FGFR3}-\textit{TACC3} fusions is yet more pronounced at the genomic level, whereby even identical fusions transcripts (\textit{FGFR3 EX17-TACC3 EX11}; \textit{FGFR3 EX17-TACC3 EX8}; \textit{FGFR3 EX17-TACC3 EX6}) harbours distinct genomic breakpoints [Supplementary Figure 2 from (Di Stefano, 2015)]. Further studies might elucidate why these translocation events involve more frequently specific genomic regions in \textit{FGFR} and \textit{TACC3} genes.

We were able to collect clinical records on the 40 patients harbouring \textit{FGFR3-TACC} fusions identified so far.

We did not observe specific differences in sex ratio and median age at diagnosis. Regarding tumor location we found that all 40 patients of this series were affected by supra-tentorial hemispheric brain tumors: none of them affected deep structures of the cerebrum, ventricles, cerebellum, brainstem and/or spine.

Given the recognized role of \textit{FGFR3} as a regulator caudo-lateral (occipito-temporal) cortex development (Thomson, 2009), we wondered if \textit{FGFR3}-
TACC3 initiated glioma occurred specifically in posterior lobes. In this series of 40 FGFR3-TACC3 positive glioma patients, we found that FGFR3-TACC3 glioma do not occur specifically in caudo-lateral lobes but rather in all cerebral lobes. However, we observed a slightly higher frequency in partieto-occipital lobes (12/40; 30%) compared to what was reported about glioma in the literature of around 17% (Larjavaara et al. 2007). This observation is affected by the small sample size of FGFR3-TACC3 cases. We plan to analyse in a larger series, with case-controlled analyses that may also illustrate if cortical location is predominant in FGFR3-TACC3 gliomas.

To determine if FGFR3-TACC3 fusion events were associated with difference on survival, we extend follow-up and survival analysis to novel identified patients of this study. In our previous study, we failed to observe difference in survival (Di Stefano, 2015). Inversely, in this second analysis performed in a larger number of patients and a longer follow-up, we found a clearly longer median survival of up to 40 months in FGFR3-TACC3 glioma patients, as compared with FGFR3-TACC3 negative-IDH-wild-type patients. Difference in overall survival resulted yet more pronouncedly in the GBM subgroup, where we observed strikingly long survival rates (longer than 24 months) in 9 patients out of the 34 GBM harbouring FGFR3-TACC3 fusions (26%), independent of the transcript isoforms.

The majority of patients in both groups received standard radio-chemotherapy at first line in both groups (Stupp, 2005). Eight GBM patients harbouring FGFR3-TACC3 fusions were recently included in a phase II trial testing AZD4547, an ATP-competitive pan-FGFR selective inhibitor, at recurrence. Enrolment in this clinical trial does not explain the longer survival we observed in FGFR3-TACC3 positive GBM because:

- patients with the longest survivals did not match with those who received or are receiving AZD4547
longest survivals correspond to patients with especially long remission intervals after first line treatment

enrolments in TARGET trial are relatively recent, starting from September 2015.

The reason for this better prognosis remains to be determined. However, this finding highlights a supplementary relevance of usefulness of specific therapies targeting FGFR3-TK in this selected subgroup of patients. We previously reported a clinical improvement and a minor response in two patients treated with an anti-FGFR ATP-competitive pan-FGFR selective inhibitor (JNJ42756493) (Di Stefano, 2015). Other anti-FGFR specific reversible and covalent inhibitors are being tested in the setting of phase I and phase II trials in selected patients harbouring activating aberrations of FGFR receptors.

In perspective, the enrichment of therapeutic armamentarium against FGFR oncogenic drivers may be particularly interesting in this subset of patients given their longer evolution, by rechallenging or combining specific therapies targeting FGFR3 that might finally lead to a relevant clinical benefit.

In our previous study, we observed that all glioma harbouring FGFR3-TACC3 fusions presented an intense and diffuse staining for FGFR3 as a result of the accumulation of the fusion protein. In this study we determined, prospectively, sensitivity and specificity of FGFR3 staining to predict the presence of FGFR-TACC fusions. While constant in all cases harbouring the fusion (100% sensitivity), FGFR3 expression predicts the presence of the fusion in only 20% of the cases.

Despite the majority of FGFR3 showing an intense and diffuse staining for FGFR3, a small fraction of FGFR3-TACC3 gliomas had a weak expression of FGFR3, with 20 to 50% FGFR3 positive tumor cells.

This data shows that IHC for FGFR3 is an efficient and reliable pre-screening method and can be integrated in histological routine work-up: when positive, it raises the attention level of pathologists, clinicians and biologists for
biomolecular analysis on frozen samples that can be performed in reference laboratories.

Finally, by the extension of molecular characterization in a larger case series with a larger panel of molecular aberrations, we confirmed that FGFR3-TACC3 positive glioma harbour a specific genomic background principally characterized by the absence of EGFR amplification and EGFRViii variant, a higher frequency of MDM2 and CDK4 amplification and 10q loss.

Overall, our findings have shown the importance and feasibility of prospective genotyping for FGFR3-TACC3 fusions and provide preliminary elements on clinical and histological phenotype of FGFR3-TACC3 glioma patients. Further studies should answer the question on radiological and histological signatures linked to this driver oncogenic alteration, on efficacy FGFR-TK inhibitors and on eventual factors of resistance to this promising target therapy.
4.3 Exploratory Analysis on Mechanism of Resistance to Anti-FGFR Therapies

FGFRs/FGFs are key molecules involved in embryogenesis, tissue homeostasis, tissue repair, wound healing, and inflammation (Powers, 2000). The main effects of the FGFR pathway include proliferation, migration, and antiapoptotic signals. Proliferation is mainly achieved through the MAPK cascade, whereas antiapoptotic signals are mediated by PI3K/AKT with cross talk between both pathways (Turner, 2010).

FGF signalling is deregulated in many cancer types. Activating mutations and amplification occurring in 50-60% of non muscle invasive bladder cancers (van Rhijn, 2002) and translocations t(4;14) (p16.3;q32.3) occurring in 15-20% in multiple myeloma are the most frequent FGFR activating alterations reported so far (Chesi, 1997).

At a therapeutic level, the most clinically advanced anti-FGFR drugs are small-molecule TKIs, targeting the ATP-binding site of the intracellular tyrosine kinase domain of FGFRs. In our previous study (Di Stefano, 2015), we treated two patients harbouring FGFR3-TACC3 recurrent glioblastoma with JNJ-42756493 an oral ATP-competitive pan-FGFR selective inhibitor that inhibits tyrosine phosphorylation of activated FGFR at nanomolar (Tabernero, 2015). We observed a clinical improvement in both patients, a stabilization of tumor growth and one minor response.

Next, 4 months after starting anti-FGFR therapy, Patient 1 showed tumor progression on MRI and underwent surgery 4 weeks after discontinuation of JNJ-42756493. Expression of FGFR3 was maintained in recurring GBM, scoring highly positive (Id 5008 in Figure 4.3.1). We confirmed the presence of FGFR3-TACC3 fusions transcript with the same breakpoint as the initial GBM FGFR3 EX17-TACC3EX6 (Id 4451) Figure 4.3.1. At a genomic level, we found
FGFR3-TACC3 in the recurrent GBM with the same breakpoint as the initial tumor (FGFR3 EX17+INT17-TACC3EX5+INT5+EX6) Figure 4.3.1.

Recent data indicates that the FGFR pathway is a potential driver of a number of mechanisms of resistance to various targeted drugs. Interestingly, Chell et al. generated a derivative of the KMS-11 myeloma cell line (FGFR(Y373C)) with acquired resistance to AZD4547 (KMS-11R cells) an inhibitor of FGFR1-3 and they identified the presence of a secondary heterozygous mutation at the gatekeeper residue, encoding FGFR3(V555M) as a mechanism of acquired resistance to FGFR inhibitors [(Chell, 2013); Figure 4.3.2 adapted from the article].

Basing on this observation we looked for new acquired mutations of FGFR3-TACC3 in the recurrent GBM.

We performed PCR and Sanger sequencing of all 18 exons of FGFR3 and 16 exons of TACC3 in the initial GBM and in the recurrent GBM after anti-FGFR therapy. By comparison of FGFR3 and TACC3 genomic DNA in exonic regions, we did not find any new acquired mutation in the recurrent GBM. In particular, no mutations at FGFR3 (V555M) were present in the primary, nor in the recurrent GBM. Only 6 common-indexed non functional SNPs were found in both the initial and the recurrent GBM (FGFR3-Exon 14: SNV G>A; FGFR3-Exon 18: SNV A>G, FGFR3-Exon 18: deletion of 2bp (TG>-); FGFR3-Exon 18: SNV C>T ; TACC3-Exon 1: SNV T>C ; TACC3-Exon 4: SNV A>G).

Additional studies, including % of FGFR3 cells expressing and exome sequencing of recurrent resistant tumors after anti-FGFR therapy are planned.
Figure 4.3.1 FGFR3 expression and FGFR3-TACC3 gene fusions in Patient 1, before and post-treatment with JNJ-42756493. In A) Post-gadolinium T1-weighted and FLAIR imaging show the target lesion on the right parietal lobe corresponding to progression disease (see Figure 4 from Di Stefano et al. (2015) for baseline imaging).

B) Representative micrographs of FGFR3 immunostaining of untreated GBM (4451) in B1 and recurring GBM after JNJ-42756493 (5008) in B2. 10 X magnification in B1 and 40 X magnification in B2.

C) Schematic representation of the FGFR3–TACC3 fusion transcripts in untreated GBM (4451) in C1 and recurring GBM (5008) in C2. The junction sequences of FGFR3 EX17-TACC3 EX 6 isoform in mRNA and the reading frame at the breakpoint are reported.

D) Schematic representation of the genomic fusions between FGFR3 and TACC3 FGFR3 EX17+INT17-TACC3EX5+INT5+EX6 in untreated GBM (4451 in D1) and recurrent GBM (5008) in D2.

The reported regions belonging to FGFR3 and TACC3 are in red and blue, respectively.

C3 and D3: results from RT-PCR screening: M, DNA ladder.
Figure 4.3.2 Analysis of FGFR3 Val555 in KMS-11R cells [A and B; images adapted from (Chell, 2013)] and untreated GBM (4451 in C) and post treatment GBM in (5008 D) after anti-FGFR-therapy ATP-binding inhibitor. While resistant KMS-11R cells after AZD4547 showed a gatekeeper mutation in FGFR3V555Met, both untreated GBM (4451) and the recurrent (5008) after JNJ-42756493 show wild-type sequence at Val555. Sequence chromatograms of genomic DNA at codon 555 (exon 13) of FGFR3 are shown.
5. SECTION 3 – DIAGNOSTIC MARKERS

In Vivo Non-Invasive Detection of 2-Hydroxyglutarate in IDH Mutated Gliomas

5.1 INTRODUCTION

Proton magnetic resonance spectroscopy (1H-MRS) is used in the metabolic research of gliomas, and as a supplemental tool to magnetic resonance imaging (MRI) in the diagnosis of gliomas (Law, 2003; Zonari, 2007). However, 1H-MRS presents certain limitations that can be attributed to the intrinsically low concentrations of metabolites yielding limited signal-to-noise ratio (SNR), to the low spectral resolution at clinical fields, and to the lack of specificity of the metabolites that have been previously proposed as biomarkers of gliomas.

In this regard, the emergence of 2-hydroxyglutarate (2 HG) as an onco-metabolite overproduced in isocitrate dehydrogenase IDH-mutated gliomas (Dang, 2010; Parsons, 2008; Yan, 2009), the substantially longer survival duration of IDH-mutated gliomas patients (Sanson, 2009) and recent pioneering studies on the feasibility of non-invasive detection of 2 HG by 1H-MRS (Pope, 2012; Andronesi, 2012; Choi, 2012), have recently drawn attention of neuro-oncologists.

Reportedly, due to severe spectral overlap with its background signal, the quantification of 2 HG is challenging (Andronesi, 2012; Choi, 2012).
5.1.1 Overproduction of 2-Hydroxyglutarate in IDH-Mutated Gliomas

Isocitrate dehydrogenase is an enzyme with three isoforms, i.e., IDH1, IDH2, and IDH3 (Dang et al. 2010). Intra-cellularly, it catalyses the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) in cytoplasm and peroxisomes (IDH1) and in mitochondria (IDH2 and IDH3) (Figure 5.1) (Arcaro, 2007; Dang, 2010).

Mutations in IDH1 were detected initially by a whole-genome sequence analysis in a small subset of glioblastoma patients (Parsons, 2008). Subsequent studies confirmed the presence of IDH mutations in 70–90% of low-grade glioma and secondary glioblastoma, in ~20% of acute myeloid leukaemia, and in intrahepatic cholangiocarcinoma, chondrosarcoma, and melanoma (Gross, 2010; Waitkus, 2016; Yang, 2012).

Glioma-specific mutations in IDH1 always affect the amino acid arginine in position 132 of the amino acid sequence belonging to a high, evolutionarily conserved region located at the binding site for isocitrate (Hartmann, 2009). Mutations in IDH2 affect arginine at position 172, which is the analogous site to arginine 132 in IDH1 (Hartmann, 2009). Mutations in both IDH1 and IDH2 are heterozygous and of somatic origin.

The predominant amino acid sequence alteration in IDH1 was R132H, accounting for 92.7% of the detected mutations. For this specific mutation, an immunostaining with a mutant protein recognition antibody has been proven to be highly sensitive and specific and successfully introduced in clinical practice (Capper, 2009). Less frequently, other amino acid sequence alteration IDH1 mutation may occur in codon 132 (R132C for 4.1%, R132S for 1.5%, R132G for 1.4% and R132L for 0.2% of all IDH1 mutations). IDH2 mutations occur with a frequency of 3% in non-glioblastoma tumors and interestingly seem to be associated with tumors with an oligodendroglial component (Hartmann, 2009).

Type and distribution of IDH1 and IDH2 mutations are given in Table 5.1.
The *IDH1* mutation is one of the earliest known genetic events in low-grade gliomas, and it is thought to be a “driver” mutation for tumorigenesis (Suzuki, 2015). Presence of the *IDH1* is associated with a more favourable prognosis compared to tumors with wild-type *IDH1* in all glioma grades and it is also predictive of response to anti-cancer therapy (Cairncross, 2014; Sanson, 2009; Houillier, 2006). The reasons for this better prognosis remain to be determined. *IDH* mutations cluster with other molecular aberrations such as codeletion of 1p19q: all the 1p19q-codeleted gliomas have been shown to be mutated on *IDH1* or *IDH2*; inversely not all *IDH* mutated gliomas present 1p19q chromosomal translocation (Labussiere, 2010).

Combinations of these two molecular alterations in the subgroup of non-GBM (grade 3 and grade) allow definition of three molecular subgroups (*IDH*mutated+1p19q codeleted/*IDH*mutated+non codeleted and *IDH* wild-type gliomas) with marked differences in survival rates ([Figure 5.2](#)) and differential response to PCV based chemotherapy in addition to radiotherapy, as recently reported (Cairncross, 2014). These observations could explain part of the relevant heterogeneity in clinical course we usually observe in non-glioblastoma patients ([Figure 5.2](#)) and elige the *IDH* mutation as a major marker for prognostication and for decisions regarding chemo-radiotherapy regimens.

From a metabolic perspective, mutations in *IDH1* and *IDH2* lead not only to the loss of wild-type enzyme activity-interconversion of isocitrate to α-ketoglutarate (α-KG), but also to a gain-of-function that results in the conversion of α-KG to the “oncometabolite” 2-hydroxyglutarate (2HG) (Dang, 2009). 2HG is a competitive inhibitor of multiple α-KG-dependent deoxygenises, such as the prolyl hydroxylases, and histone demethylases, and the TET family of methylcytosine hydroxylases (Xu et al. 2010). As a result, *IDH1/2* mutant cells undergo extensive epigenetic modifications that ultimately result in tumorigenesis (Lu, 2012; Turcan, 2012; Sasaki, 2012)
According to previous published studies, 2HG accumulation may be measured on snap frozen tumor samples by liquid chromatography - mass spectrometry (LC/MS) (Figure 5.3). IDH mutant tumors have been shown to present more 2HG than IDH wild-type (range from 5 and 35 μmol of 2HG per gram of tumor) by 100 fold, and 0.045 to 0.68 μmol/gr in IDH wild-type gliomas. No differences have been observed for α-KG levels and other metabolites (Dang, 2009), Figure 5.3.

5.1.2 Noninvasive Detection of 2HG by 1H-MRS

To date, a total of only 9 studies reported (Pope, 2012; Andronesi, 2012; Choi, 2012; Natsumeda, 2014; de la Fuente, 2016; Lazovic, 2012; Emir, 2016; Heo, 2016) on in-vivo 1H-MRS of 2HG in gliomas including 2 animal studies (Lazovic, 2012; Heo, 2016). 5 different 1H-MRS methods have already been proposed (Table 5.2).

The 2HG molecule has 5 non-exchangeable protons (5-spin system) with complicated J-coupling interactions (Choi, 2012). These interactions refer to inter-spin interactions through electron bonds in a molecule (de Graaf RA, 2007; Allen, 1997). Such an indirect interaction between spins rather than a direct interaction through space is termed as J-coupling; the spins involved in the interaction are said to be J-coupled (de Graaf RA, 2007; Allen, 1997). J-coupling results in peak splitting (e.g., a singlet into a multiplet) and changes in line shape and signal amplitude as a function of time (typically, echo time [TE]).

The majority of the 1H-MRS-detectable metabolites have coupled spins such as 2HG, Glu, Gln, gammaaminobutyric acid (GABA), and N-acetylaspartylglutamate (NAAG) (Govind, 2015). On the other hand, water and creatine (Cr) are representative metabolites with uncoupled spins only.

The spectral characteristics of 2HG are determined by J-coupling in combination with the resonance frequencies of the 5 protons that are determined by chemical
environments of the protons in the molecule (chemical-shift), giving rise to three, non-field-dependent, multiplets centred at ~4.0, ~2.3, and ~1.9 ppm (contributed by 1, 2, and 2 spins, respectively) (Figure 5.4) (Choi, 2012). The spins resonating at ~1.9 ppm are J-coupled with the spin resonating at ~4.0 ppm, as well as with those resonating at ~2.3 ppm (Choi, 2012). The multiplet at ~4.0 ppm is relatively small in amplitude as it is contributed by only one proton, it overlaps with signals from lactate (Lac) and myo-inositol (mI), and is close to the strong PCr (~3.9 ppm) and water (~4.7 ppm) signals. The multiplet at ~1.9 ppm also overlaps with signals from other metabolites (Figure 5.4), in particular, with N-acetylaspartate (NAA). The multiplet at ~2.3 ppm has the largest signal and is therefore widely used as a target signal for 2 HG quantification (Figure 5.4). However, it also overlaps widely with signals from at least 4 other metabolites (Figure 5.4) (Andronesi, 2012; Choi, 2012).

Techniques for detection of 2 HG, published so far can vary insofar as:

- pulse sequence (PRESS, LASER, COSY): series of radio frequency pulses applied to the sample, which may vary in number, duration, and shape and are related to the characteristic frequencies of the target signals
- echotime (short TE or long TE): the time between the application of the radiofrequency excitation pulse and the peak of the measured signal (spin-echo).
- different editing method, which allows the signals of specific metabolites in a target spectral region to be cancelled.
- single or multivoxel: single or multiple region(s) of interest for spectral detection.
- size of voxel (ranging from 1.8 and 8 cm$^3$)
- magnetic field (3 to 7T)
Results from the nine previous studies on 2 HG in-vivo detection by 1H-MRS, including 2 animal studies, are resumed in Table 5.2.

Examples of in vivo single-voxel localized PRESS spectra from IDH mutated gliomas fro (Choi, 2012) are shown in Figure 5.5.
Figure 5.1 Adapted from (Kim, 2016). Simplified metabolic pathways associated with isocitrate dehydrogenase (IDH) and 2-hydroxyglutarate (2 HG). Mutations of IDH1 and IDH2 result in overproduction of 2 HG. 1H-MRS-visible metabolites including 2 HG are marked in bold. Acetyl-CoA = acetyl coenzyme A, Gln = glutamine, Glu = glutamate, GSH = glutathione, GSSG = glutathione disulfide, H2O = water, H2O2 = hydrogen peroxide, IDH-MT = mutant isocitrate dehydrogenase, NAD = nicotinamide adenine dinucleotide, NADH = nicotinamide adenine dinucleotide hydrate, NADP = nicotinamide adenine dinucleotide phosphate, NADPH = nicotinamide adenine dinucleotide phosphate hydrate, α-KG = alpha-ketoglutarate.
Table 5.1 Adapted from (Hartmann, 2009). Type of 716 IDH1 and 31 IDH2 mutations and frequency among mutations in 1,010 WHO grades II and III astrocytomas, oligodendrogliomas and oligoastrocytomas. N (%) number of tumors and percentage of mutation among all mutations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH1</td>
<td>G395A</td>
<td>R132H</td>
<td>664 (92.7%)</td>
</tr>
<tr>
<td></td>
<td>C394T</td>
<td>R132C</td>
<td>29 (4.2%)</td>
</tr>
<tr>
<td></td>
<td>C394A</td>
<td>R132S</td>
<td>11 (1.5%)</td>
</tr>
<tr>
<td></td>
<td>C394G</td>
<td>R132G</td>
<td>10 (1.4%)</td>
</tr>
<tr>
<td></td>
<td>G395T</td>
<td>R132L</td>
<td>2 (0.2%)</td>
</tr>
<tr>
<td>IDH2</td>
<td>G515A</td>
<td>R172K</td>
<td>20 (64.5%)</td>
</tr>
<tr>
<td></td>
<td>G515T</td>
<td>R172M</td>
<td>6 (19.3%)</td>
</tr>
<tr>
<td></td>
<td>A514T</td>
<td>R172W</td>
<td>5 (16.2%)</td>
</tr>
</tbody>
</table>
Figure 5.2 Overall survival of 206 grade III gliomas from Pitié Salpêtrière database, according \textit{IDH} mutation status and the presence of 1p19q codeletion. Rounds correspond to censored patients.
Figure 5.3 Adapted from (Dang, 2010). A) Malignant human gliomas containing R132 mutations in IDH1 contain increased concentrations of 2HG. Human glioma samples obtained by surgical resection were snap frozen, genotyped to stratify as wild-type (WT) (N=10) or carrying an R132 mutant allele (Mutant) (n=12) and metabolites extracted for LC-MS analysis. Among the 12 mutant tumors, 10 carried a R132H mutation, one an R132S mutation, and one an R132G mutation. Each symbol represents the amount of the listed metabolite found in each tumor sample. Red lines indicate the group sample means. The difference in 2HG observed between WT and R132 mutant IDH1 mutant tumors was statistically significant by Student’s t-test (p<0.0001). There were no statistically significant differences in αKG, malate, fumarate, succinate, or isocitrate levels between the WT and R132 mutant IDH1 tumors. B) The clinical characteristics, IDH1 mutation status, levels of metabolites measurement in the clinical specimens. 2HG levels ranged from 5 to 32 µmol/gr in IDH mutated gliomas and from 0.045 to 0.68 µmol/gr in IDH wild-type gliomas.
Figure 5.4 Adapted from (Kim, 2016). Simulated spectra of 2 HG and its background metabolites at 3T. Concentration ratio of 5:9.25:4.5:1.5:1.5 (mM) was assumed for 2 HG:Glu:Gln:GABA:NAAG. Line widths of all spectra were broadened to mimic in-vivo spectra. GABA = gamma-aminobutylic acid, Gln = glutamine, Glu = glutamate, NAAG = N-acetylaspartylglutamate, 2 HG = 2-hydroxyglutarate
Figure 5.5 Adapted from (Choi, 2012). In vivo single-voxel localized PRESS spectra from normal brain (a) and tumors (b-f), at 3T, are shown together with spectral fits (LCModel) and the components of 2HG, GABA, glutamate, and glutamine, and voxel positioning (2x2x2 cm3). Spectra are scaled with respect to the water signal from the voxel. Vertical lines are drawn at 2.25 ppm to indicate the H4 multiplet of 2HG. Shown in brackets is the estimated metabolite concentration (mM) ± standard deviation. Abbreviations: Cho, choline; Cr, creatine; NAA, N-acetylaspartate; Glu, glutamate; Gln, glutamine; GABA, γ-aminobutyric acid; Gly, glycine; Lac, lactate; Lip, lipids. Scale bars, 1 cm.
Table 5: 1H-MRS Methods and Their Performance in Identifying IDH Mutational Status in Gliomas.

<table>
<thead>
<tr>
<th>Method</th>
<th>Authors (Ref)</th>
<th>Subject</th>
<th>Number of Subjects</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Cutoff</th>
<th>Reference</th>
<th>Pulse Sequence</th>
<th>TE1 (ms)</th>
<th>TE2 (ms)</th>
<th>TR (ms)</th>
<th>Scan Time (min)</th>
<th>Voxel Volume (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANDRONESI et al.</td>
<td>Human</td>
<td>2</td>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>20</td>
<td>Cho + Gin</td>
<td>Siemens</td>
<td>1.0</td>
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<tr>
<td>CHOI et al.</td>
<td>Human</td>
<td>22</td>
<td>-</td>
<td>77</td>
<td>100</td>
<td>19</td>
<td>Water</td>
<td>Philips</td>
<td>3.0</td>
<td></td>
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<tr>
<td>Pap et al.</td>
<td>Human</td>
<td>9</td>
<td>13</td>
<td>-</td>
<td>100</td>
<td>78</td>
<td>NA</td>
<td>Philips</td>
<td>3.0</td>
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<tr>
<td>NAITOMO et al.</td>
<td>Human</td>
<td>25</td>
<td>7</td>
<td>-</td>
<td>100</td>
<td>72</td>
<td>35</td>
<td>Water</td>
<td>d2</td>
<td></td>
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<tr>
<td>LEONARD et al.</td>
<td>Mouse</td>
<td>3</td>
<td>7</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>20</td>
<td>Cho + Gln</td>
<td>Siemens</td>
<td>7.0</td>
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<tr>
<td>Short TE with baseline</td>
<td></td>
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<tr>
<td>CHOI et al.</td>
<td>Human</td>
<td>15</td>
<td>13</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>19</td>
<td>Water</td>
<td>Philips</td>
<td>3.0</td>
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<tr>
<td>CHOI et al.</td>
<td>Human</td>
<td>22</td>
<td>-</td>
<td>7</td>
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<td>100</td>
<td>19</td>
<td>Water</td>
<td>Philips</td>
<td>3.0</td>
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<tr>
<td>LEONARD et al.</td>
<td>Human</td>
<td>22</td>
<td>5</td>
<td>-</td>
<td>91</td>
<td>100</td>
<td>50</td>
<td>Water</td>
<td>d2</td>
<td></td>
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<tr>
<td>EMR et al.</td>
<td>Human</td>
<td>10</td>
<td>1</td>
<td>5</td>
<td>NA</td>
<td>98</td>
<td>30</td>
<td>Water</td>
<td>Siemens</td>
<td>7.0</td>
<td></td>
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<tr>
<td>ANDRONESI et al.</td>
<td>Human</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>20</td>
<td>Cho + Gin</td>
<td>Siemens</td>
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<td>Short TE</td>
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<tr>
<td>CHOI et al.</td>
<td>Human</td>
<td>15</td>
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<td>-</td>
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<td>19</td>
<td>Water</td>
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<td>CHOI et al.</td>
<td>Human</td>
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<td>19</td>
<td>Water</td>
<td>Philips</td>
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<tr>
<td>LEONARD et al.</td>
<td>Human</td>
<td>22</td>
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<td>91</td>
<td>100</td>
<td>50</td>
<td>Water</td>
<td>d2</td>
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<tr>
<td>EMR et al.</td>
<td>Human</td>
<td>10</td>
<td>1</td>
<td>5</td>
<td>NA</td>
<td>98</td>
<td>30</td>
<td>Water</td>
<td>Siemens</td>
<td>7.0</td>
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<tr>
<td>ANDRONESI et al.</td>
<td>Human</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>20</td>
<td>Cho + Gin</td>
<td>Siemens</td>
<td>3.0</td>
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</table>

†Specificity estimated from IDH wild-type tumor voxels + healthy voxels. ‡Cut off Cramer-Rao lower bound for successful spectral fitting. §For 2HG concentrations of ≥ 1 mM. ¶For 2HG concentrations of ≥ 1 mM. Cho = choline, Cr = creatine, Gln = glutamine, Glu = glutamate, Healthy = healthy brain tissue, IDH = isocitrate dehydrogenase, MT = tissue from IDH-mutated tumor, NA = not available, sensitivity estimated from IDH-mutated tumor voxels, TE1 = 1st echo time, TE2 = 2nd echo time, TR = repetition time, WT = tissue from IDH-wild-type tumor.
5.2 AIMS

The main objective of this study is to setup and optimize non-invasive detection of 2HG in 1H-MRS by a novel difference spectroscopy sequence (MEGA-PRESS; Mescher-Garwood Point-Resolved Echo Spectroscopy Sequence) in a prospective cohort of patients before surgery for a suspected glioma.

Secondary objectives will be to determine sensitivity and specificity of this technique by comparison with genomic analysis of tumor samples and to correlate with 2HG tumor levels measured by gas chromatography-tandem mass spectrometry (GC-MS/MS).

Association with grade and genomic background will also be explored.

A supplementary cohort of IDH mutant glioma patients will be examined after surgery in order to determine sensitivity of MEGA-PRESS in this setting and to explore 2HG variations during anti-cancer therapies.
5.3 Diagnostic value of 2-hydroxyglutarate detection by 1H-MR spectroscopy before surgery in patients with glioma: correlations with tumor phenotype and tissue dosage.

5.3.1 INTRODUCTION

The overproduction of the oncometabolite 2-hydroxyglutarate (2 HG) in IDH mutated gliomas can be detected non-invasively by magnetic resonance spectroscopy (MRS) (Andronesi, 2012; Choi, 2012).

IDH mutations (involving IDH1 and IDH2 isoforms) occur in 70–90% of grade II and grade III gliomas, and depict a molecular background and biological behaviour which differ significantly from IDH-wild type gliomas without dependence on the grade (Parsons, 2008; Sanson, 2009).

For these reasons, IDH mutational status has been recently integrated in WHO classification of brain tumor (Louis, 2016) as a determinant molecular factor together with 1p19 codeletion for histological diagnosis. Moreover, IDH mutant protein may become druggable targets of new therapies that can inhibit the mutant protein.

For these reasons, detection of IDH mutations is crucial for diagnosis, prognosis and treatment planification.

Previous studies reported the feasibility of non-invasive detection of 2 HG by MR spectroscopy for concentration >1mM with different spectroscopic methods based on conventional sequences optimized for detection of 2 HG, or spectral editing (Pope, 2012; Andronesi, 2012; Choi, 2012).

Detection of 2 HG by MRS in a preoperative setting proved to be challenging and IDH mutant case series reported so far are relatively small. In addition, few data are available on correlations with 2 HG measured in tissue samples, as well as the molecular status and histological phenotype.
In this study, we used a MEGA-PRESS difference spectroscopy sequence (Mescher-Garwood Point-Resolved Echo Spectroscopy Sequence; (Mescher, 1998)), for 2 HG detection in a group of 27 patients before surgery for a suspected grade II and grade III glioma. We assessed specificity and sensitivity and we related the results to 2 HG concentration in tumor as well as tumor molecular status.

5.3.2 METHODS

Patients

We prospectively studied adult patients with intracranial gliomas who went to the Pitié-Salpêtrière Department of Neurosurgery for surgical resection or brain biopsy of their brain tumors. Collection of patient samples and clinico-pathological information was undertaken with informed consent and ethical board approval in accordance with the tenets of the Declaration of Helsinki. All subjects gave written informed consent. All patients displayed measurable disease on magnetic resonance imaging (MRI) for which surgical resection was warranted. Clinical classification and grading of the tissue was performed by a board-certified neuropathologist (Louis, 2016).

MRI/MRS Protocol

Acquisitions were performed using a 3-T whole-body system (MAGNETOM Verio, Siemens, Erlangen, Germany) equipped with a 32-channel receive-only head coil. The protocol included T2-weighted FLAIR and T1-weighted sequences for voxel placement and tissue segmentation. MRS data were acquired using a single-voxel MEGA-PRESS sequence optimized for 2 HG detection (TR=2s, TE=68ms, 128 averages, scan time = 9 min) with editing pulses applied at 1.9 and 7.5 ppm, for the edited and non-edited condition respectively, which allows for the measure of the 2 HG signal at 4.05 ppm.
Water suppression was performed using VAPOR and outer volume suppression techniques (Tkac, 1999). A non-water suppressed scan was acquired for quantification of absolute metabolite concentrations. Typical VOI size was 2x2x2 cm³ (Fig. 5. 1c, f). VOI size was adapted to tumor size in order to minimize partial volume effects, keeping a minimum size of 6 cm³.

**MRS Post-Processing**

Frequency and phase corrections were performed on single spectra based on the total choline signal at 3.2 ppm, using in-house written Matlab routines. Spectral quantification was performed using LCModel (Provencher, 2001) based on the water reference scan, assuming a bulk water concentration in tumors of 55.5 mM, and correcting for the water transverse relaxation time T2 (150 ms) (Madan, 2015). The Cramer Rao lower bounds (CRLB) threshold for reliable 2 HG detection was set to 30%.

**Tumor analysis and 2 HG tissue dosage**

Automated IHC was performed on 4-μm-thick FFPE sections with an avidin–biotin–peroxidase complex on Benchmark XT (Ventana Medical System Inc, Tucson AZ, USA) using the Ventana Kit including DAB reagent to search for the expression of \textit{IDH1} R132H (Dianova, H09), P53 (DAKO, DO.7), and \textit{ATRX} (SIGMA, polyclonal). Labeling was defined as positive (at least one cluster of positive tumor cells) or negative (no positive tumor cells detected) (Tabouret, 2016).

The QIAamp DNA Mini Kit was used to extract tumor DNA from frozen tumors, as described by the manufacturer (Qiagen, Courtaboeuf, France). DNA was extracted from blood samples using a conventional saline method.
Mutational status of \textit{IDH1}, \textit{IDH2} was determined using the Sanger technique, as previously described (Sanson, 2009). All cases in this series scoring negative for IDH1 R-132H immunostaining were analyzed for \textit{IDH1} and \textit{IDH2}.

The presence of 1p19q codeletion was assessed by CNV analysis by SNP array (9 cases) (Gonzalez-Aguilar, 2012) and a customs next-generation sequencing (NGS) targeted gene capture (11 cases).

\textbf{Metabolite Extraction and Analysis}

2 HG tissue levels were measured by gas chromatography-mass spectrometry (GC-MS/MS) using a Scion TQ instrument (Brüker) in full scan or MRM modes depending on metabolite concentration. Tissue samples were homogenized in bidistilled water and soluble protein concentration was measured by the BCA assay. All samples were treated by organic (ethylacetate) extraction and by a standard silylation protocol (BSTFA + 1% TMCS). Stable isotope internal standards were purchased from Cambridge Isotope Laboratories (2,3,3-D3-2 \textit{HG}). Inter-series coefficients of variation and linearity for 2 HG were <6% and >99% in the ERNDIM external quality control programs (http://www.erndimqa.nl). "D vs. L stereoisomer determination was performed by chiral derivatization and GC-MS/MS in MRM mode as previously reported (Janin, 2014).

\textbf{5.3.3 RESULTS}

\textbf{Patients}

Between October 2014 and July 2016, we prospectively included 27 consecutive patients that were suspected of having a grade II or grade III glioma prior to surgery at \textit{Pitié Salpêtrière} Department of Neurosurgery (26 patients) and \textit{Hopital Foch} (1 patient).
Of the 27 patients enrolled in this study, 25 were found to have good quality MRS: one patient was excluded because of a poor compliance during MR and one patient was excluded because of a small residual volume requiring voxel size<6 cm³ established as a minimum size for VOI in this study.

Patient’s characteristics and findings are summarized in Table 5.3.1. 24 patients underwent MRS before a median interval of 1 day before surgery. Only in one patient (Patient 19) was surgery delayed by 6 months because of the occurrence of pulmonary embolism and the start of anticoagulant therapy. All patients but one were included before their first surgery at initial diagnosis of a suspected glioma. Patient 21 was included before second surgery for a known oligodendroglioma, which was diagnosed and operated on in 2007. Recurring in 2010, he was treated with chemotherapy by temozolomide for 24 months and radiotherapy; after an interval of two years without any treatment he recurred and was then included in this study before undergoing his second surgery.

With the exception of Patient 21, no patients received any cancer therapy before inclusion in this study. Among the 25 patients, all underwent subtotal resection excepted two patients (Patient 23 and Patient 24) who were biopsied. Because of the small size of tumor biopsy, frozen tissue for 2 HG tissue dosage was not available for these two patients.

Median age at diagnosis was 38 years old (range 22-63); sex ratio was 1.2.

Genotyping assay for IDH found 21 IDH-mutant and 4 IDH wild-type. In the IDH mutant group integrated diagnosis according to WHO 2016 (Louis, 2016), resulted in diffuse astrocytoma grade II (7 patients), anaplastic astrocytoma (7 patients), oligodendroglioma (2 patients), anaplastic oligodendroglioma (3 patients), and glioblastoma IDH-mutant (2 patients) (Table 5.3.1). One patient (Patient 17) showed a particular phenotype IDH mutant, ATRX maintained, INA positive, p53 negative, hTERT C228T mutated, highly suggestive the presence of 1p19q which was finally not proved by NGS. In the IDH wild-type subgroup, one was a ganglioglioma, two were diffuse astrocytoma grade II and one was a glioblastoma.
Regarding the type of IDH mutation, twenty patients harbored IDH1 mutations (19 patients R132H, 1 patient R132G), one patient (Patient 20) harbored an IDH2 R172K mutation.

**MRI and MRS Studies**

Because of the principal inclusion criteria consisting of the suspicion of grade II or grade III glioma, operative structural MRI imaging characteristics were generally indistinguishable between IDH mutant and wild-type tumors as shown in Figure. 5.3.1 in C and D, respectively. Tumor size, surrounding edema and mass were not associated with IDH mutational status.

Localized MR spectroscopy using MEGA-PRESS of the areas of tumor revealed the presence of measurable 2 HG at 4 ppm.

Principal criteria to score patients as 2 HG positives was 2 HG concentration CRLB <30%.

20 patients of this series presented measurable levels of 2 HG and CRLB < 30%: they were then finally scored as 2 HG positives in the pre-operative setting. After surgery, each of these 20 patients were confirmed to harbor an IDH mutant glioma.

For these patients, range of 2 HG concentrations by MRS was comprised between 1.42 and 8.56 mM (median 3.34mM) (Figure 5.3.3 in A).

Inversely none of the four IDH wild-type glioma patients show any measurable 2 HG with high CRLB (99% in two patients, 65% and 66% in the remaining two).

CRLB value (31%) at limit of sensitivity were found in the remaining IDH1 R132H mutant patients (Patient 1): in this case, MRS was finally scored negative for 2 HG MRS detection (Table 5.3.1. and Figure 5.3.4.).

No false positive for IDH mutation were recorded by MRS.
Example of MEGA-PRESS spectra for one $IDH$-mutant and one $IDH$ wild-type are shown in Figure 5.3.1.

Twenty $IDH$ mutant patients had measurable levels of 2 HG by MRS, with low CRLB values, suggesting that the measurement of 2 HG was accurate. They were then scored as “2 HG positive” in MRS according to our quality criteria. Inversely, no false positive was recorded in this series (Table 5.3.1). MEGA-PRESS optimized for 2 HG detection show a sensitivity of 95% and specificity of 100% of prediction of the $IDH$ mutation in the tumor. The positive predictive value of the presence of the $IDH$ mutation for MEGA-PRESS was 100% (VPN 80%) (Table 5.3.2).

**Quantitative Measure of 2 HG and Other Metabolites**

Quantitative measures of 2 HG were available by MRS for 21 cases. Levels were comprised between a minimum of 1 nmol/mg to a maximum of 613 nmol/mg. At the tissue level, gas chromatography-tandem mass spectrometry (GC-MS/MS) quantitation of tumor-derived 2 HG shows higher levels of 2 HG in $IDH$ mutant compared to $IDH$ wild-type samples (Figure 5.3.3 in B). Range of tissue 2 HG was comprised between 1.7 nmol/mg and 613 nmol/mg in $IDH$ mutant (median 130 nmol/mg). The two $IDH$ wild-type for which a tissue dosage was available show levels at 1.0 and 2.9 nmol/mg. To note, known ranges from the previous report (Dang, 2009) had 2 HG levels ranging from 5 to 32 µmol/gr in $IDH$ mutated gliomas and from 0.045 to 0.68 µmol/gr in $IDH$ wild-type gliomas (Figure 5.3). Excepting for Patient 1, (presented in Figure 5.3.4), which is the only $IDH1$ mutant in this series with 2 HG lower than the range reported by Dang et al., all other $IDH$ mutant in this series displayed levels of 2 HG $\geq$ 18 nmol/mg.
2 HG tissue dosages by GC-MS/MS resulted then consistent with 2 HG MRS assessment because Patient 1 is the only IDH mutant patient scoring negative by MRS.

Regression analysis showed a significant correlation between 2 HG by MRS and tissue dosage ($r^2=0.27$; $P=0.015$) (Figure 5.3.2).

Next, to determine if histo-molecular background influences 2 HG accumulation we compared 2 HG detectable levels by the IDH mutant-1p19 codeleted gliomas ($IDH^+/1p19q+$) versus IDH mutant-non codeleted gliomas ($IDH^+/1p19q-$).

We did not find significant differences in 2 HG levels measured by MRS nor in the tissue between $IDH^+/1p19+$ and $IDH^+/1p19-$. MRS 2 HG levels resulted 2.8 mM $IDH^+/1p19+$ (range 1.67-8.5 mM; 4 patients) versus 3.8 mM in $IDH^+/1p19-$ (range 1.76-6.8 mM; 13 patients) (Figure 5.3.3 in C).

By GC-MS/MS, median tumor 2 HG levels resulted in 79 nmol/mg in $IDH^+/1p19+$ (range 45.9-154.0 nmol/mg; 5 patients) versus 135 nmol/mg in $IDH^+/1p19-$ (range 1.7-613 nmol/mg; 16 patients) (Figure 5.3.3 in D).

Finally, to determine if glioma grade affected 2 HG levels, we compared detectable levels of 2 HG between glioma grades. We observed a higher median level in grade IV (7.7 mM) than in grade III (3.3 mM) and grade II (3.2 mM). We could not perform the Mann-Whitney test because of the number of grade IV samples <3. A t-test showed a significant difference of means of 2 HG- $P=0.0001$ (grade IV versus grade II) and $P=0.003$ (grade IV versus grade II)-by MRS (Figure 5.3.3 in E).

From the tissue dosages analysis, we observed a trend to higher concentration of 2 HG in $IDH$ mutant GBM (median 371 nmol/mg; range 130-613 nmol/mg) versus grade III (median 125 nmol/mg; range 23.80-336 nmol/mg) and grade II (median 117 nmol/mg; range 1.7-316 nmol/mg), but these differences did not
raise level of significativity ($P=0.06$ and $P=0.08$ respectively) (Figure 5.3.3 in F). To note, Patient 25 harboring an $IDH$ wild-type glioblastoma showed low levels in the tissue (2.9 nmol/mg) and no measurable 2 HG by MRS.

**Impact of Surgery and Anti-Cancer Therapies in a Supplementary Cohort**

In order to explore reliability of non-invasive 2 HG measure by MRS in post-operative management of $IDH$ mutant glioma patients, we extended our analysis to a supplementary group of 35 patients known to be affected by an $IDH$ mutant glioma.

We analyzed these 35 patients by the same MRS protocol, as used in the pre-operative setting and previously describe (15 grade II, 15 grade III and 4 grade IV at the initial tumor).

Sixteen patients were scanned after surgery and before starting an anticancer therapy, 19 patients were scanned post-treatment during or after radiotherapy or chemotherapy.

Interestingly, we observed that sensitivity of non-invasive detection of 2 HG accumulation dramatically decreased to 43%.

Measurable levels of 2 HG, according to good quality criteria (CRLB<30%) were found in 15 patients out of 35.

Among cases scoring positive for 2 HG by MRS, we noticed that a higher fraction of patients 9/15 (60%) were scanned in a pre-treatment phase versus 7/20 (35%) in the group scoring negative of 2 HG at MRS.

Even if insignificant, this trend was consistent with a supplementary finding in the first patient who completed a follow-up period before and during radiotherapy followed by chemotherapy protocol.

This representative case is shown in Figure 5.3.6. The patient was affected by an anaplastic oligodendroglioma harboring $IDH$ mutation (Figure 5.3.6) and 1p19q codeletion and was treated by radiotherapy and adjuvant PCV after surgery. First MRS scan was performed after surgery and before starting radiotherapy, and then
repeated after radiotherapy and during chemotherapy protocol. During the follow-up we observed a striking and progressive reduction of 2 HG levels with regression of detectable levels of 2 HG after radiotherapy (4.1 mM with CRLB 10% at baseline; 2.2 mM with CRLB 24% 1 month after radiotherapy and 1.37 mM CRLB 35%, 5 months after radiotherapy and during PCV chemotherapy) as it showed by subsequent spectra 1 month after radiotherapy and during chemotherapy Figure 5.3.6.

Similarly, 9 patients in this series were included in a phase 1 escalating dose trial with specific inhibitors of IDH1 (AG120) and IDH2 (AG221) (Agios). Among them, 6 show measurable levels of 2 HG by MRS at baseline and then continued the follow-up with close MRS scans during the first month after the start of the treatment.

Average levels of 2 HG at different time points are illustrated in plot G in Figure 5.3.7 and 1 representative for the anti-IDH1 and the anti-IDH2 protocols are illustrated in Figure 5.3.7. The follow-up of these representative cases and of mean levels of 2 HG show an important and permanent decrease of 2 HG rapidly after the start of the specific anti- IDH target therapy.

Finally, in order to expand our analysis to a putative effect of genomic background of 2 HG levels, we combined 2 HG measurements by MRS in patients harboring glioma with IDH mutation from the pre-operative cohort (17 patients) and the post-operative cohort (35 patients).

In this larger series we confirmed the absence of significant differences in 2 HG concentrations by MRS between 1p19 codeleted gliomas (median 2 HG 3.8; range 1.6-9.2mM) versus 1p19 non codeleted gliomas (median 3.9; range 1.76-6.8) but, remarkably, we found that three patients (Patient 20 from the preoperative cohort) and two patients from the post-operative cohort harboring IDH2 K172 displayed significantly higher levels of 2 HG (median 2 HG 6.8mM, range 4.3-9.2mM) than IDH1 mutant (3.79mM; range 1.67-8.5mM) (P=0.03). Despite the small sample size (only 3 IDH2 mutant), differences resulted as being statistically significant and results are shown Figure 5.3.5.
Table 5.3.1. Clinical and histo-molecular features of the cohort with corresponding 2 HG by MRS and tissue dosage. CRLB means Cramer Rao lower bounds; na=not available.

<table>
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<tr>
<th>Pt</th>
<th>Sex</th>
<th>Age at diagnosis</th>
<th>Histological diagnosis</th>
<th>IDH status</th>
<th>1p/19q codetection</th>
<th>ATRX immunostaining</th>
<th>MRS 2 HG concentration (mM)</th>
<th>CRLB (%)</th>
<th>Tumor 2 HG concentration (mmol/g)</th>
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<td>31</td>
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<td>6</td>
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Table 5.3.2. Predictive value of 2 HG non-invasive detection by MRS in a prospective cohort of 25 patients scanned before surgery. IDH mutation status was assessed in IDH1 and IDH2. MRS by MEGA-PRESS proved to be highly specific (100%) and sensitive (95.2%). Positive predictive value was 100% and the negative predictive value 80% (chi2 19.05).
Figure 5.3.1. Examples of MEGA-PRESS Spectra, Voxel positioning and Sanger sequence of *IDH1* codon 132 of the tumor after resection, of one *IDH1* mutated glioma patient (in A, C and E) and one *IDH1* wild-type glioma patient in (B, D and F). MRS spectrum after differential editing shows a peak of 2 HG at 4ppm (in A) and the absence of the 2 HG peak in B. Sanger sequencing show the presence an hetozygote R-132H mutation of *IDH1* characterized by the appearance of a green peak in chromatogram corresponding to adenine at guanine position (black peak) in F *IDH1* wild-type sequence at codon 132.
Figure 5.3.2. Regression plot of 2 HG concentrations measured by MRS (X axis) and in the tumor GC-MS/MS (Y axis) in the subgroup of 21 patients with measurable levels of 2 HG on MRS (and CRLB<30%). Green Dots correspond to grade II, blue dots to grade III and red dots to grade IV. 2 HG concentrations in MRS display a significant correlation with concentration in the tumor ($r^2=0.27; P=0.015$).
Figure 5.3.3. 2-HG measurable levels in the tissue, and by MRS according to IDH status (A and B), molecular subgroup (C and D) and grade (E and F). Each symbol represents the amount of the listed metabolite found in each tumor sample. Black bars indicate the group sample means.

In plot A, B, C, D green dots correspond to grade II, blue dots correspond to grade III and red dots correspond to grade IV. In plot E and F violets dots correspond to IDH wild-type sample. Plot A, C, D corresponds to dosages by MRS. Only measurable levels of 2-HG with CRLB<30% are reported.

Plots D, E, and F correspond to dosages by GC-MS/MS analysis.

In A and B gliomas are stratified according to the IDH status.

In C and D, gliomas are stratified according to the molecular subgroup (IDHwt versus IDH mut1p19q codeleted versus IDH mutant 1p19q non codeleted).

In E and F, gliomas are stratified according the grade.

In grade IV, by MRS, 2-HG mean level was significantly higher than grade III and grade II by t-test ($P=0.0001$ and $0.003$ respectively).

In grade IV, tumor 2-HG shows a trend to higher concentrations (median 371 nmol/mg; range 130-613 nmol/mg) versus grade III (median 125 nmol/mg; range 23.80-336 nmol/mg) and grade II (median 117 nmol/mg; range 1.7-316 nmol/mg) ($P=0.06$ and $P=0.08$ respectively).

No differences were observed according to the molecular subgroups IDH+/CodeL+ versus IDH+/CodeL- subgroups.
Figure 5.3.4: MRS and Spectra obtained by Mega-Press in Patient 1. Detection of 2 HG peak is at limit of sensitivity (CRBL 31%) and then MRS was scored negative for 2 HG detection. This patient correspond the only false negative case of this series (Tab2). Interestingly, the average percentage of tumor cells expressing the mutant form of IDH1 R132H was around 10%, with heterogeneous areas scoring from 50% and 1% labeled tumor cells (100% of tumor cells). Contamination by non-tumor cells was present. Molecular analysis showed a small peak in chromatogram corresponding to the mutant allele of IDH1 in codon 132 and NGS analysis assessed a fraction of 0.048% (37/761) mutant reads. Molecular and histological findings in this patient suggest a sort of dilution with wild-type IDH1 DNA and protein and could explain in part the negative result of MRS.
Figure 5.3.5. 2 HG measurable levels by MRS in 32 IDH mutant gliomas (17 from the pre-surgery and 15 from the post-surgery subgroups) according to IDH mutation. Levels distribution analysis show significant higher levels in the 3 patients harboring IDH2 K172 mutations (median 2 HG in IDH1 mutant 3.79mM versus 6.80 mM in IDH2 mutants; P=0.03)
Figure 5.3.6 2 HG permanent decrease during radiochemotherapy. A representative case of a 50-year-old patient affected by an IDH mutant 1p19q codeleted anaplastic oligodendroglioma that was scanned by MRS after surgery and then every month during anticancer treatment.

In A) SNV plot of tumor DNA showing a complete loss of small arm of 1p and long arm of 19q (indicated by the arrows), In B) Sanger sequence showing the hetozygous mutation in codon 132 of IDH1.

Axial and coronal FLAIR imaging before starting radiotherapy and 1 months later and 5 months later during concomitant chemotherapy by PCV (are shown in C, D and E respectively).

MRS Spectra obtained by Mega PRESS before RT (in F), 1 month after radiotherapy and 4 5 months after radiotherapy shows a significant decrease of 2 HG from 4.1 mM (CRLB 10%) to undetectable levels.
Figure 5.3.7 2 HG decrease specific anti IDH1 and anti IDH2 therapies.
In the left block a representative case of a 36 patient harboring an IDH mutant glioma. In A Flair imaging at baseline and in B 2 HG levels measured at different time points are consistent with the reduction of 2 HG peak observed rapidly at 3 days (C2) and 29 days (C3).
A similar results observed in Patient Idaspe 14 (54 years old) harboring an IDH2 mutation and treated by an anti IDH2. Baseline and subsequent spectra (F1, F2, F3 and F4) showed a prompt and persistent reduction of 2 HG at 4 ppm.
Average levels of the 6 patients included in specific anti-IDH therapies are reported in G, showing the permanent reduction of 2 HG after the start of target therapies.
5.3.4 DISCUSSION

The quantification of the 2 HG metabolite through MRS represents an appealing imaging biomarker in glioma because it directly measures the product of the mutant enzyme, which is highly useful for diagnosis, treatment planification and prognosis. Moreover, the fascinating perspective of realizing the pharmacodynamics measure of activity of anti-IDH therapies at the tumor level in real time makes this technique appealing despite its technical challenges.

A number of approaches and methodologies have been reported (PRESS; LASER, COSY) with high levels of sensitivity (100%) and lower levels of specificity 70-90% (see Table 5.3.2).

In this study we report an original technique for 1H-MRS non-invasive detection of 2 HG by MEGA-PRESS as a reliable tool to predict IDH mutation. Using stringent criteria as CRLB<30%, MEGA-PRESS proves to be highly precise (100%) and with sensitivity of around 95%.

Consistently, 2 HG levels assessment by MEGA-PRESS resulted in a good correlation with 2 HG tissue dosages.

Based on our data and previous reports (Dang, 2010; Andronesi, 2012) most tumor levels of 2 HG seem to be high enough to grant detection by in vivo MRS, however there may be a number of tumors in which levels could be around or below the 1-mM detection threshold, conclusively affecting sensitivity. Indeed, one IDH mutant glioma in our series showed 2 HG levels and CRLB at the lower limit of detectability and was finally judged as negative by MRS. In this patient, we found a very low level of 2 HG in the tissue and an important contamination by non-tumor cells at histological and genomic level. Consistently with our observation, density of tumor cells has been recently suggested as a factor influencing 2 HG measurement by MRS (de la Fuente, 2016).
This point may intuitively explain the dramatic reduction of sensitivity at 43% of MEGA-PRESS in detection of 2 HG after surgery, as we observed in our supplementary series of post-operative patients.

We investigated other potential factors correlating with 2 HG levels and we observed that high-grade IDH mutant gliomas show higher levels of 2 HG as well as IDH2 mutation. The 1p19 codeletion does not seem to influence 2 HG levels. However, the rare IDH2 mutation, which is associated with 1p19q codeletion, appears associated with higher levels of 2 HG.

Finally, our preliminary result on radiological follow-up of IDH mutant patients during radiochemiotherapy suggests that one of the major factors that affect 2 HG level are cancer therapies, as shown in our representative cases and also other reports (Andronesi, 2012). Further studies might elucidate duration of such a decrease, the presence of differential response of 2 HG to specific chemo-radiotherapy regimens and more interestingly if 2 HG decreases may be a predictor of tumor response, clinical benefit and/or tumor progression.

More fascinatingly, we report here our preliminary results on 2 HG as dramatically and rapidly decreasing with anti-IDH therapies. This observation proves that MEGA-PRESS is a reliable and reproducible method for 2 HG assessment and open fascinating field of research on the value of this variation in IDH mutant response and resistance to target therapy.
6. CONCLUSIONS AND PERSPECTIVES

To date, driver oncogenes remain the most appealing targets for development of innovating therapies and new diagnostic tools.

In this study, we dichotomized the field of research in the two main nosological entities identified in glioma so far: the *IDH* mutant and *IDH* wild-type, and we developed a novel approach for target therapies and non-invasive molecular diagnosis.

In the first study we proposed a molecular prognostic classification of gliomas basing on *IDH* and *pTERT* mutational status and we also reported a striking parallelism of germ line variants which are differently associated to the risk of developing specific subtypes of glioma mainly according the *IDH* status.

In the second study focused on *IDH* wild-type gliomas, we reported an unbiased screening assay for *FGFR-TACC* fusions that occur in grade II and III glioma harboring wild-type *IDH1*, with frequency similar to glioblastoma (GBM), therefore providing a clue to the aggressive clinical behavior of this glioma subtype in glioma.

Our RT-PCR based assay overcomes the great variability of variants that are generated by *FGFR-TACC* chromosomal translocation in human cancer. Thanks to the largest screening performed so far, we identified the highest number of cases harboring *FGFR-TACC* gene fusions and we were able to show that *FGFR-TACC* fusions display a specific genomic background, which is mutually exclusive with *IDH* and *EGFR* amplification, but co-occurs with *CDK4, MDM2* amplification and 10q loss.

We showed that *FGFR-TACC*-positive glioma displays strikingly uniform expression of the fusion protein and, judging from the high specificity of FGFR3 staining, we found that FGFR3 IHC could be considered a reliable method for prescreening. Preclinical experiments with *FGFR3-TACC*-positive glioma cells treated with an anti-FGFR TK inhibitor (JNJ-42756493) showed strong anti-tumor effects and treatment of two patients with recurrent GBM harboring
FGFR3-TACC3 resulted in clinical improvement and radiological tumor reduction.

We also showed that FGFR3-TAC3 fusions are associated with longer survival in GBM and given the promising therapies we are testing in this setting, rechallenge and concomitant administration of target TK inhibitors may be a relevant option to be tested to produce a clinically relevant benefit in this selected subgroup of patient.

In perspective, we will then move to analysis of clinical efficacy of anti-FGFR specific target therapies and of eventual mechanism of resistance.

In the third study, we developed and validated novel methods for 2 HG detection in IDH mutant tumors and we showed that this technique is highly specific and sensitive in pre-operative stages and that it could be integrated in diagnostic work-up.

In the post-operative phase 2 HG seems to be, in a number of patients, an interesting new imaging marker to monitor. Further studies will answer the question on the predictor value of this variation of response and survival.

In conclusion, our findings may have contributed to enrich the repertoire of target driver oncogenes of therapeutic relevance in glioma and improve access towards innovating diagnostic tools in the field of neuro-oncology.
7. ANNEXES

In this section, we report twelve articles and studies where we contributed, from November 2013, as authors or co-authors, and that are annexes to the PhD research project on molecular markers of gliomas presented in this thesis.
Predictive biomarkers in adult gliomas: the present and the future

Laure Thomas*, Anna L. Di Stefano¹, and François Ducray³⁶

Purpose of review
This review summarizes recent studies on the predictive value of molecular markers in adult gliomas, including 1p/19q codeletion, MGMT methylation, IDH mutation and markers identified using omics and next-generation sequencing studies.

Recent findings
The long-term results of the Radiation Therapy Oncology Group and European Organization for Research and Treatment of Cancer trials in anaplastic oligodendroglioma gliomas have shown that the 1p/19q codeletion predicts an overall survival benefit from early PCV (procarbazine CCNU vincristine) chemotherapy. This benefit can also be predicted using gene expression-based molecular subtypes of gliomas while the predictive value of the IDH mutation in this context requires further study. In elderly patients with glioblastoma, the analysis of MGMT methylation status in two phase III trials suggests that this alteration may guide treatment decisions; however, this finding still needs confirmation in prospective studies. Omics and next-generation sequencing studies have identified additional potential predictive markers. In particular, IDH mutations, BRAF V600E mutations and FGFR gene fusions might predict efficacy of therapies targeted against these alterations.

Summary
Currently, the 1p/19q codeletion is the only well-established predictive marker with clinical utility.
However, it is likely that other molecular markers such as MGMT methylation, IDH mutation and those identified using omics and next-generation sequencing studies will further guide treatment decisions in adult gliomas.

Keywords
1p/19q codeletion, glioma, IDH mutation, MGMT methylation, predictive biomarker

INTRODUCTION
Until recently, treatment decisions in glioma were based on clinical, radiological and histological factors with little use of predictive molecular markers [1]. When treating anaplastic gliomas, many neurooncologists used the 1p/19q codeletion status for care decisions but this strategy was not supported by strong evidence [2]. In glioblastoma patients less than 70 years, MGMT methylation predicts a stronger benefit of temozolomide radiochemotherapy, but is not decisional in this population, as unmutated patients can benefit from this regimen [3].

Now, the long-term results of the Radiation Therapy Oncology Group (RTOG) and European Organization for Research and Treatment of Cancer (EORTC) trials have clearly established that the 1p/19q codeletion predicts a benefit for early chemotherapy in anaplastic gliomas, and two phase III trials suggest that MGMT methylation should guide treatment decisions in elderly patients with glioblastoma [4-7]. In addition, omics and next-generation sequencing studies have identified gene expression signatures and molecular markers that could predict treatment efficacy, particularly when using targeted therapies [8-10].

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In the mid 1990s, two phase III studies—one by the RTOG and the other by the EORTC—were initiated to test if adding PCV (procarbazine, CCNU, vincristine) chemotherapy to radiotherapy as part of the initial treatment might improve the overall survival (OS) of patients with anaplastic oligodendroglial tumors (AOTs). The first results of these two studies, published in 2000, showed that PCV and radiotherapy increased progression-free survival (PFS) but did not affect OS and that this outcome was true irrespective of 1p/19q status [29, 30]. After a median follow-up of 11 years, the long-term results of both studies now clearly demonstrate that 1p/19q codeletion is not only a major prognostic factor but also a major predictive factor of early chemotherapy benefit [4, 5]. In patients with 1p/19q codeleted tumors, PCV and radiotherapy resulted in a nearly three-fold increase in median PFS compared with radiotherapy alone (8.4 years versus 2.9 years in the RTOG study; 13 years versus 4.1 years in the EORTC study) and in a two-fold increase of the median OS (14.7 years versus 7.3 years in the RTOG study; median OS not reached versus 9.3 years in the EORTC study). In contrast, early PCV was associated with only a modest increase in PFS with no effect on OS in non1p/19q-codeleted tumors. Therefore, the updated results of the RTOG and EORTC study define radiotherapy and PCV as the new standard of care for newly diagnosed 1p/19q-codeleted AOTs.

However, the survival curves of patients with 1p/19q-codeleted AOTs treated with radiotherapy alone or radiotherapy and PCV only began to diverge at 5 years, which suggests that some 1p/19q-codeleted tumors are not sensitive to PCV. As such, additional biomarkers need to be identified to help predict the benefit of early PCV chemotherapy in these tumors. Although radiotherapy and PCV did not improve median OS in non1p/19q-codeleted tumors, the survival curves indicated a higher rate of long-term survival in this group compared with those in the group that received radiotherapy alone. Because noncodeleted tumors that harbor IDH mutations are associated with a far better prognosis than non1p/19q-codeleted tumors that express wild-type IDH, assessing the value of IDH status to predict early chemotherapy benefits in non1p/19q-codeleted tumors would be interesting [16]. In the EORTC study, robust conclusions could not be made because of the limited number of patients with AOTs that were both non1p/19q-codeleted and IDH mutated. Further molecular analyses from the EORTC study are expected. Interestingly, a translational study on the EORTC study demonstrated that the benefit of early PCV chemotherapy could be more reliably predicted by tumor gene expression profiles [8]. One hundred and forty-four AOTs were assigned to main glioma subtypes (called intrinsic glioma
subtypes (LGs) previously identified based on unsupervised gene expression analysis. Tumors assigned to ICG-9 (which was enriched in 1p/19q codeleted tumors) significantly benefited from adjuvant PCV. A similar (although nonsignificant) trend was observed for tumors assigned to IGS-17 (which was enriched in IDH mutated but non 1p/19q codeleted tumors).

In low-grade gliomas (LGGs), 1p/19q codeletion also has a strong prognostic value. The median survival duration is 12–15 years in the presence of the 1p/19q codeletion and 3–8 years in the absence of the codeletion [1]. In the largest published series of patients treated with up-front temozolomide, the 1p/19q codeletion was associated with both a higher response rate (72% versus 46%) to chemotherapy and a longer duration of response (>40 versus 20 months) [31]. However, its predictive value needs to be confirmed in prospective clinical trials [32]. Analysis of the outcome according to the 1p/19q codeletion in the RTOG phase III trial (radiotherapy versus radiotherapy and PCV in unfavorable LGGs) and in the EORTC/NCIC phase III trial (up-front radiotherapy versus up-front temozolomide in progressive LGGs) are expected to clarify the predictive and clinical values of 1p/19q in LGGs.

**O6-METHYLGUANINE-DNA METHYL TRANSFERASE**

MMMT silencing through methylation of its promoter induces low expression of MGMT protein and decreases DNA repair activity, which increases sensitivity to alkylating agents. MGMT methylation occurs in approximately 40% of glioblastomas. In 2005, it was identified as both a strong prognostic and predictive marker in glioblastomas treated with temozolomide radiochemotherapy [33]. However, assessment of MGMT methylation was not incorporated into treatment decisions because MGMT methylation was only demonstrated to be a quantitative predictive marker. Indeed, when the effect of concomitant adjuvant temozolomide versus radiotherapy alone was evaluated in the EORTC/NCIC 26981/22981 glioblastoma trial, the benefit of radiochemotherapy was higher in MGMT-methylated patients (23.4 versus 15.3 months, *P* = 0.004) than in MGMT-unmethylated patients (12.6 versus 11.8 months, *P* = 0.035). However, the latter group still benefited from this treatment, particularly in terms of long-term survival [34].

A retrospective analysis of MGMT methylation was recently performed in two phase III trials, the NOA-8 and the Nordic trials, which evaluated the effect of radiotherapy versus temozolomide chemotherapy in elderly glioblastoma patients [67**,77**]. MGMT methylation was analyzed in 56 and 69% of patients in the NOA-8 and the Nordic trial, respectively. In the NOA-8 trial (patients >65 years), MGMT-methylated patients had a longer PFS when treated with temozolomide as opposed to radiotherapy (8.4 versus 4.6 months, *P* = 0.01), whereas MGMT-unmethylated patients had a longer PFS when treated with radiotherapy than with temozolomide (4.6 versus 3.3 months, *P* = 0.01). A similar yet not significant trend was observed for OS. The Nordic trial (patients >60 years) showed a nonsignificant trend toward longer OS in MGMT-methylated patients when treated with temozolomide rather than with radiotherapy (hazard ratio = 0.64, *P* = 0.07), but MGMT-unmethylated patients did not fare better when treated with radiotherapy than with temozolomide. PFS data were not available for this trial, as these data were deliberately not collected. These results, together with another retrospective study, suggest that MGMT methylation could guide treatment decision in elderly patients with glioblastoma [35**]. However, the data are not yet robust enough to be translated into the clinic and need prospective validation. The results of the NCIC/EORTC phase III trial (accelerated radiotherapy versus accelerated radiotherapy and temozolomide) are awaited to clarify the predictive value of this alteration in elderly patients with glioblastomas and the place of combined radiochemotherapy in this population.

Until now, the use of MGMT methylation as a predictive factor has also been limited by the fact that the optimal technique to study MGMT methylation is still debated. In a recent study, the rate of MGMT-methylated patients varied from 33 to 60% depending on the method that was used [36**]. Methylation-specific PCR has been used as a standard to study MGMT methylation, but this method is only qualitative and lacks automation. Therefore, alternative semiquantitative and quantitative techniques have been developed. However, these techniques do not study exactly the same regions of the MGMT promoter. Therefore, as the methylation pattern of the promoter can be heterogeneous, some patients are classified as methylated or as unmethylated depending on the technique used [36**]. Two recent studies suggested that pyrosequencing was the most reliable technique and had the best predictive value [36**,**37**]. Interestingly, both the methylation-specific PCR and the pyrosequencing technique analyze a region of the MGMT promoter, whereas methylation was recently shown to display the highest negative correlation with MGMT expression and the highest positive correlation with survival [36**].
Brain and nervous system

In contrast to glioblastomas, MGMT methylation in anaplastic gliomas appears until now only as a prognostic factor without predictive value [39,40]. This may be explained by the fact that, in these tumors, MGMT methylation is tightly associated with IDH mutation and a diffuse hypermethylated phenotype [38**-41].

**IDH1/IDH2 MUTATIONS**

Isocitrate dehydrogenase mutations have a strong diagnostic and prognostic value in adult diffuse gliomas but no clear predictive value [42]. Whether IDH mutated non-p19q-codelated anaplastic gliomas benefit from early PCV chemotherapy remains to be determined. The IDH mutation has been suggested to predict the response to first-line temozolomide chemotherapy in low-grade gliomas, but this finding requires confirmation [43]. An analysis of the outcome according to the IDH mutation in the RTOG phase III trial (radiotherapy versus radiotherapy and PCV in unfavorable LGGs) and the EORTC/NCIC phase III trial (up-front radiotherapy versus up-front temozolomide in progressive LGGs) is expected to clarify the predictive value of IDH mutation in LGGs. However, IDH mutation might predict the efficacy of mutant IDH inhibitors in the future. Important advances have been achieved in the understanding of the pathophysiology of IDH mutations. As suggested by the distribution of its mutations, the mutated IDH1 protein has been shown to be oncogenic [44**]. IDH1 mutations result in the abnormal production of 2-hydroxyglutarate (2-HG) that is structurally similar to α-ketoglutarate. 2-HG competitively inhibits multiple α-ketoglutarate enzymes leading to histone and DNA hypermethylation, altered cell differentiation and activation of enzymes implicated in hypoxia-inducible factor (HIF) degradation [45**-47**]. Most interestingly, a selective R132H-IDH1 inhibitor has recently been shown to specifically impair the growth of IDH1-mutant glioma cells and promote their differentiation [48**].

**NEW PREDICTIVE BIOMARKERS IDENTIFIED THROUGH OMICS AND NEXT-GENERATION SEQUENCING STUDIES**

Omics studies have enabled the classification of gliomas into molecularly homogeneous subgroups that are of prognostic value, independently of histology [49]. These subgroups could also be of predictive value. Erdem-Esenalp et al. [50] demonstrated that the gene expression profiles of anaplastic glioma could be used to predict the benefit of early PCV chemotherapy. In glioblastomas, Verhaak et al. [50] suggested that the molecular classification into four subclasses (classical, mesenchymal, pro-neural, and neural) predicts the benefit of a more intensive treatment. Patients with mesenchymal and classical glioblastomas showed increased OS when treated with temozolomide radiochemotherapy and/or more than three cycles of adjuvant temozolomide, which was not the case for patients with proneural glioblastomas. The classical subclass has also been suggested to be associated with a higher response rate to first-line chemotherapy [51]. However, these results require validation in prospective studies. Extensive molecular studies have also identified recurrent alterations that could predict the efficacy of targeted therapies. BRAF alterations were first identified in the majority of pilocytic astrocytomas suggesting that these tumors might be candidates for BRAF inhibitors [52,53]. In these tumors, the most frequent alteration is a KIAA1549-BRAF fusion transcript. This alteration is also found in a small subset of diffuse gliomas, in particular 1p/19q-codelated oligodendrogliomas [54]. Subsequently, activating BRAF V600E mutations were found in two-thirds of pleomorphic xanthoastrocytomas and gangliogliomas as well as in 50% of epithelioid glioblastomas (an uncommon glioblastoma variant not recognized in the WHO 2007 classification) [55,56,57,58,59]. However, this mutation is rare (<5%) in other diffuse gliomas, including glioblastomas. Interestingly, this mutation can be easily assessed using a V600E mutation-specific antibody and a small case study suggested that vemurafenib (a potent BRAF inhibitor with clinically meaningful activity against BRAF-mutated metastatic melanoma) might be active in adults with recurrent pleomorphic xanthoastrocytomas that showed a BRAF mutation [60,61**]. MET is amplified in approximately 5% of glioblastomas and could predict the efficacy of crizotinib, an oral small-molecule inhibitor of the MET and anaplastic lymphoma kinase (ALK) tyrosine kinases that is highly effective in lung cancers with ALK translocation [62]. Oncogenic EGFR alterations are much more frequent (40%), but anti-EGFR therapies have failed to demonstrate clinical activity until now. However, novel EGFR tyrosine kinase inhibitors could be more effective [63**]. Finally, high-throughput RNA sequencing studies identified recurrent activating EGFR fusion transcripts in a small subset of glioblastomas (3%) that might be good candidates for the use of anti-EGFR-targeted therapies [64,65**].

**CONCLUSION**

Major advances have been achieved in the molecular characterization of adult gliomas, which led to
the identification of strong diagnostic and prognostic markers. Predictive biomarkers are more difficult to identify and need to be validated in prospective clinical trials before being translated into the clinic. Currently, the 1p/19q codeletion is the only well-established predictive marker with clinical utility. However, it is likely that other molecular markers, such as MGMT methylation, IDH mutation and those identified using omics and next-generation sequencing studies, will further guide treatment decisions for gliomas in the near future. Because many of these new predictive biomarkers will most likely be present in only small subsets of patients, facilities that can perform prospective high-throughput molecular analyses of gliomas will be necessary [64].

Acknowledgements
A.I.D.S. is supported by investigator fellowship from Collegio Gliomai, Turin, Italy.

Conflicts of interest
There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of outstanding interest
3. This study describes the patterns of treatment over time in a retrospective study of 130 patients with oligodendroglial tumours from 1987 and 2007.
4. Within M. Dicrew, the MGMT status is in a monolayer of two, two
5. van der Milt FB, Brandt MA, Taphoorn MJ, et al. Adjunct radiation,
6. Monti, and concurrent chemotherapy in newly diagnosed anaplastic
7. oligodendrogliomas: a long-term follow-up of the EORTC tumor group study 26951.
8. This study examined the use of radiation and PCV in the new standard of care for newly diagnosed 1p/19q codeleted anaplastic
9. oligodendroglioma, and demonstrated that the 1p/19q codeletion is a strong predictor of early PCV chemotherapy benefit in these tumors.
12. The long-term results of the RO10 6403 trial defined radiotherapy and PCV as the new standard of care in newly diagnosed 1p/19q codeleted anaplastic oligodendroglioma. This study demonstrated that 1p/19q codeleted anaplastic oligodendrogliomas are associated with a long-term survival benefit in the overall population.
13. This study demonstrated that 1p/19q codeleted anaplastic oligodendrogliomas are associated with a long-term survival benefit.
Anna Luisa Di Stefano
Acute late-onset encephalopathy after radiotherapy: An unusual life-threatening complication
Paul J. Regal, Anna L. Di Stefano, Giulia Berzero, et al.
Neurology 2014;82;1102
DOI 10.1212/WNL.0000000000000201

This information is current as of March 24, 2014

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://www.neurology.org/content/82/12/1102/full.html

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ACUTE LATE-ONSET ENCEPHALOPATHY AFTER RADIOTHERAPY: AN UNUSUAL LIFE-THREATENING COMPLICATION

Paul J. Regal, Newcastle, Australia; Di Stefano et al. reported 5 patients who developed severe cognitive encephalopathy 9 months to 17 years after whole-brain radiotherapy for brain tumors. MRI, CSE, EEG, and other laboratory tests excluded almost all other potential causes for encephalopathy. The favorable response to IV methylprednisolone in 2-4 days further restricted the field of potential causes. The authors decided that brain biopsy, a potential gold standard, was not necessary. Brain autopsy on the 2 patients who died 2-2.5 years after autopsy was not undertaken. I wonder whether the authors estimated the denominator—the number of patients treated with whole-brain radiotherapy—to yield these 5 cases. In addition, it would be interesting to know the incidence of other forms of encephalopathy after radiotherapy. From my research in the Central Coast Australia Dementia Intervention Study—a prospective randomized controlled trial for subjects age 65—both the informant-rated instrumental activities of daily living and informant-rated apathy evaluation score declined significantly in the days prior to delirium. Perhaps the authors could provide the cognitive scores before encephalopathy, on admission, and after recovery.

Author Response: Anna L. Di Stefano, Giulia Berzera, Enrico Marchioni, Davide, Italy. We thank Dr. Regal for his comments. Peculiar characteristics of acute late-onset encephalopathy after radiotherapy (ALERT) syndrome are acute onset and rapid response to steroids. We agree with the value of neurocognitive findings in the setting of unusual complications of radiation therapy. These were not available in our retrospective case series. Two patients did not show MRI abnormalities attributable to brain biopsy. In the remaining patients, brain biopsy was not performed because of patients’ clinical conditions at the time of ALERT syndrome and the subsequent improvement and MRI normalization after high-dose steroids. In this setting, we judged brain biopsy as an invasive procedure exposing patients to a disproportionate risk.

Patients 1 and 3 died in hospital not within our one of acute respiratory distress due to pulmonary infection. They had no more neurologic symptoms or MRI abnormality. Although autopsy could have been informative, it was not performed. We intend to collect neuropsychological specimens from patients presenting with similar symptoms from all sites wishing to collaborate with us. As a retrospective study, neuropsychological tests were not strictly performed. From 1998 to 2011, patients were admitted into 4 different neurologic sites at the onset of neurologic symptoms. We cannot correctly estimate the number of patients receiving whole-brain radiotherapy in origin departments but we suggest that a more informative denominator would be the number of long-surviving patients after brain irradiation. To answer this, we are conducting a prospective clinical study on long survivors after brain irradiation to explore the underlying mechanisms of acute encephalopathy and its relationship with dementia postirradiation complications.

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WHITE MATTER HYPERINTENSITIES ON MRI IN HIGH-ALTITUDE U-2 PILOTS

Julian Hoffmann-Regen, Kim Hinkelmann, Francenca Regen, Berlin, Germany; McGuire et al. presented imaging data on the prevalence and location of white matter hyperintensities (WMHs) in MRI scans of high-altitude pilots. The authors demonstrated that WMHs differ between the pilot group and a matched group of healthy controls. They concluded that these differences may be the result of hypoxia in the pilot group. While
Acute late-onset encephalopathy after radiotherapy: An unusual life-threatening complication
Paul J. Regal, Anna L. Di Stefano, Giulia Berzero, et al.
Neurology 2014;82:1102
DOI 10.1212/WNL.000000000000201

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Parametric Response Maps of Perfusion MRI May Identify Recurrent Glioblastomas Responsive to Bevacizumab and Irinotecan

Domenico Aquino¹, Anna Luisa Di Stefano¹,², Alessandro Scotti³,², Lucia Cuppini⁴, Elena Anghilieri⁵, Gaetano Finocchiaro⁴, Maria Grazia Bruzzone⁴, Marica Edo²

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Abstract

Background: Perfusion weighted imaging (PWI) can be used to measure key aspects of tumor vascularity in vivo and recent studies suggest that perfusion imaging may be useful in the early assessment of response to angiogenesis inhibitors. Aim of this study is to compare Parametric Response Maps (PRMs) within the Region Of Interest (ROI) approach in the analysis of tumor changes induced by bevacizumab and irinotecan in recurrent glioblastomas (rGBM), and to evaluate if changes in tumor blood volume measured by perfusion MRI may predict clinical outcome.

Methods: 42 GBM patients with KPS ≥50 were treated until progression, as defined by MRI with RANO criteria. Relative cerebral blood volume (rCBV) variation after 8 weeks of treatment was calculated through semi-automatic ROI placement in the same anatomic region as in baseline. Alternatively, rCBV variations with respect to baseline were calculated into the evolving tumor region using a voxel-by-voxel difference. PRMs were created showing where rCBV significantly increased, decreased or remained unchanged.

Results: An increased blood volume in PRM (PRM rCBV) higher than 18% (first quartile) after 8 weeks of treatment was associated with increased progression free survival (PFS) 24 versus 13.9 weeks, p = 0.045) and overall survival (OS) 38 versus 25 weeks, p = 0.016). After 8 weeks of treatment, ROI analysis showed that mean rCBV remained elevated in non responsive patients (4.81 ± 0.9) versus 5.1 ± 1.2, p = 0.38), whereas decreased in responsive patients (4.2 ± 1.3 versus 3.5 ± 1.6, p = 0.044), and re-increased progressively when patients approached tumor progression.

Conclusions: Our data suggest that PRMs can provide an early marker of response to antiangiogenic treatment and warrant further confirmation in a larger cohort of rGBM patients.

Introduction

Glioblastomas (GBM) are highly vascularized tumors, leading to development of the angiogenesis-targeting antiangiogenic drugs (1). Bevacizumab, a monoclonal antibody targeting the vascular endothelial growth factor (VEGF), has recently entered into the clinical arena and it represents one of the most promising available antiangiogenic drugs (2). Despite the number of studies based on GBM treatments with bevacizumab, there is no combination with other drugs, in vivo modifications induced by treatment are poorly defined (3). Moreover, although the highly variable response to bevacizumab, currently, there are no prospectively validated predictive or prognostic biomarkers for it (4).

Perfusion weighted imaging (PWI) can be used to measure key aspects of tumor vascularity in vivo and recent studies suggest that perfusion imaging may be useful in the early assessment of response to angiogenesis inhibitors. Sommers, studying recurrent GBM patients treated with bevacizumab, an inhibitor of the VEGF receptor-positive glioblastomas, calculated a “vascular normalization index” by combining the change in the contrast agent (CA) microvascular volume and circulating collagen IV and found that this index increased 1 day after treatment initiation, and became associated with overall survival (OS) in recurrent GBM patients (5).
are better correlated with treatment response than enhancing tumor size [9].

In 16 patients with recurrent GBM treated with bevacizumab, Bonelli observed that mean rCBV, mean leakage coefficient, and hyperperfusion volume (HPV), defined as the fraction of tumor with an rCBV above a pre-specified threshold, correlate with time to progression [2].

Parametric Response Maps (PRM) are voxel-wise analytic approach to quantify significant regional changes in tumor physiology after therapy [8,9].

The aim of this work is to compare PRMs with the classical Region Of Interest (ROI) approach [10] in the assessment of tumor changes induced by bevacizumab and nimotuzumab in recurrent GBM, and to evaluate if changes in tumor blood volume measured by perfusion MRI may predict clinical outcome [11].

Methods
Ethics statement
All patients in the current work are part of a study carried out according to the Italian Decree Law of May 6th, 2003 allowing treatment of patients with no other therapeutic option, with drugs not yet approved by the Italian Regulatory Agency, but with evidence of efficacy in phase II clinical trials [11]. The protocol was approved by the Ethics Committee of the Neurological Institute “Carlo Besta” of Milan and registered in the Institute database (Protocol 08/2005). All patients gave written informed consent. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Patients
Forty-six of the 50 patients who underwent the same MRI protocol, were enrolled [11]. All patients underwent prior surgery and radiochemotherapy according to the Stupp’s protocol [12], followed by second or third line chemotherapy.

Magnetic Resonance Imaging (MRI) was performed with a 1.5-T MR Unit (Magnets: Avanto Siemens, Erlangen, Germany) before starting therapy and followed up every 6 weeks until tumor progression or treatment discontinuation.

RANO criteria were used to assess tumor response and tumor progression; however, an assessment of FLAIR hyperintensity, a threshold of 25% or more of the maximal trans-sagittal area was used [13]. Baseline tumor volumes were determined on 3D post-gadolinium T1-weighted images by manually outlining the enhancing portion of the lesion using MRIcro (http://www.micro Imaging.ere, oxford. UK). The total enhancing volume was obtained as the product of the number of enhancing voxels and the voxel volume.

We used the following four criteria to assess MR patients of disease at baseline and at progression [15]: Local disease, multifocal, contiguous with the primary site or resection cavity or within a 5 cm margin; Distant disease: a second non-contiguous lesion in addition to disease at the primary site. Multifocal disease: three or more non-contiguous lesions including the primary site (parenchymal fluid spread of disease was also defined as multifocal disease). Progression: more than 3 cm beyond the primary site with poor or indifferent contrast enhancement or FLAIR margins.

Acquisition protocol
The radiological MRI protocol included: 1) a 3D post-contrast MPRAGE T1-weighted sequence (TR=1100; TE=2.25 ms, matrix=304×312, voxel size=0.67×0.67×0.9 mm, 120 slices, AT=1.42 min); 2) a 3D FLAIR sequence (TR=5000; TE=177 ms, matrix=256×256, voxel size=1×1×1, 160 slices); 3) PWI was performed with a Dynamic Susceptibility Contrast (DSC) GRE echo-planar (EP) sequence (TR/TE=50/15 ms, flip angle=30°, matrix size=128×128, voxel size=2×2×5 mm, 50 dynamic volumes of 17, AT=4.62 min). Acquisitions were carried out during the injection of the gadolinium-based contrast agent Gadobutrol (Bayr, 0.5 mol/L). A bolus injection of 9 mL was administered at 3 mL/s using an automated injector. To minimize TI shortening effects, a contrast agent predose of 3 cc was used to saturate the blood-borne gadolinium contrast. Post-processing

dEvac MRI volume was spatially co-registered to the first one at baseline by an affine 12 DOF registration. Post-contrast T1-weighted images were co-registered and resampled to match the spatial resolution of the DSC volume of reference. DSC-MRI data were processed to create rCBV maps. Norel/Eric (http://www. noreloneuroinfo.com) was used for perfusion processing, including a CA leakage correction by blood-brain barrier disruption, and an automated gamma-variate fitting of time-resolved CA concentration curves.

The following two methods were used to evaluate rCBV variation during treatment.

Region Of Interest (ROI). Three separated ROIs were placed in regions of highest perfusion seen on the rCBV color maps at baseline. Size and morphology of ROIs were maintained constant (around 40 mm² area) and the maximum value recorded. Reference ROI was drawn on contralateral normal white matter, with the same size and position [10,16]. rCBV variation after 6 weeks of treatment was calculated through semi-automatic ROI placement in the same anatomic region as baseline.

Parametric Response Maps (PRMs). This technique was previously used to assess rCBV variation at two endpoints for each patient after radiotherapy in GBM [17] and create rCBV maps of patients with grade III and IV gliomas receiving concurrent radiochemotherapy [8].

Two ROIs were drawn by an experienced neuroradiologist on contrast-enhanced T1-weighted images the first including all tissues into the enhancement area, the second covering the hyperintense area that was judged to be necrotic. This second volume was then subtracted from the first one and the selected region defined as the "necrotic area". A control ROI of the same size was drawn contralaterally on normal, white matter. Perfusion changes over time were quantified by using a voxel-by-voxel analysis in the tumor region, drawn at baseline [17]. In case of progression another ROI was drawn on the new tumor volume and the sum of this ROI and baseline ROI was considered as definitive ROI. The rCBV values of each voxel within the tumor at week 6 and at time of progression were compared with baseline values. To evaluate their different two thresholds were set: they were determined to be the 55% confidence interval (CL) (1.56SD) obtained comparing the rCBV values of the two time points in the normal contralateral white matter. The tumor region was then subdivided in three regions represented with different colors: 1) areas with rCBV greater than the upper threshold (increased rCBV, ICBV, represented in red); 2) areas with rCBV lower than the lower threshold (decreased rCBV, oCBV, represented in blue); 3) areas unchanged (ICBV, represented in black) (Figure 1). Colored maps were then overlaid/merged on T2 reference images, allowing a qualitative assessment of perfusion changes in pathologic areas. The procedure was repeated at each time point until progression.

F100 ONE | www.f100one.org 2 March 2014 | Volume 9 | Issue 3 | e16535
Figure 1. Parametric Response Maps creation process. 1) computation of the CBV values of a ROI placed on the normal white matter between the baseline and the time point under examination; 2) classification of increased, decreased and unchanged difference values in the tumor area on the basis of the previously determined thresholds; 3) Chromatic representation of the difference map obtained by the subtraction of the baseline map from the time point one. Red vessels indicate an increase of CBV, blue a decrease and black vessels are unchanged.

doi:10.1371/journal.pone.0090535.g001

Statistical analysis
PSF and OS were calculated from treatment onset until disease progression or death/last follow-up, if censored. Kaplan-Meier analysis estimated PSF and OS. The log rank test assessed differences in progression or survival in patients with different clinical or radiological parameters. These parameters were set at the 25th, 50th, 75th, 90th percentiles and separately evaluated in all patients.

Correlations between radiological and clinical parameters or treatment response were assessed using the Mann-Whitney exact U test. The Wilcoxon rank sum test evaluated differences among radiological parameters at baseline, week 8 or progression. All p-values were two-sided.

A multivariate analysis and a Cox proportional hazard regression model analysis were performed on variables showing statistically significant differences at univariate analysis to investigate their independent prognostic role. In particular, iCBV variation was used as a dichotomous parameter. All statistical analyses were performed using the R software (www.r-project.org).

Results

Clinical results

Patients’ clinical and demographical baseline characteristics are reported in Table 1. In particular, MRI showed in 31 cases (74%) local disease, in 6 (14%) multifocal and in 3 (12%) distant disease. No patient was previously treated with bevacizumab or other antiangiogenic drugs.

During treatment three patients discontinued irinotecan before progression due to low tolerance and continued bevacizumab as monotherapy. Tumor volumes at baseline were significantly lower in patients presenting local disease than in patients presenting distant or multifocal disease (median 12.4 versus 36.7 and 40.0 cm³, respectively; Mann-Whitney, p = 0.01).

Median follow-up was 33.5 weeks (range 9–111 weeks). At the time of this analysis, four of 62 patients were progression-free; three died before disease progression and four were alive.

Median OS was 35.0 weeks (CI 23.5–44.5); OS at 6 months was 66.6%; CI 33.9–99.0%) and OS at 22 months 22% (CI 9–56.8). Median PFS was 20.0 weeks (CI 11.8–28.2); PFS at 6 months was 40%.

Median OS and median PFS were not significantly different when considering sex, age ≥ 40 versus < 40, age ≥ 60 versus < 60, KPS ≥ 70 versus < 70, and partial response versus stable/progressive disease according to RANO criteria [12].

Patients with local pattern of disease at baseline had longer PSF and OS than patients with distant or multifocal disease at baseline: PFS 20.0 versus 9.0 weeks, p < 0.001; OS 56.0 versus 18.0 or 15.0 weeks respectively, p < 0.001. Figure 2.

All patients underwent MRI at baseline and 8 weeks after treatment onset; 32 patients were also assessed 16 weeks after treatment onset.

Magnetic resonance results

MRI at 8 weeks after treatment onset showed partial responses in 9 cases, stable disease in 21 patients and progressive disease in 10. In this report we define patients showing progression at 8 weeks as non-responders, and patients radiologically improved or with stable disease as responders. No other partial or complete response was observed later.

The analysis of tumor responses to treatment at 8 weeks or later time points was available in 32 patients; other 10 patients died or interrupted treatment before progression or had incomplete scanning.

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Table 1. Patients characteristics at baseline.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of pts</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>27</td>
<td>64</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>36</td>
</tr>
<tr>
<td>Median age at start (range)</td>
<td>53 (15-91)</td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>40-60</td>
<td>25</td>
<td>59</td>
</tr>
<tr>
<td>&gt;60</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Median (all pts) (range)</td>
<td>70 (50-100)</td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>70-100</td>
<td>20</td>
<td>78</td>
</tr>
<tr>
<td>&gt;100</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>De novo GBM</td>
<td>36</td>
<td>86</td>
</tr>
<tr>
<td>Secondary GBM</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Disease recurrence</td>
<td>27/72</td>
<td>64/36</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>42/119</td>
<td>100/19</td>
</tr>
<tr>
<td>Tumor volume, cm³ (range)</td>
<td>22.39 (0.97-132.56)</td>
<td></td>
</tr>
<tr>
<td>Tumor volume, cm³ (range)</td>
<td>31</td>
<td>74</td>
</tr>
<tr>
<td>Multifoc. (Sarcomatoid/medul. ch.)</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Diffuse</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Involvement</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Twenty patients (16 with local and 4 with multifocal disease) did not show changes when compared to baseline. Seven patients converted to a diffuse pattern of disease (8 patients starting from local and 1 from distant pattern; whereas 3 converted to multifocal disease (2 from local and 3 from distant disease).

Analysis with the ROI method

Results are detailed in Table 2. Mean cCBV of all patients at baseline was 4.4 ± 1.2 SD (Table 2a).

Patients with local disease at baseline showed significantly lower cCBV than patients with distant or multifocal disease at baseline (p = 0.004).

In all patients mean cCBV decreased significantly at 8 weeks (4.4 ± 1.2 SD versus 4.1 ± 1.9 SD, p = 0.004), but was statistically unchanged at 16 weeks (3.7 ± 1.3 SD; ns). The serial measurement of cCBV at all time points until progression, performed in 24 patients, showed that cCBV changes differently within time according to treatment response. After 8 weeks of therapy, 32 responsive patients showed a significant decrease of cCBV with respect to the baseline (4.2 ± 1.5 versus 3.8 ± 1.6, p = 0.04), whereas 10 non-responsive patients, who progressed after 8 weeks maintained decreased cCBV (4.2 ± 0.9 versus 4.1 ± 1.2, p = 0.03) (Figure 3a).

Patients who progressed after 8 weeks showed an initial significant decrease of cCBV, followed by a progressive tendency to increased cCBV as long as they approached tumour progression.

In particular, when progression occurred at 16 weeks (9 cases) a new increase in cCBV at 16 weeks occurred after a significant decrease at 8 weeks (Figure 3a); a decrease of 8 weeks in cCBV was also observed in patients who progressed at 24 weeks or later (6 cases), but in the following MR performed before progression a light continuous increase of cCBV was observed (Figure 3c).

The radiological pattern of disease at progression might influence cCBV changes. While patients with local, distant or multifocal disease at progression showed an initial cCBV decrease followed by a new increase, patients with diffuse disease at progression maintained a low cCBV (Table S1 in File S1).

Parametric response maps (PRM)

Increased or decreased blood volume in PRM was defined as PRM_{25%}, or PRM_{50%}, respectively, as in the work of Grifanti et al. At 8 weeks we observed a mean PRM_{25%} of 30% ± 16% and a PRM_{50%} of 15% ± 21% in all patients. Similar data were obtained at 16 weeks of treatment: PRM_{25%} was 31% ± 19% and PRM_{50%} was 17% ± 14% (Table 2b).

If we consider PRM results dividing patients into non-responsive and responsive, at 8 weeks we found a lower PRM_{25%}, value in non-responsive than responsive: 23% ± 15% versus 30% ± 14%, not significant (Table 2b). Most of the non-responsive presented PRM_{25%}, <15% (first quartile, p = 0.04).

We also examined perfusion at progression in 24 patients using PRMs: PRM_{25%}, was 30% ± 20%, and PRM_{50%}, 30% ± 10%. No significant difference in PRM at progression was found dividing patients according to treatment response or pattern of disease at progression (see Table S2 in File S1).

Correlation with survival

Median PFS and OS were longer in responsive patients (PFS: 8 versus 17 months, p = 0.0001; OS: 16 versus 20 months, p = 0.0001).

Tumor volume higher than 35th percentile (44.3 cm³) was associated with significantly shorter PFS (14 versus 18 weeks, p = 0.0023) and OS (25 versus 39.00 weeks, p = 0.0009).

Using the classical ROI method cCBV values (at baseline and 8 weeks) did not correlate with PFS or OS. PRM analysis, on the
Figure 2. Correlations between PRM_{TV} higher than 18% and baseline magnetic resonance disease patterns and PFS/OS. A: Patients with PRM_{TV} higher than the first quartile, 18%, had longer survival than the others. B: Patients with local pattern of disease at baseline had longer PFS and OS than those with distant or multifocal disease.

doi: 10.1371/journal.pone.0090359.g002

Table 2. a) Mean rCBV max at different timepoints b) mean PRM_{TV} and PRM_{ABC} at different time points.

<table>
<thead>
<tr>
<th></th>
<th>No. of pts</th>
<th>Baseline rCBV</th>
<th>8 week rCBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>42</td>
<td>4.6±1.2^b</td>
<td>4.1±1.6^c</td>
</tr>
<tr>
<td>Non-respond</td>
<td>19</td>
<td>4.8±1.8</td>
<td>5.1±1.3</td>
</tr>
<tr>
<td>Respond</td>
<td>32</td>
<td>4.3±1.3^f</td>
<td>3.8±1.6^g</td>
</tr>
<tr>
<td>MRI pattern at baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local</td>
<td>31</td>
<td>4.2±1.9^p</td>
<td>4.0±1.5</td>
</tr>
<tr>
<td>Multifocal or Extent</td>
<td>11</td>
<td>4.9±1.6^q</td>
<td>4.2±1.9</td>
</tr>
</tbody>
</table>

a = b - p = 0.05 c - d = 0.01 e - f = 0.04

<table>
<thead>
<tr>
<th></th>
<th>No. of pts</th>
<th>PRM_{TV}</th>
<th>PRM_{ABC*}</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>42</td>
<td>18%±4%</td>
<td>30%±16%</td>
</tr>
<tr>
<td>Non-respond</td>
<td>19</td>
<td>12%±11%</td>
<td>29%±14%</td>
</tr>
<tr>
<td>Respond</td>
<td>32</td>
<td>19%±15%</td>
<td>33%±14%</td>
</tr>
<tr>
<td>MRI pattern at baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local</td>
<td>31</td>
<td>19%±15%</td>
<td>31%±16%</td>
</tr>
<tr>
<td>Multifocal or Extent</td>
<td>11</td>
<td>15%±10%</td>
<td>29%±16%</td>
</tr>
</tbody>
</table>

a - b - p = 0.05 e - f = 0.04
century, did show correlations with survival. Patients with PRMS<18% had 18% (hazard ratio) showed a significantly longer survival (Figure 2). Median OS was 30.0 weeks in patients with PRMS<18% and 21.3 weeks in patients with PRMS>18% (p=0.016). Median PFS was 26.3 weeks in patients with PRMS<18% and 13.1 weeks in patients with PRMS>18% (p=0.045).

Multivariate analysis

A multivariate analysis and a Cox proportional hazards regression analysis were performed on variables showing statistically significant differences at univariate analysis to investigate their independent prognostic value. Multifocal and distinct pattern of disease at baseline and PRMS<18% were independent predictors of shorter PFS (HR 3.2, p=0.001) and HR 5.2, p=0.003, respectively). Multifocal and distinct pattern at baseline were the only independent predictors of OS (HR 3.3, p=0.035) (Table 3).

Discussion

GBM is a tumor characterized by heterogeneous features with different regional expression of potential therapeutic targets such as RGDGR and VEGFR [18,19]. The pattern of microvascular proliferation can be various within the tumor with both simple, epithelial-like capillary loops and irregular, chaotic, large collections of capillaries with partially thrombosed slit-like lumens, microvascular hyalinosis, resulting in minimal perfusion to the surrounding tumor tissue [20] [17].

MRI and Positron Emission Tomography (PET) can give detailed information about tumor heterogeneity. In particular, advanced MRI techniques could lead to a better microstructural and functional characterization of gliomas. Diffusion MRI giving information about the degree of cellularity in the different portions of tumoral and peritumoral areas could be predictive and prognostic in gliomas and seems to correlate with survival in patients treated with bevacizumab [17,21-23]. Spectroscopy MRI (1H-MRS) can inform about metabolite concentration in the tumoral portion and could be an early indicator of response to antiangiogenic therapy [24,25].

Dynamic Susceptibility Contrast-MRI (DSC-MRI) gives information about microvascular density and antiangiogenic therapy efficacy and could be helpful in tumor grading. In particular, rCBV may provide a prognostic information complementing histopathology [16,26].

In our work we used DSC-MRI to evaluate the hemodynamic response over time in patients affected by recurrent GBM and treated with bevacizumab and irinotecan. We chose this technique because of its current use in the clinical practice and due to the characteristics of GBM. Indeed rCBV is a reliable indicator of microvascularization [27] and can be used to assess glioma grade [16,28] and distinguish progression from pseudo-progression [29].

Moreover, some studies demonstrated that rCBV correlates with overall survival [27,28,30].

The most common methods to evaluate rCBV over time are the ROI-based and the histogram-based. The first one is highly user-dependent but allows a precise identification of the portion of the tumor to be analyzed, on the other hand, it cannot accurately characterize the hemodynamic heterogeneity of high grade gliomas. The histogram-based method is less user-dependent and allows a better representation of the tissue heterogeneity, with similar sensitivity but higher specificity than the ROI method [6,14]. Its main limitation is spatial localization: it gives information about glioma heterogeneity and might give indications about glioma grade, but it is not able to spatially localize regions where rCBV changes occur.

In this work the ROI method was used in comparison to PRMs. The PRMs [9,10] is a voxel-wise technique estimating point by point the rCBV differences over time to better inquire the

Table 3. Univariate and multivariate analysis.

<table>
<thead>
<tr>
<th>Model</th>
<th>Progression-Free Survival</th>
<th>Overall Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate p values</td>
<td>Multivariate p values</td>
</tr>
<tr>
<td>Volume</td>
<td>0.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>Multifocal and Distinct pattern</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>PRMS&lt;18%</td>
<td>0.03</td>
<td>0.12</td>
</tr>
</tbody>
</table>

DOI:10.1371/journal.pone.0090535.g003
The first method is biased by the user ROI selection and it only takes into consideration the region of maximum CBV. Thus, mean CBV after 8 weeks of treatment remained elevated in non-responsive patients, but decreased in responsive patients, followed by a progressive tendency to increase as long as patients approached tumor progression. On the contrary, the PRM method is less user-dependent as it considers the whole enhancement region, being unaffected by the selection criteria.

As previously reported [30], the PRMs technique is preferable because it offers the same sensitivity but a higher specificity than the ROI classical approach. Interestingly, its non-responsive patients no major modification of perfusion was observed after treatment, suggesting that non-responsive patients may progress before the VEGF dependence [33].

Even if the PRMs approach is affected by intrinsic limitations [17], such as the need of a high quality image registration, it provided relevant information. Its potential to predict survival in recurrent GBM treated with bevacizumab and irinotecan, adds to previous results in high grade gliomas treated with radiotherapy [8], making it a promising prognostic biomarker. Moreover, the technique, though with the analysis of ADC maps [17], could be used in combination with other kinds of sequences, such as the Dynamic Contrast Enhanced-MRI (DCE-MRI) to obtain more detailed informations about the biology of the disease under treatment.

Supporting Information
File S1 Supplementary tables. Table S1: Mean rCBV changes according to radiological disease status at progression. Table S2: Mean PRMΔCBV and PRMΔrCBV changes during treatment. (DOCX)

Acknowledgments
We thank Dr. Stefano Costabile (Fundazione IRCCS Istituto Neurologico C. Besta, Milano, Italy) for her support in collecting data; Dr. Serena Pellegri (Fundazione IRCCS Istituto Neurologico C. Besta, Milano, Italy) for digitizing figures; Rocio Aga, Basel, Switzerland and Hospita Italia Srl (Sassuolo, Italy) for providing the drugs.

Author Contributions
Conceived and designed the experiments: DA ALDIZ AS ME MB. Performed the experiments: DA ALDIZ AS ME. Analyzed the data: DA ALDIZ AS LC MF MG ME. Contributed reagents/materials/analysis tools: DA ALDIZ AS EA. Wrote the paper: DA ALDIZ LC MF ME. Performed clinical follow-up of patients: EA ME.

References
21. Due to the limitations in the image provided, the text content is not fully discernible. It appears to be a page from a document with text in multiple sections, possibly related to a scientific or medical field. For a full understanding, the text needs to be transcribed accurately and contextually.
Clinical Study

Facing Contrast-Enhancing Gliomas: Perfusion MRI in Grade III and Grade IV Gliomas according to Tumor Area

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Received 14 February 2014; Accepted 6 March 2014; Published 3 April 2014

Academic Editor: Loinard Guiseppe

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Tumoral neangiogenesis characterizes high grade gliomas. Relative Cerebral Blood Volume (rCBV), calculated with Dynamic Susceptibility Contrast (DSC) Perfusion-Weighted Imaging (PWI), allows for the estimation of vascular density over the tumor bed. The aim of this study was to characterize putative tumoral neangiogenesis via the study of maximal rCBV with a Region of Interest (ROI) approach in three tumor areas—the contrast-enhancing area, the nonenhancing tumor, and the high perfusion area on CBV map—in patients affected by contrast-enhancing glioma (grades III and IV). Twenty-one patients were included: 13 were affected by grade IV and 8 by grade III gliomas. Maximal rCBV values for each patient were averaged according to glioma grade. Although rCBV from contrast-enhancement and from nonenhancing tumor areas was higher in grade IV glioma than in grade III (3.58 and 2.46; 3.58 and 2.2, resp.), the differences were not significant. Instead, rCBV recorded in the high perfusion area on CBV map, independently of tumor compartment, was significantly higher in grade IV glioma than in grade III (3.58 versus 3.78, P = 0.0363). In conclusion, neangiogenesis encompasses different tumor compartments and CBV maps appear capable of best characterizing the degree of neovascularization. Facing contrast-enhancing brain tumors, areas of high perfusion on CBV maps should be considered as the reference areas to be targeted for glioma grading.

1. Introduction

Gliomas are the most common brain primary neoplasms and are classified based on histologic parameters including atypia, vascular endothelial proliferation, necrosis, and mitosis [1, 2]. A common histopathological characteristic for both grade III and grade IV glioma is vascular endothelial barrier disruption and tumoral neangiogenesis [3, 4] as evidenced by contrast-enhancement observed using conventional Magnetic Resonance Imaging (MRI). Although neuro-radiological necrosis is a hallmark of glioblastoma (GBM; grade IV glioma), it is not a constant finding. Therefore it can be difficult to distinguish between grade III and grade IV gliomas using conventional MRI.

Dynamic Susceptibility Contrast (DSC) Perfusion-Weighted Imaging (PWI) measures concentration of a paramagnetic contrast material in the organ, providing reliable information
on blood flow and vasculization. Among other perfusion parameters such as Cerebral Blood Flow (CBF) and Mean Transit Time (MTT), Cerebral Blood Volume (CBV) was shown to best correlate with tumoral neangiogenesis and subsequently gliomas grading. In particular, Relative Cerebral Blood Volume (rCBV), reflecting increased capillary density, was shown to be significantly higher in high grade gliomas (grade III and grade IV glioma) than low grade gliomas (grade II glioma) and with higher values in grade IV [5].

Although rCBV increase is recognized as a surrogate marker of malignancy [6-8], differentiation between grade III and grade IV glioma is not consistently reproducible [8-11].

Furthermore, rCBV increases in glioblastoma multiforme (GBM) peritumoral area [11] and the peculiar pattern of rCBV increase surrounding glioblastoma contrast-enhancing tumour bed [12, 13] raises the question of whether measuring rCBV values may best distinguish between grade III and grade IV gliomas.

In this study we investigated rCBV differences in patients with grade III and grade IV glioma using a Region of Interest (ROI)-based method in three different tumor areas: the contrast-enhancing area, the nonenhancing tumor, and the high perfusion area on CBV map.

The aim of the study was to determine which tumor compartments showed rCBV differences related to glioma grade.

2. Materials and Methods

We examined perfusion MRI from 21 patients affected by histologically proven high grade gliomas: fifteen patients were affected by grade IV glioma and 6 patients by grade III glioma.

Twelve patients underwent a DSC perfusion examination before surgery and 9 with residual tumor were examined after surgery after a median of 6.8 months. All patients undergoing perfusion after surgery had residual tumor and were not in progression at the time of DSC perfusion examination. All patients in this study were affected by primary disease and in particular grade IV glioma patients were all affected by primary GBM.

Demographical characteristics of patients are detailed in Table 1. Glioma grading was ascertained by an experienced neuroradiologist according to WHO criteria [12]. The study was approved by the local Institutional Review Board and all patients provided informed consent.

All participants were scanned using a 1.5T Philips Intera Gyroscan (Philips Medical System, Best, The Netherlands) with a maximum slew rate of 150 Tm s⁻¹ and a maximum gradient amplitude of 30 mT m⁻¹. All scans were performed using an 8-channelSENSE (sensitivity encoding parallel imaging) head coil.

The scanning protocol included the following:

An axial 2D spin-echo (SE) T2-weighted Fluid Attenuated Inversion Recovery (FLAIR) image; echo time (TE)/repetition time (TR)/inversion time (TI) = 460/2550/2800 ms, slice thickness = 5 mm, gap = 1 mm, and matrix size = 256 × 192, FOV = 200 × 200 mm² (for an in-plane resolution of 0.9 mm × 1.3 mm). Slice thickness = 5 mm, gap = 1 mm, and number of slices = 24.

An axial 3D perfusion weighted gradient echo (GRE) sequence (Principles of Echo Shifting with a Train of Observations, PRESTO) for Dynamic Susceptibility Contrast MRI: TE/TR = 50/68 ms (effective TE = 23.71 ms), FA = 7°, ETL = 5, acquisition matrix = 256 × 256, FOV = 220 × 220 mm² (in-plane resolution of 0.88 mm × 0.88 mm), slice thickness = 3 mm, NEX = 1, and number of slices = 30 with 60 temporal localizations. This sequence was acquired with a standard dose of 0.2 mmol/Kg body weight of gadopentetate dimeglumine (Gd-DTPA) contrast agent (Gadovist) which was injected at a rate of 4 mL/s, followed by a 20 mL continuous saline flush. Using a 0.05 mmol/Kg dose, presaturation of the baseline signal prior to the PWI acquisition was done to reduce T1 effects as well as potential contrast leakage effects due to blood brain barrier disruption.

Table 1: Patients’ clinical data and tumor diagnosis.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Number of patients</th>
<th>Age (years) (median, range)</th>
<th>Sex ratio (male/female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>6</td>
<td>69 (24-66)</td>
<td>6.0</td>
</tr>
<tr>
<td>IV</td>
<td>15</td>
<td>63 (25-80)</td>
<td>1.5</td>
</tr>
</tbody>
</table>

An axial 3D T1-weighted fast field echo (FFE) sequence after the PWI: TE/TR = 4.4/255 ms, FA = 30°, ETL = 1, acquisition matrix = 256 × 256, FOV = 220 × 220 mm² (in-plane resolution of 0.98 mm × 0.98 mm), slice thickness = 1.6 mm, gap = 0, NEX = 1, and number of slices = 170.

Postprocessing was performed using Olea Medical PerfScape software (version 2.0). The DSC acquisition was corrected for patient motion using the built-in feature of PerfScape. The T1 SE, FLAIR, and T1 3D FFE images were then coregistered and resampled into the space of the DSC MRI. It is well known that disruption of the blood brain barrier, as is common in high grade tumors, can lead to inaccuracy in measures of CBV [14]. As such, the correction for leakage effects option in PerfScape was employed.

Relative CBV (rCBV) was calculated from three separate ROIs that were placed in three different compartments: the area of contrast-enhancement, the nonenhancing tumor, and high perfusion area seen on the CBV color overlay maps. In the contrast-enhancement area, necrosis was excluded from CBV measure. The nonenhancing surrounding tumor ROIs corresponded to areas of T2/FLAIR hyperintensity outside contrast-enhancement. We ensured that T2/FLAIR ROIs were not placed in areas of contrast-enhancement as all images were coregistered. Placement of ROIs on the CBV map was performed in high perfusion areas independently of the contrast-enhancement and T2/FLAIR ROI locations.

Morphology and size of the ROIs were constant (elliptical 40 mm² area) and the maximum value was recorded for each compartment according to Law et al. [15].
Table 2: Mean rCBV values according to histological grading. Relative Cerebral Blood Volume (rCBV) was measured with the ROI-based approach in three distinct areas: the high perfusion area on CBV map ("CBV map" in the table), the contrast-enhanced area ("CE" in the table), and the nonenhancing tumor ("Non-CE" in the table). Only rCBV values measured in the high perfusion area in CBV map showed significant difference between grade III and grade IV gliomas.

<table>
<thead>
<tr>
<th>WHO glioma grade</th>
<th>CBV map ( \mu ) (SD)</th>
<th>CE ( \mu ) (SD)</th>
<th>Non-CE ( \mu ) (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioma grade III</td>
<td>3.78 (1.79)</td>
<td>3.08 (1.02)</td>
<td>2.20 (1.73)</td>
</tr>
<tr>
<td>Glioma grade IV</td>
<td>7.51 (3.84)</td>
<td>5.58 (5.48)</td>
<td>2.68 (2.93)</td>
</tr>
</tbody>
</table>

All values were normalized to a corresponding ROI placed in contralateral normal brain parenchyma. All ROIs were placed by two operators (A.D., N.B.) via consensus. Maximal rCBV values for each patient were averaged according to glioma grade. Differences between grades and tumor areas were tested using t-test. ANOVA was used in order to compare rCBV values in different subgroups of patients. Contingency analysis was performed by Fisher’s exact test. In all analyses we considered a \( P \) value of 0.05 (two-sided) as being statistically significant.

3. Results

Twelve patients underwent a DSC perfusion examination before surgery (3 patients affected by grade III glioma and 9 patients affected by grade IV glioma) and 9 patients with residual tumor were examined after surgery after a median of 6.8 months (3 patients affected by grade III glioma and 6 by grade IV glioma). Distribution of patients according to the timing of perfusion MRI (pre-surgery versus post-surgery) was not significantly different between grade III glioma and grade IV (\( P = 0.67 \)).

In the grade III glioma subgroup, 2 patients were affected by anaplastic oligodendroglioma and 4 patients by anaplastic astrocytoma. In the grade IV glioma subgroup all patients were affected by glioblastoma.

Mean rCBV values from patients are detailed in Table 2. In the grade III glioma subgroup, mean rCBV was higher in the contrast-enhanced area than nonenhancing tumor (3.01 and 2.20, resp., \( P = 0.11 \)); rCBV recorded in CBV map, independently of tumor compartment as seen on conventional MRI, was 3.78.

In glioma grade IV, mean rCBV was higher in contrast-enhanced area than nonenhancing tumor (5.38 and 2.68, resp., \( P = 0.04 \)); the mean rCBV recorded in the CBV map was 7.51.

Between glioma grade III and glioma grade IV, no significant differences in rCBV were observed in the contrast-enhancement area and in the nonenhancing tumor (\( P = 0.27 \) and 0.71, resp.). Conversely, mean rCBV was significantly higher in grade IV gliomas than in grade III (\( P = 0.036 \)) in the high perfusion area of CBV map independently of tumor compartment, as seen on conventional MRI (Figure 1).

4. Discussion

Neovascular proliferation is a hallmark of malignant gliomas and PWI is useful in glioma grading through detection of vascular density and of the grade of tumor-associated neovascularization [7, 16].

The measure of rCBV is commonly used in order to predict glioma grade or to differentiate radionecrosis from tumor recurrence in a diagnostic setting. Several reports on rCBV increase in peritumoral area of glioblastoma [10, 13] have suggested that there is a mismatch between the extension of effective vascular proliferation and area of contrast-enhancement.

In this work we mapped rCBV maximal increase in two different compartments of glioma grade III and glioma grade IV— the contrast-enhancing area and nonenhancing tumor with a ROI-based method. rCBV was also recorded in high perfusion area of CBV map independently of corresponding tumor area on conventional MRI.

Values of rCBV recorded in this work are consistent with the other reports in the literature [6]. As expected we found a significantly higher rCBV in contrast-enhanced area than in nonenhancing tumor in the grade IV glioma subgroup.

Concerning rCBV differences according to the tumor grade, we did not find significant differences of rCBV values recorded in contrast-enhancing area or nonenhancing tumor between grades III and IV.
Only measures of rCBV in the high perfusion area on the CBV map showed a significant difference between grade III glioma and grade IV, with higher values in grade IV. Taken together, these results support the idea that neangiogenesis heterogeneously encompasses both contrast-enhancing and nonenhancing tumor areas. The contrast-enhancing areas appear to reflect a higher degree of neangiogenesis, although the difference with respect to nonenhancing areas was significant only in grade IV glioma subgroup.

Interestingly, we did not find significant differences in maximal rCBV in either of these two areas when comparing grades III and IV. This suggests that basing rCBV measurements on signal characteristics of conventional MRI may not be sufficient to distinguish between grade III and grade IV gliomas.

Glioblastoma has been shown to present with a more heterogeneous neovascularization than grade III glioma [12]. In particular glioblastoma present, more so than with lower grades, areas with low perfusion due to necrosis, area of focal rCBV increase, and also increased rCBV values in peritumoral normal-appearing parenchyma [14, 17].

In particular, a special pattern of rCBV increase in peritumoral area can occur in a “striped like” fashion which has been termed a “striped sign.” This feature has been described as mostly represented in glioblastoma rather than lower grade gliomas and in particular with respect to grade III glioma [12, 13]. The same authors showed that this specific pattern of rCBV in peritumoral area was significantly associated with normalized choline increase and with the subsequent appearance of contrast-enhancement in the same area [12, 13]. A similar example of mismatch between high perfusion area from CBV map and contrast-enhancement observed in our patients is shown in Figure 2. Histopathologically, these patterns of rCBV may reflect diffuse migration of glioma cells along vascular channels of the white matter tracts spreading beyond the visible tumor border [18].

Taken together, these results support the hypothesis that only the rCBV map represents extensively the neovascular phenomena, its extension into apparently normal surrounding parenchyma, and its quantitative difference among glioma grades.

Limits of the study are the small sample size and potential sampling differences from the ROI-dependent method of measure which may increase interobserver variability. Nevertheless, the latter is the most used in clinical routine. Additionally, all ROIs were placed in consensus by two authors. The fact that some patients were scanned before surgery and other patients afterwards presents an additional confound. However, we did not find any significant differences between the pre-/posttreatment groups (results not shown). Nevertheless, we cannot rule out the possibility that surgical intervention in some patients may have influenced the results.

In conclusion, maximal rCBV values measured directly on the CBV map seem to best characterize the extensive neangiogenesis phenomena of high grade gliomas and quantitative difference of microvascular density between grade III and grade IV glioma. Such measurements should be considered as the reference map for glioma grading and potentially for serial measures of rCBV modification during antiangiogenic treatment.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.
Acknowledgments

The study was supported by a Grant of the National Ministry of University and Research, PRIN 2008 “2010/2009 M & K. 003”. The authors are grateful to OLEA Medical for providing OLEA’s PerfScape Software for postprocessing perfusion analysis. Dr. Di Stefano is supported by an Investigator Fellowship from Collegio Ghislieri, Pavia, Italy.

References

Research Article

An ANOCEF Genomic and Transcriptomic Microarray Study of the Response to Irinotecan and Bevacizumab in Recurrent Glioblastomas

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Received 14 February 2014; Accepted 25 February 2014; Published 2 April 2014

Academic Editor: Giuseppe Lombardi

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Background. We performed a retrospective study to assess whether the initial molecular characteristics of glioblastomas (GBMs) were associated with the response to the bevacizumab/taxane chemotherapy regimen given at recurrence. Results. Comparison of the genomic and gene expression profiles of the responders (n = 12) and nonresponders (n = 13) demonstrated only slight differences and could not identify any robust biomarkers associated with the response. In contrast, a significant association was observed between GBM molecular subtypes and response rates. GBMs assigned to molecular subtype 1G8.10 and 1G8.11 had a lower response rate than those assigned to other subtypes. In an independent series of 33 patients, neither EGFR amplification nor CDR2NA deletion (which are frequent in 1G8.10 and classical GBMs) was significantly associated with the response rate, suggesting that these two alterations are unlikely to explain the lower response rate of these GBMs molecular subtypes.

Conclusion. Despite its limited sample size, the present study suggests that comparing the initial molecular profiles of responders and nonresponders might not be an effective strategy to identify biomarkers of the response to bevacizumab given at recurrence. Yet it suggests that the response might differ among GBMs molecular subtypes.
1. Background

In recurrent glioblastomas (GBMAs), studies have shown a high response rate (30–50%) to bevacizumab, a human monoclonal antivascular endothelial growth factor (VEGF) antibody, administered alone or in combination with irinotecan, demonstrating a 15-30% estimated 6-month progression-free survival (PFS) [1-3]. Simple biomarkers that would help in selecting patients most likely to benefit from bevacizumab would be very helpful, but no such markers are available to date. In the present study, we hypothesized that the response to bevacizumab plus irinotecan given at recurrence might be related to the molecular characteristics of the initial tumor. To identify predictive biomarkers, we compared the initial GBM genomic and gene expression profiles of responders and nonresponders to bevacizumab plus irinotecan chemotherapy. This study was approved by the ANOCF review board. All patients who underwent a genetic analysis of tumor samples collected for this study signed a written informed consent form. The patients' clinical characteristics are summarized in Table 1 and see additional Table 1 in Supplementary Material available online at http://dx.doi.org/10.1038/s13555-014-0258-0. All of the 25 patients included in this study had de novo GBM according to the 2007 World Health Organization Classification [4] and were initially treated according to the Stppe regimen [5]. To exclude patients with possible pseudoprogression, only those patients with a progression occurring more than 3 months after the end of the radioschemotherapy treatment were selected [6]. Patients received bevacizumab (10 mg/kg) plus irinotecan (225 mg/m²) every two weeks either at the first (n = 15), second (n = 9), or third (n = 1) recurrence (chemotherapy details are available in additional Table 1). To identify clinically meaningful biomarkers of the response, the patients were considered to be responders if they achieved a complete or partial response according to RANO criteria [6] and presented more than 6-month progression-free survival (PFS); the patients were considered to be nonresponders if they progressed within 4 months.

2. Methods

2.1. Patients. We retrospectively identified responders and nonresponders to bevacizumab/irinotecan chemotherapy. This study was approved by the ANOCF review board. All patients who underwent a genetic analysis of tumor samples collected for this study signed a written informed consent form. The patients' clinical characteristics are summarized in Table 1 and see additional Table 1 in Supplementary Material available online at http://dx.doi.org/10.1038/s13555-014-0258-0. All of the 25 patients included in this study had de novo GBM according to the 2007 World Health Organization Classification [4] and were initially treated according to the Stppe regimen [5]. To exclude patients with possible pseudoprogression, only those patients with a progression occurring more than 3 months after the end of the radioschemotherapy treatment were selected [6]. Patients received bevacizumab (10 mg/kg) plus irinotecan (225 mg/m²) every two weeks either at the first (n = 15), second (n = 9), or third (n = 1) recurrence (chemotherapy details are available in additional Table 1). To identify clinically meaningful biomarkers of the response, the patients were considered to be responders if they achieved a complete or partial response according to RANO criteria [6] and presented more than 6-month progression-free survival (PFS); the patients were considered to be nonresponders if they progressed within 4 months.

2.2. Samples. The samples were provided as snap-frozen sections of the areas immediately adjacent to the region used for the histopathological diagnosis. Only samples representative of the tumor and from which high-quality DNA and/or RNA could be obtained were selected (n = 25). A total of 28 samples were available for the genomic Illumina SNP array study, which included samples from 8 responders and 13 nonresponders. The gene expression array study was performed on 23 samples (including 19 samples common to the SNP array study): 11 responders and 12 nonresponders.

2.3. Genomic and Gene Expression Data

2.3.1. RNA and DNA Extraction. Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen), and DNA was extracted using the QiAamp DNA Mini Kit (Qiagen) following the manufacturer’s instructions. Both the RNA and DNA were assessed for integrity and quantity, following stringent quality control criteria (CIT program protocols http://citc.gatech.edu/). The genomic and gene expression analyses were performed using R software (http://www.R-project.org/).

2.3.2. Gene Expression Arrays. The gene expression arrays were performed using the IGBMC microarray platform (Strasbourg, France). Total RNA was amplified, labeled, and hybridized to the Affymetrix Human Genome U133 plus2 GeneChip, following the manufacturer’s protocol (Affymetrix, Santa Clara, CA, USA). The microarrays were scanned using an Affymetrix GeneChip Scanner 3000, and the raw intensities were quantified from the subsequent images using GCOS 1.4 software (Affymetrix). The data were normalized using the robust multichip average method implemented in the R package affy [9].

Unsupervised hierarchical clustering analysis was performed using the Pearson correlation metric. Only probesets with an Affymetrix annotation class A and located on autosomes were considered. Differences between the sample clusters were tested using the Chi-squared test, and genes differentially expressed between the tumors of responder and nonresponder patients were assessed using the t-test followed by Benjamini and Hochberg correction. The analyses of the gene sets using KEGG and Biocarta pathways and Gene Ontology terms, Molecular Signature Database gene sets, and Stanford Microarray Database gene sets were performed on the 1000 most differentially expressed genes (590 genes upregulated in responders and 590 genes upregulated in nonresponders) using hypergeometric tests [10]. We used the published centroid-basedclassifier of Verhaak et al. to classify our samples according to their system [7]. Samples were assigned to one of the six molecular subtypes of gliomas (called intrinsic glioma subtypes (IGS) described by Greenedel et al. [8] using ClusterDepro (an R package: http://cran.r-project.org/package=clusterDepro) [11].

2.3.3. Genomic Arrays. The genomic arrays were performed using the Integrigen Platform (Evry, France). DNA was hybridized to Illumina SNP HumanCNV370 chips according to the instructions provided by the array manufacturer (Illumina, San Diego, CA). The raw fluorescent signals were imported into Illumina BeadStudio software and normalized as previously described [12] to obtain the log R ratio (LRR) and B Allele Frequency (BAF) for each SNP. A supplemental normalization procedure TQN [13] was applied to correct for dye bias. The genomic profiles were then segmented using the circular binary segmentation algorithm (DNAcopy package, Bioconductor) [14] into the LRR and BAF data separately, as previously described [13, 15]. The absolute copy number and genotype status of the segments were then determined using the genome alteration print (GAP) method [15].
The data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/). ArrayExpress accession: E-MTAB-953.

2.3.4. RT-PCR. The gene expression of NPTX2, EPHA7, SOCS2, PDGFD, PRKLCZ, and ENPP4 in the tumors and nonmalignant control tissue were analyzed using UPL probe real-time quantitative polymerase chain reaction (QPCR) analysis. The reference gene was PPIA. The sequences of the primers and probes are listed in additional Table 2. The real-time QPCR reactions were performed as follows: iX LightCycler 480 Probes Master (Roche Applied Science), 4 pmoles each primer, 2 pmoles Universal ProbeLibrary Set, Human, and 8 ng cDNA. The real-time QPCR cycles were as follows: initial denaturation at 95°C for 10 minutes and 45 cycles of 95°C for 10 seconds and annealing at 60°C for 30 minutes. The 2-DeltaΔCT method was used to determine the relative expression levels. The calculation of the relative expression levels of each gene is described in the calibration RNA.

2.4. Independent Data Set. An independent series of 33 GBMs from the Salpêtrière database treated with the bev/cis/ir combination at recurrence (31 out of 33 were treated at first recurrence) was used to assess the impact of the CDKN2A homozygous deletion and EGFR amplification. These alterations were assessed in the initial tumor using CGH arrays as previously described [16]. The response according to RANO criteria was assessable in 29 of the patients. RNA was available for 7 of the responders and 11 nonresponders and was used to study NPTX2, EPHA7, SOCS2, PDGFD, PRKLCZ, and ENPP4 gene expression using RT-PCR.

### 3. Results

#### 3.1. Patients’ Characteristics. Twelve responders and thirteen nonresponders were included. All of the patients exhibited an evaluable disease at the initiation of bev/cis/ir treatment. The patients’ characteristics are shown in Table 1. After bevacizumab/irinotecan onset, the responders had a longer progression-free survival (PFS) and overall survival (OS) than the nonresponders. The OS since diagnosis was also significantly longer for the responders (Table 1).

#### 3.2. Responders and Nonresponders Have Very Similar Genomic and Gene Expression Profiles. The comparison of the genomic profiles (gains, losses, homozygous deletions, and amplifications) of the responders (n = 11) versus nonresponders (n = 13) demonstrated only slight genomic differences (Figure 1, additional Tables 3a and 3b), with the most consistent being an entire chromosome 20 gain that was significantly more frequent in the nonresponders (Fisher’s exact test P = 0.004). EGFR amplification (9/13 in nonresponders versus 4/8 in responders) and CDKN2A locus homozygous deletion (8/13 in nonresponders versus 4/8 in responders) were also more frequently observed in nonresponders, but the difference was not significant. Similarly, the comparison of the gene expression profiles of the responders (n = 11) and nonresponders (n = 12) demonstrated only few differences. Sixty probe sets (fifty-one in responders and nine in nonresponders) were differentially expressed, with a t-test P-value < 0.05 and a fold change above 2, though with a very high (95%) false discovery rate (additional Table 4). Neither the expression of VEGF nor its receptors were associated with the response to the treatment. Using RT-PCR we studied the expression of 6 genes implicated in angiogenesis and overexpressed in responders (ENPP4, PRKLCZ, and EPHA7) or nonresponders (NPTX2, SOCS2, and PDGFD) in an independent series of 7 responders and 11 nonresponders. EPHA7 [17] is implicated in endothelial
tubulogenesis, and PRX2 has been implicated in VEGF transcriptional activation [18]. NPTX2 has been shown to be overexpressed in edematous versus noneedematous gliomas in the absence of increased VEGF expression [19]. FGFR3 is a proangiogenic factor [20], and SOCS2 is involved in IGF/IR signaling and is also a proangiogenic factor [21]. However, with the exception of SOCS2, we failed to confirm similar overexpression in the responders/nonresponders that was significant in this independent series (additional Table 5).

Lastly, the pathway analysis performed on the 1000 genes that were most differentially expressed (500 genes upregulated in responders and 500 genes upregulated in nonresponders) demonstrated that these gene lists were significantly enriched in genes with different ontologies (additional Tables 6 and 7). The list of upregulated genes in the responders was significantly enriched in genes upregulated in the normal brain, whereas the list of upregulated genes in the nonresponders was enriched in genes that have been shown to be upregulated during hypoxia [22] and also in genes that might be targets of the transcription factor HIF1.

3.3. GBMs Molecular Subtypes Are Associated with Different Response Rates. As responders and nonresponders had very similar gene expression profiles, we hypothesized that there might be several subgroups of responders and nonresponders. To test this hypothesis, we performed an unsupervised hierarchical clustering analysis of the 23 GBMs included in the gene expression study. As shown in Figure 2, three main subgroups were identified. This clustering was robust and conserved across different gene lists and clustering methods. However, none of the three clusters was enriched in responders or nonresponders, and some responders and nonresponders could have very similar gene expression profiles. Therefore, to assess whether transcriptional subgroups of GBMs previously identified in larger series of patients were associated with a specific pattern of response to the bevacizumab/tirapazam regimen, we classified our 23 samples according to the transcriptional classifications of Gravendeel et al. [8] and of Verhaak et al. [7] and estimated the response rate in each subgroup. According to Gravendeel et al. [8], 14 GBMs were assigned to molecular subtype B (IGS-55), 3 to molecular subtype 22 (IGS-22), and 6 to molecular subtype 23 (IGS-23). According to Verhaak et al. [7], 9 GBMs were classified as classical, 6 as mesenchymal, 5 as proneural, and 3 as neural. The 9 classical GBMs were also assigned to IGS-18 which in addition consisted of 3 neural and 2 proneural GBMs. Interestingly, the GBMs assigned to IGS-18 were more frequently not responsive than the GBMs assigned to IGS-22 or IGS-23 (10/14 versus 2/9; Fisher’s exact test P value = 0.03) and a similar trend was observed for classical versus nonclassical GBMs (7/9 versus 5/14, Fisher’s exact test P value = 0.09). Conversely, IGS-18 GBMs had a shorter PFS after bevacizumab/tirapazam than IGS-22/23 GBMs (3.2 months versus 9.4 months, P = 0.01) and classical GBMs had a shorter PFS than nonclassical GBMs (2.2 months versus 8.3 months, P = 0.003) (Figure 3). Overall survival after bevacizumab/tirapazam also tended to be shorter in IGS-18 than in IGS-22/23 GBMs and in classical than nonclassical GBMs (7 months versus 18.9 months, P = 0.06 and 6.6 months versus 14.3 months, P = 0.06).

3.4. Neither EGFR Amplification Status Nor CDKN2A Locus Homozygous Deletion Status Is Associated with the Response Rate, the Progression-Free Survival, or the Overall Survival after Bevacizumab/Tirapazam Initiation. Because, in our series, EGFR amplification and CDKN2A homozygous deletion were more frequent in IGS-18 GBMs than in IGS-22/23 GBMs (10/14 versus 0/5, Fisher’s exact test P value <0.01)
Figure 2: Unsupervised hierarchical clustering of the 23 GBMs. The heatmap was constructed using the 2365 probesets (quantile 0.95), with the greatest robust coefficient of variation between the tumor samples. The samples and genes were clustered using Ward’s linkage and Pearson’s correlation coefficient. For each probe set, the lowest and highest intensity values are displayed in blue and red, respectively. Response: black = responder, white = nonresponder. Verhaak = class according to Verhaak et al’s classification [7]; neural = green, classical = red, mesenchymal = blue, and proneural = orange.

Figure 3: Progression-free survival according to Gravendeel et al. [8] and Verhaak et al. [7] molecular subtypes. GBMs assigned to IGS-18 (dashed line) had a shorter PFS after bevaxumab/titotecan than those assigned to IGS-22 and IGS-23 (plain line) (3.2 months versus 9.4 months, \( P = 0.01 \)). GBMs classified as classical (dashed line) had a shorter PFS than those classified as nonclassical (2.2 months versus 8.3 months, \( P = 0.003 \)).
and 10/14 versus U5 Fisher's exact test \( P \) value = 0.1, resp.) and also more frequent in classical than in nonclassical GBMs (5/23 versus 2/30, Fisher's exact test \( P \) value < 0.01 and 10/14 versus U5 Fisher's exact test \( P \) value < 0.02, resp.), we decided to evaluate the impact of these two genomic abnormalities in an independent series, in order to assess if these genomic abnormalities contribute to the lower response rate of IGS-18 and classical GBMs. This independent series comprised 33 GBMs from the Sulpâtère database treated with the combination of bevacizumab/irinotecan at recurrence and for whom the CDKN2A locus homozygous deletion and EGFR amplification status were available in the initial tumor. The patients' characteristics are shown in Table 2. However, we did not observe any significant association between EGFR amplification and/or CDKN2A deletion status and the response rate to bevacizumab/irinotecan, the PFS, or the OS after bevacizumab/irinotecan initiation.

### 4. Discussion

Several studies have identified radiological, histological, and clinical markers of the response to bevacizumab [23–25]. The objective of the present study was to identify biomarkers predictive of the response to bevacizumab/irinotecan given at GBM recurrence based on the transcriptional and genomic characterization of the initial tumor. Given the dramatically different clinical and radiological response patterns to this treatment, we hypothesized that the comparison of a limited series of well-selected responders and nontresponders would be sufficient to identify robust and clinically useful biomarkers if such markers do exist. However, although the responders and nontresponders had dramatically different response patterns, we found that the two groups of patients had very similar genomic and gene expression profiles and we failed to identify any robust predictive biomarker. There are several possible hypotheses to explain this finding. First, the genomic and transcriptional characteristics of the initial tumor might not be predictive of the response to bevacizumab/irinotecan given at recurrence because the molecular profile of recurrent GBMs might have significantly changed. However, Sutharssummetree et al. demonstrated that the expression of VEGF and CA9 (a marker of hypoxia) assessed by immunohistochemistry in the initial GBM was associated with the response and survival, respectively, in patients receiving bevacizumab and irinotecan at recurrence [26]. Interestingly, we similarly found that the profile of nontresponders was enriched in genes upregulated during hypoxia, though not influenced by VEGF expression. A second hypothesis to explain the absence of major difference between the profiles of responders and nontresponders is that the criteria used for defining the responders and nontresponders in the present study were not appropriate. These criteria were chosen to discover biomarkers that might be clinically meaningful and that might identify responders that achieve both a radiological response and prolonged PFS (>6 months) and to differentiate these patients from those who progress rapidly, regardless of the radiological response. Another hypothesis (and we suggest the most likely) is that the comparison of responders and nontresponders (regardless of the criteria) might not be the best strategy to identify biomarkers of the response. Indeed, this strategy assumes that all of the responders and nontresponders share common characteristics, which might be inappropriate if there are not one but several subgroups of responders/nontresponders with different mechanisms of response or resistance. In fact, both Verhaak et al. and Gravendeel et al. demonstrated that this is likely to be the case, as they identified transcriptional subgroups of GBMs that seem to display different patterns of response according to the treatment used [7, 8]. Furthermore, we previously found that mesenchymal GBMs were more likely to respond to radiotherapy, whereas classical GBMs were more likely to respond to first-line alkylating chemotherapy [10]. In our series, though it was not designed to study this association, we observed an interesting association between GBMs molecular classes and the response rates. Using Gravendeel et al. classification, GBMs assigned to IGS-18 had a lower response rate to bevacizumab/irinotecan than the GBMs assigned to IGS-22 and IGS-23 [8]. Using Verhaak et al. classification a similar trend was observed for classical GBMs [7] when compared to nonclassical GBMs. This is in agreement with the fact that IGS-18 GBMs are generally assigned to the classical subtype (9 out of 14 cases in our series) [27]. As EGFR amplification and CDKN2A deletion status are two genomic hallmarks of IGS-18 and classical GBMs, we next studied the impact of these two genomic abnormalities in an independent series.
of 33 patients. However, we did not identify any significant association with the response rate to bevacizumab/irinotecan suggesting that EGFR amplification and CDKN2A deletion are not responsible for the lower response rate of IGS-18 and classical GRMs to bevacizumab/irinotecan.

Taken together, our findings suggest that comparing the initial genomic and gene expression profiles of responders and nonresponders might not be an effective strategy to identify robust biomarkers of the response to bevacizumab/irinotecan given at recurrence. Yet, they also suggest that GRMs molecular subclades are associated with the response to this treatment. This result however needs to be validated in a prospective and larger series of patients.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Julien Laflaire and Anna Luisa Di Stefano contributed equally to this work.

Acknowledgments

The authors thank Fabien Petel for his help in managing the microarray database of the CINIT. They thank Pims French for his help in assigning their samples to IGs. This work is part of the Cartes d'Identité des Tumeurs (CIT) national program funded and developed by the Ligue Nationale Contre le Cancer and was also supported in part by the Institut National du Cancer (INCa, ref 2109-126). Di Stefano is supported by an investigator fellowship from Collegio Ghislieri, Pavia, Italy. Pr Figarella-Branger is supported by a Grant INCa-DGOS-Inserrno 6038. Frozen specimens from the AP-HM institution were stored and then provided by the AP-HM tumour bank (authorization no. AC-2013-1796). Frozen specimens from Lyon were stored in NeuroBioTIC, Bron, France.

References


SHORT REPORT

Herpes simplex encephalitis in glioma patients: a challenging diagnosis

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ABSTRACT

Objectives In recent years, herpes simplex encephalitis (HSE) has been reported with increasing frequency in settings of immunosuppression, such as acquired immunodeficiency, transplantation and cancer. As observed, in immunocompromised individuals HSE presents peculiar clinical and pathological features, and poorer prognosis.

Methods Here we describe a retrospective series of seven cases of HSE in patients with high-grade glioma (HGG), collected among three institutions in a 5-year period (during this time, a total of 1750 patients with HGG were treated).

Results Diagnosis of the condition was particularly challenging due to the confusing clinical presentation and the atypical biological findings. As a result, antiviral treatment was started with a sharp delay compared with immunocompetent hosts. Prognosis was poor, with high short-term mortality and severe residual disability in survivors.

Conclusions The substantial incidence of HSE observed in our centres together with the difficulty in diagnosing the condition suggest that the incidence of this complication may be highly underestimated. The aim of our report is to strengthen the observation of HSE in patients with HGG and outline the key elements that may allow its diagnosis.

INTRODUCTION

Herpes simplex encephalitis (HSE) is the most common form of sporadic encephalitis in the general population, with an incidence of 2–4 cases per million people annually. Although most commonly seen in immunocompetent hosts, in recent years several cases of HSE in settings of immunosuppression—such as acquired immunodeficiency, transplantation and cancer—have now been reported.2,3 In immunocompromised patients, HSE may present with atypical clinical and pathological features,3 making the diagnosis challenging even for expert neurologists. In patients with brain tumours, the diagnosis of HSE is made even more difficult by the presence of neurological signs and symptoms attributable to the tumour itself.

Single cases of HSE in high-grade glioma (HGG) patients are scattered through literature, but the actual incidence of this complication is unknown. Rarity and the difficulty in diagnosing the condition might cause underreporting of the occurrence of HSE in patients with HGG.

We report seven cases of HSE in patients with HGG, collected among three institutions in a 5-year period, and review the existing literature. The aim of our report is to strengthen the observation of HSE in high-grade glioma patients, describe the peculiar clinical and pathological profile of HSE in this setting and outline the key elements that may allow its diagnosis.

CASE SERIES

Here we describe a series of seven patients with HGG who developed HSE during cancer treatment, collected among three institutions (C. Mondino National Institute of Neurology Foundation, Pavia, Italy; C. Besta Neurological Institute, Milan, Italy; AP-HS Group CHU Hospital Pitié-Salpêtrière, Paris, France). Every centre conducted an Internal Review Board-approved retrospective study using an institutional database of all patients receiving a diagnosis of 'herpes simplex encephalitis' and 'glioma' from the period between 1 January 2008 and 30 September 2013. During this time, a total of 1750 patients with HGG were treated in the three institutions.

Patients developed HSE during different steps of HGG evolution: one patient 2 weeks after surgery (patient 7), four patients during the SNPP protocol (patients 1, 2, 5 and 6) and the remaining two (patients 3 and 4) 4 and 13 months after its conclusion while receiving a first-line chemotherapy.

Clinical and pathological findings in our seven patients are detailed in Table 1.

All the patients were receiving daily steroids, and four of the seven patients showed a grade II or III lymphopenia (CTCAE V3.0). The main clinical features of HSE included fever (1/7), stupor/coma (4/7) and partial seizures (6/7). In two patients (patients 2 and 4), an encephalitis was suspected due to the clinical and electroencephalographic appearance of a new epileptic focus contralateral to the tumour site, and in three others (patients 1, 3 and 7) due to persistent consciousness alteration. In all cases, systemic infections and tumour progression were excluded on the basis of chest X-rays, crine and blood testing, and brain CT scan. Cerebrospinal fluid (CSF) analysis revealed slight blood-brain barrier damage in all seven patients, but lymphomnocytopneutropenia in only three. The diagnosis of HSE was confirmed by CSF analysis and PCR for herpes simplex virus 1 (HSV-1).
## Table 1: Clinical and pathological findings in our seven patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Type</th>
<th>Lesion size (cm)</th>
<th>Cancer stage</th>
<th>Cohort</th>
<th>Lymphopenia (mm&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Clinical Features</th>
<th>Toxicity</th>
<th>CTP score</th>
<th>WBC result (x 10&lt;sup&gt;9&lt;/sup&gt;/L)</th>
<th>Prothrombin ratio (%)</th>
<th>Days to treatment start</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72F</td>
<td>GBM</td>
<td>D</td>
<td>Engaging, 10 weeks</td>
<td>Glioblastoma</td>
<td>160</td>
<td>525</td>
<td>Fever, constipation, nausea, vomiting</td>
<td>Grade 3</td>
<td>40</td>
<td>13.5</td>
<td>70</td>
<td>7</td>
<td>Death (1st ED)</td>
</tr>
<tr>
<td>2</td>
<td>50M</td>
<td>GBM</td>
<td>D</td>
<td>Engaging, 30 weeks</td>
<td>Glioblastoma</td>
<td>160</td>
<td>1700</td>
<td>Fever, constipation, nausea, vomiting</td>
<td>Grade 3</td>
<td>40</td>
<td>13.5</td>
<td>70</td>
<td>7</td>
<td>Death (1st ED)</td>
</tr>
<tr>
<td>3</td>
<td>55M</td>
<td>GBM</td>
<td>D</td>
<td>Completed, 12 months</td>
<td>Glioblastoma</td>
<td>160</td>
<td>550</td>
<td>Fever, constipation, nausea, vomiting</td>
<td>Grade 3</td>
<td>40</td>
<td>13.5</td>
<td>70</td>
<td>7</td>
<td>Death (1st ED)</td>
</tr>
<tr>
<td>4</td>
<td>60M</td>
<td>GBM</td>
<td>D</td>
<td>Completed, 4 months</td>
<td>Glioblastoma</td>
<td>160</td>
<td>800</td>
<td>Fever, constipation, nausea, vomiting</td>
<td>Grade 3</td>
<td>40</td>
<td>13.5</td>
<td>70</td>
<td>7</td>
<td>Death (1st ED)</td>
</tr>
<tr>
<td>5</td>
<td>65M</td>
<td>GBM</td>
<td>D</td>
<td>Engaging, 40 weeks</td>
<td>Glioblastoma</td>
<td>160</td>
<td>500</td>
<td>Fever, constipation, nausea, vomiting</td>
<td>Grade 3</td>
<td>40</td>
<td>13.5</td>
<td>70</td>
<td>7</td>
<td>Death (1st ED)</td>
</tr>
<tr>
<td>6</td>
<td>70M</td>
<td>GBM</td>
<td>D</td>
<td>Engaging, 40 weeks</td>
<td>Glioblastoma</td>
<td>160</td>
<td>500</td>
<td>Fever, constipation, nausea, vomiting</td>
<td>Grade 3</td>
<td>40</td>
<td>13.5</td>
<td>70</td>
<td>7</td>
<td>Death (1st ED)</td>
</tr>
<tr>
<td>7</td>
<td>75M</td>
<td>GBM</td>
<td>D</td>
<td>Stage not performed</td>
<td>Glioblastoma</td>
<td>160</td>
<td>500</td>
<td>Fever, constipation, nausea, vomiting</td>
<td>Grade 3</td>
<td>40</td>
<td>13.5</td>
<td>70</td>
<td>7</td>
<td>Death (1st ED)</td>
</tr>
</tbody>
</table>

Note: CTP = cerebral tumor progression; CTP = chemotherapic tumor progression; WBC = white blood cell count; m = months; cm = centimeters; mm<sup>3</sup> = cubic millimeters; ED = emergency department; + = positive; - = negative.
positive in all the patients with high CSF/serum replication ratio. MRI showed unilateral or bilateral typical HSE alterations in all cases. Apparent diffusion coefficient (ADC) maps were positive, indicating restricted diffusion in temporoparietal basal areas, in all four patients in whom diffusion weighted imaging (DWI) was acquired (Figure 1). Despite treatment intravenous acyclovir, 10 mg/kg every 8 h daily, four patients died of the infection, while the three who survived were left with severe residual cognitive impairment and disability.

**DISCUSSION**

This retrospective study represents the largest series of HSE in high-grade glioma patients and describes the peculiar clinical and paraclinical profile of HSE in this population. HSE clinical presentation was dominated by the presence of mild to moderate hyperthermia, proptosis and recurrent seizures up to status epilepticus. Diffusion-weighted MRI was constantly altered, and resulted in an early and helpful tool to direct the following diagnostic work-up, CSF profile was misleading due to absence or poor evidence of CNS inflammatory markers. This finding may be possibly addressed to an aCGF immune compartmental cellular response and has been reported in patients receiving brain irradiation and prolonged steroids. All seven cases were eventually confirmed by PCR testing on CSF, emphasizing the importance to perform this test even in the absence of pleocytosis. In addition to the peculiarities attributable to the background of immunosuppression, in patients with HGG the diagnosis of HSE is made even more difficult by the broad spectrum of alternative causes that can produce similar clinical phenotypes: tumour progression, early-onset radiation-induced encephalopathy and systemic infections or metabolic diseases. Antiviral treatment was started as soon as diagnosis HSE was evoked. Still, the diagnostic delay was remarkable in our series: the median time from admission to starting antiviral treatment was 3 days in our series compared with the average of 7.5 days in the series of the report in immunocompetent hosts. The delay in diagnosis may have possibly contributed to the poor prognosis we observed. On the basis of our experience, we report three key elements that, occurring alone or in combination, should prompt early suspicion of HSE in glioma patients: hyperthermia, acute consciousness alteration rapidly progressing to coma and the appearance of new epileptic focal not corresponding to the site of the primary tumour. In these scenarios, diffusion-weighted MRI and PCR for HSV on CSF, followed by empirical treatment with acyclovir, should be promptly undertaken.

In recent years, the literature is enriching with reports of HSE in immunocompromised patients, including patients with systemic neoplasms and primary CNS tumours. We conducted a review of the literature and found 14 previously reported cases of HSE in patients with gliomas, with details reported in online supplementary table S2. Consistently with that found in our series, HSE occurred during different steps of cancer history (in five patients perioperatively, in six patients during...
radiotherapy, and in the last three patients within 3 months of its completion. 12,13), and evolution was characterized by high mortality or severe morbidity despite antiviral treatment. Regarding aetiology, patients with HGG are usually exposed to a number of conditions predisposing to infections, such as chronic steroid treatment, chemotherapy-induced myelosuppression and the tumour itself. Steroids are known to have profound effects on the distribution and function of lymphocytes. The related risk of infections is proportionate to the dose and duration of steroid administration and patients with HGG usually require steroid treatment for the entire disease duration, and particularly during combined radiochemotherapy. The Snup protocol 14 in since 2003 the standard of care in patients with HGG. In our experience, tenosynovitis provides neuropenia during the concomitant and adjuvant phase, and a peculiar protracted lymphopenia during the concomitant phase, when associated to chronic steroids. In the case of HSE, lymphopenia is most likely the effect to promote the infection and was observed in four of the seven patients in our series. Additionally, the impairment of cell-mediated immunity induced by steroids and chemotherapy is worsened by the local and systemic immunosuppressive effect of HGG-induced cytokines. 15 Irradiation is known to have a great impact on neurons and immune cells, and all patients with HGG receive radiotherapy as part of the Snup protocol. 16 Considering HSV latent tropism towards neuroepithelial structures, it is remarkable that half of the patients reported in literature and in our series suffered from a temporal neoplasia, thus suggesting that direct local irradiation may play a role in promoting viral reactivation. This may also be part of the reason why, of all major viral infections, HSE is the most frequently reported in patients with brain cancer.

On the basis of our observations, we would like to raise the attention of neurologists and oncolgists on HSE as an underestimated, rare but non-negligible complication in patients with HGG. Snup protocol is already recognized to be a time of susceptibility for opportunistic infections, and prophylactic antimicrobial treatment is recommended by a number of oncological guidelines 17 to prevent Pneumocystis carinii pneumonia. Conversely, invasive viral infections are considered a rare event in patients with solid tumours and, therefore, antiviral prophylaxis is not recommended. Still, compared with other patients with cancer, HGG are contemporary exposed not only to a severe cell-impaired immunity but also to the local insult of radiotherapy. Antiretroviral prophylaxis with aciclovir has proven effective and is already recommended in a number of haematological malignancies during the period of latency to prevent herpesvirus reactivation. 18 Aciclovir is usually well tolerated, and major side effects are very rare. On these grounds, considering the poor prognosis HSE has shown in patients with HGG, a beneficist discussion for the introduction of aciclovir prophylaxis may be pertinent in the neuro-oncology community.

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Acknowledgments. The authors would like to thank Georgia Buselli for her valuable assistance in radiological data collection and commentary.

Contributors. All authors made substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. All authors contributed to drafting the work or revising it critically for important intellectual content. All authors approved the final version published. All authors are accountable for the accuracy and integrity of any part of the work. Specific contributors include the following: patient management: EDA, GB, CD, MN, FG, CD, ALLE, MC, AS. Neurooncology: FM, LM, neurosurgery: GB, management: GB, data analysis: GB, MC, AS. Funding. This study was supported by Italian Ministry grant "Becchi Corriere 2012. AIDS is supported by an investigator-initiated grant from the Citi/North Shore, Pavia, Italy.

Conflict of interest. None.

Patient consent. Obtained.

Ethics approval. Approved by the Institutional Ethics Committee.

Provenance and peer review. Not commissioned, externally peer reviewed.

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J Neurol Neurosurg Psychiatry 2015 86: 374-377 originally published online May 29, 2014
doi: 10.1136/jnnp-2013-307198

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Anti-Tumour Treatment

Systemic treatments for brain metastases from breast cancer, non-small cell lung cancer, melanoma and renal cell carcinoma: An overview of the literature

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A R T I C L E   I N F O
Article History:
Received 18 April 2014
Received in revised form 13 May 2014
Accepted 19 May 2014

A B S T R A C T
The frequency of metastatic brain tumours has increased over recent years: the primary tumour most involved are breast cancer, lung cancer, melanoma and renal cell carcinoma. While radiation therapy and surgery remain the mainstay treatment in selected patients, new molecular drugs have been developed for brain metastases. Studies so far report interesting results. This review focuses on systemic cytotoxic drugs and, in particular, on new targeted therapies and their clinically relevant activities in brain metastases from solid tumours in adults.

I N T R O D U C T I O N

Metastatic brain tumours are the most common intracranial neoplasms in adults and are a significant cause of deleterious effects on many critical neurological functions. Moreover, morbidity and mortality rates are higher for patients who develop brain metastasis (BM); over the last few years, the frequency of BM has increased due to longer survival of patients through more effective systemic treatment and earlier BM detection by improved neuro-imaging.

Estimates of BM incidence vary from 20% to 50% [1]; analyses of patient data from the Metropolitan Detroit Cancer Surveillance System showed a total incidence proportion of BM of 9.6% [1]; the incidence proportion of BM was highest for lung cancer (15.2%), followed by 6.5% for melanoma, 6.5% for renal cancer, 5.1% for breast cancer. However, as described in various studies, the incidence of BM may be higher than observed, due to asymptomatic BM [2].

Radiation therapy and surgery remain the cornerstones of treatment in selected patients, while cytotoxic drugs have a limited impact. On the other hand, in recent years, advances in the understanding of the biology of BM have led to the development of new targeted therapies and interesting results have been obtained so far.

In this review, we analysed systemic treatments, both cytotoxic and, in particular, new molecular drugs for BM from solid tumours in adults, such as breast cancer, lung cancer, renal cancer and melanoma.

B R A S T C A N C E R

Recent improvements in systemic therapy have increased the overall survival of breast cancer (BC) patients, including metastatic patients. In the context of controlled systemic disease, the prevalence of BM from BC is increasing. BC is the second leading cause of BM after lung cancer and accounts for 17–20% of all cases. BM treatment options currently include whole-brain radiotherapy
Cytotoxic drugs

In the setting of newly or recurrent BM from BC, few prospective trials have evaluated the benefit of cytotoxic agent administration. At the onset of BM, in combination with radiotherapy, the use of cisplatin and vinorelbine was associated with 76% of objective brain response rate (ORR) for 25 pts. However, median progression free survival (PFS) remained modest (3.7 months) while median overall survival (OS) was 6.5 months, and half of patients presented with non-hematological grade 3–4 toxicities [3]. In another phase II trial [4], combination of cisplatin and etoposide with radiation therapy (RT) for 56 patients with BM was associated with 13% of complete response and 14 partial responses (23.5%); however, mMPR and mOS was attained modest (4 and 8 months, respectively). In these first-line studies, despite encouraging response rates, effect of combination of RT and cytotoxic agents remained modest.

In another phase II study [5], the authors evaluated temozolomide activity with alternating weekly, dose-dense temozolomide, in pretreated patients with BM, stratified by primary tumor type. In this study, 51 BC patients presented with a mPFS of 1.9 months while mOS was not reached. The disease control rate (responses + stable diseases) was 56%; while ORR was 45.

In a phase I trial [6], 24 newly or recurrent patients with BM from BC, were treated with temozolomide plus capcitabine. In this study, 51 BC patients presented with a mPFS of 1.9 months while mOS was not reached. The disease control rate (responses + stable diseases) was 56%; while ORR was 45.

In a phase I trial [7] for 15 patients with recurrent BM from BC, ORR was 13.3%, mPFS and mOS were 1.4 and 5.3 months, respectively; these modest results led to premature stopping of enrollment.

Finally, in a prospective trial has evaluated the potential benefit of hormonal therapy for patients with BM from BC.

Human epidural growth factor receptor (HER) targeted therapies

HER2 is overexpressed in approximately 20% of breast cancer tissue and it represents one of the main molecular targets in the development of new therapies. Trastuzumab, a monoclonal antibody targeting HER2, was approved for metastatic breast cancer in 1998; Lapatinib is a dual tyrosine kinase inhibitor of both HER1 and HER2, approved by the FDA in 2007. Finally, pertuzumab, a monoclonal antibody that blocks dimerization of HER2 with HER1, 3 and 4, was approved in 2012.

Overexpression of HER2 is an independent factor for development of BM, which may likely be due to a more aggressive subtype of HER2-positive breast cancer, or to the fact that these patients treated with HER2-targeted therapies live longer. Moreover, these targeted drugs have limited potential to cross the blood–brain-barrier. Hence, in the setting of well-controlled extraneural disease and BM, the best treatment is still unknown and several clinical trials to determine the optimal treatment for BM are ongoing.

In the treatment of BM, several studies have suggested activity of trastuzumab for treatment of BM from BC. However, all studies, except one [8], were retrospective and even in the prospective study data concerning BM were retrospectively collected. In this study [8], use of trastuzumab was associated with better OS (17.5 vs. 3.8 months). Despite the absence of a prospective and randomized trial, use of trastuzumab at the time of BM could represent an interesting option. Trastuzumab was used in intracleral treatment in several case reports in association with intracleral methotrexate or cytarabine. This strategy was associated with stabilization of multiple BM in one case [9,10]. However, the absence of a larger cohort or prospective trial did not allow any conclusion about the potential benefit of the use of intracleral trastuzumab.

Several studies have evaluated the activity of lapatinib for BM in BC and 5 of these were prospective phase II trials [11]. The first reported on lapatinib for recurrent BM from BC. In this single-arm phase II study, 30 patients were enrolled. By “Response Evaluation Criteria in Solid Tumors” (RECIST) assessment, the ORR was only 2.6%, but by volumetric analysis, 10 pts (26%) achieved at least 10% of volumetric reduction. In this study, mPFS was 3.0 months [11]. In 2009, Lin et al. published a second phase II trial evaluating lapatinib for patients with BC and BM progressing after RT [12]. In this study, lapatinib refractory-patients were treated with lapatinib plus capcitabine. Overall response or volumetric reduction of lesions was observed in 5% and 21% of patients, respectively. In patients with the extension of lapatinib plus capcitabine, ORR and volumetric reduction were seen in 20% and 40% of patients, respectively, leading to the preferential use of this association. For patients with lapatinib alone or lapatinib plus capcitabine, mPFS were 2.4 and 3.0 months, respectively, while mOS for the entire cohort was 6.4 months [12].

In a recent randomized phase II trial, the combination of lapatinib and capcitabine versus lapatinib plus topotecan was analyzed [13]. However, this trial was prematurely stopped due to excess toxicity and lack of efficacy in the lapatinib plus topotecan arm (ORR – 0%). In the LANDSCAPE trial [14], the combination of
lapatinib plus cetuximab for the treatment of untreated brain metastases from HER2-positive breast cancer was evaluated. Sixty-six percent of patients had objective brain partial response, delaying the initiation of radiotherapy. In this trial, mPFS was 5.5 months and mOS was 17 months.

At the onset of BM, another phase I trial [15] evaluated the association of lapatinib and RT for newly BM from BC in 35 pts. ORR was 79% by volumetric criteria. In this study, mPFS was 4.8 months and mOS was 19 months. However, this study did not meet the primary objective of feasibility because of toxicity.

Moreover, combination of lapatinib and trastuzumab for BM from BC was only evaluated in retrospective studies. Use of both anti-HER2 agents could be associated with an interesting effect on patient survival [16,17].

Finally, no study is available to date on the evaluation of pertuzumab for BM from BC.

**Non-small cell lung cancer**

In patients with non-small cell lung cancer (NSCLC), brain metastases develop in approximately 30% of cases [18]. In the literature, BM from NSCLC was treated with various cytotoxic drugs or new molecular drugs with or without RT.

**Cytotoxic drugs**

Recently, many chemotherapeutic regimens have been tested in phase II or phase III trials for the treatment of brain metastases from NSCLC (see Table 2).

Pramo et al. [4] analyzed 116 patients receiving cisplatin 100 mg/m² on days 1 and Etoposide 100 mg/m² on days 1, 3, and 5 or on days 4, 6, and 8 every 3 weeks. The distribution of primary tumor type was adenocarcinoma in 56 patients (52%) and NSCLC in 43 (48%). Among 43 patients with NSCLC, 3 achieved CR (7%), 10 achieved CR, 15 had SD, 7 had PD, and 8 had insufficient treatment or evaluation. Another trial [19] evaluated the efficacy of carboplatin–cetuximab plus concurrent WBRT in patients with BM from lung adenocarcinoma. Forty-two patients were enrolled in this study. The incidence of BM from newly diagnosed NSCLC with BM and Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2 received up to six cycles of cisplatin and pemetrexed (75 and 500 mg/m², respectively) every 3 weeks in association with WBRT 30 Gy during the first cycle. Concerning brain lesions, RR was 63.6%, PFS was 10.6 months and OS was 12.6 months. A Spanish study [20] evaluated the activity of paclitaxel–cetuximab with vinorelbine or gemcitabine as front-line therapy in BM from NSCLC. Whole-brain irradiation was offered early in case of progression and later as consolidation treatment. The median OS for all patients was 21.4 years and the median DFS was 12.8 years. Paclitaxel and cisplatin combined with vinorelbine or gemcitabine as front-line therapy in brain metastases seem to achieve a response similar to that for extracranial disease; many tumors RR was observed in 38% of the patients. Kleibaum et al. [21] analyzed the response to high dose cisplatin. Twenty-four consecutive patients with BM of lung carcinoma were included in this study. The total dose of cisplatin (200 mg/m²) was divided into 5 equal daily fractions, infused over 6 h. Failure was observed in 17 cases, ORR in 7 cases (2 cases without injection contrast in the tumor, 3 partial regressions, 2 complete regressions). In conclusion, 30% of patients exhibited an ORR with low toxicity.

In the study by Quantini et al. [22], 23 previously untreated patients suffering from NSCLC BM were prospectively included in this feasibility study. Treatment consisted of three cycles of WBRT (18 Gy in 10 fractions) and vinorelbine, 30 mg/m² on days 1 and 8, docetaxel 1.5 mg/m² daily from day 1 through day 3, and cisplatin 100 mg/m² on day 2. A cycle restarted every 28 days. Specific evaluation of brain response demonstrated complete response for 7 patients, and partial response in 6 (ORR 56%). Median OS from start of protocol was 7.6 months.

In a multicentric phase III trial, Neubauer et al. [23] analyzed OS, local response and PFS of patients with BM from NSCLC and small cell lung cancer treated with RT alone or RT plus ipotecan. The data showed no significant advantage for concurrent radiochemotherapy; however, the recruited number of patients was too low to exhibit advantage of combined treatment.

Temozolomide is an orally administered prodrug that is converted spontaneously to the active alkylating agent. In patients with newly-diagnosed BM or with progression after RT, temozolomide demonstrated an interesting activity.

A phase II study [5] evaluated the efficacy of alternating weekly, dose-dense temozolomide in pretreated patients with BM previously stratified by patient performance status. This study analyzed 53 patients with NSCLC. PFS was 66 days and OS was 172 days. Thrombocytopenia was the most common adverse event causing dose modification or treatment discontinuation.

Giorgi et al. [24] evaluated in a phase II study the efficacy and safety of temozolomide in 30 NSCLC patients pre-treated with WBRT and at least one previous line of chemotherapy for metastatic brain disease. Three patients (10%) achieved an objective response of BM with 2 complete remissions. Stable disease and progressive disease were achieved in 3 (10%) and 24 patients (80%), respectively.

**Epidermal growth factor receptor (EGFR) inhibitors**

Targeted therapies are undergoing active development as a means to improve treatment efficacy in selected patient populations. Novel agents, such as EGFR tyrosine kinase inhibitors (TKI), have now been included in standard non-small-cell lung cancer.

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**Table 2**

Clinical trials of cytotoxic treatments for brain metastases in lung cancer.

<table>
<thead>
<tr>
<th>Author</th>
<th>REG</th>
<th>PFS (%)</th>
<th>mPFS (mes)</th>
<th>mOS (mes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fransiti et al. [4]</td>
<td>43</td>
<td>Cisplatin–etoposide</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Cotie et al. [25]</td>
<td>36</td>
<td>Cisplatin–irinotecan</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>Coessens et al. [26]</td>
<td>31</td>
<td>Cisplatin–gemcitabine</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>Fukuoka et al. [27]</td>
<td>30</td>
<td>Cisplatin–bevacizumab–oxaliplatin</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>Ergin et al. [28]</td>
<td>42</td>
<td>Paclitaxel–cetuximab</td>
<td>68</td>
<td>10.6</td>
</tr>
<tr>
<td>Klebaum et al. [21]</td>
<td>24</td>
<td>Cisplatin</td>
<td>30</td>
<td>NA</td>
</tr>
<tr>
<td>Stroia et al. [29]</td>
<td>33</td>
<td>TMZ</td>
<td>NA</td>
<td>66 days</td>
</tr>
<tr>
<td>Gregorio et al. [30]</td>
<td>30</td>
<td>TMZ</td>
<td>50</td>
<td>8.4</td>
</tr>
<tr>
<td>Quantini et al. [22]</td>
<td>23</td>
<td>RT + vinorelbine–irinotecan–cisplatin</td>
<td>30</td>
<td>NA</td>
</tr>
</tbody>
</table>

PFS: patients; RR: response rate; PFS: progression-free survival; OS: overall survival; RT: radiation therapy; BR: brain radiotherapy; NA: not available; TMZ: temozolomide; OPF1: oxaliplatin.

Anna Luisa Di Stefano
treatments. In a small subset of patients harboring EGFR-activating mutations, erlotinib and gefitinib administration was followed by RR and a longer PFS and OS than that obtained with standard chemotherapy. In recent years, several authors have reported a growing number of cases of partial and complete response in BM patients treated with EGFR TKIs (see Table 3). Data from retrospective series and phase II studies also suggest that a response can be obtained using EGFR tyrosine kinase inhibitors treatment for patients with BM, especially those harboring EGFR mutations.

Kim et al. [25] analyzed the response of 23 never-smoking Korean BM patients treated with erlotinib during gefitinib therapy until disease progression. RR was 69.6% and disease control rate 82.6%. Intracranial RR was observed in 17 patients.

Gefitinib is an orally active and reversible inhibitor of EGFR tyrosine kinase. Chu et al. conducted a prospective study with 78 patients with NSCLC and presence of BM treated with gefitinib showing a RR of 33%. PFS and OS were 4 and 9.9 months, respectively. Severity of skin toxicity was associated with tumor response and patient survival [26].

Moreover, EGFR inhibitors can be safely administered concomitantly with WBRT: in fact, a recent phase II randomized study [27] in BM from NSCLC compared WBRT plus gefitinib vs. WBRT plus temozolomide. In this randomized phase II trial, patients with BM from NSCLC were randomly assigned to 30 Gy WBRT with either concomitant gefitinib 250 mg/day continuously or temozolomide 75 mg/m² for 21 days every 28 days. The primary end-point was OS but the study failed to show any advantage for gefitinib: 6.3 months in the gefitinib arm and 4.9 months in the temozolomide arm (p not significant).

Ma et al. [28] analyzed the efficacy of temozolomide and osimertinib in the treatment of BM. In this study, 21 patients were enrolled. Osimertinib was administered at a dose of 80 mg/day. No primary end points were safety and OS. Concomitant treatment was well tolerated, and 4 and 13 patients had a complete and partial response, respectively. Patients with BM at baseline have shown OS, which means that concomitant treatment seems to be well tolerated with a significant improvement of quality of life in these population.

In a recent study, Hsiao et al. [29] analyzed the predictive role of EGFR mutations in BM treatment. In this study, 180 of 505 lung adenocarcinoma patients with BM during BM treatment and 139 patients including 89 EGFR-mutant and 50 EGFR wild-type patients were identified for analysis. Among patients eligible for evaluation of treatment response, up to 85% received RT and the remaining took EGFR TKIs. EGFR-mutant patients compared with EGFR wild-type patients had significantly greater intracranial RR of BM and a longer median OS after BM diagnosis.

Erlotinib is a low-molecular weight, orally bio-available drug that selectively and reversibly inhibits the tyrosine kinase activity of EGFR. Weich et al. [30] analyzed in a phase II trial the median OS of patients with BM from NSCLC treated with erlotinib plus WBRT.

Eligible patients had BM from NSCLC, regardless of EGFR status. 40 patients completed erlotinib + WBRT. Median OS of 17 patients with known EGFR status was 9.3 months and 19.1 months for EGFR wild-type and EGFR mutated, respectively.

In another retrospective study [31], 40 NSCLC patients with BM were treated with erlotinib until disease progression or death, or intolerable side effects. Intracranial disease, partial response was observed in 4 patients (10%), stable disease in 21 (52.5%), and progressive disease in 15 (37.5%), with an RR of 15% and a disease control rate of 62.5%. PFS and OS were 3.4 months and 9.2 months, respectively.

Cappuzzo et al. [32] evaluated the activity and safety of gefitinib in 41 NSCLC patients with BM. Thirty-seven patients had received prior chemotherapy and 18 patients had been treated previously with WBRT completed at least 3 months before entering the trial. Partial response was observed in 4 patients (10%), and stable disease in 7 cases, for a disease control rate of 27%. Median duration of partial response was 13.5 months. PFS was 3 months. Toxicity was mild and consisted of grade 1–2 skin toxicity and diarrhea, occurring in 24% and 10% of patients, respectively.

Wu et al. [33] evaluated the activity of gefitinib in 44 NSCLC patients with BM. Of these patients, 30 were previously treated with WBRT. Partial response was observed in 14 patients (31.8%) and stable disease in 21 (47.7%). PFS and OS were 9 and 13 months, respectively. The difference in disease control rate between the patients who had previous WBRT and those without was not significant (p not significant).

Antiangiogenic drug bevaciuzumab

One of the targeted approaches most widely studied in the treatment of NSCLC is the inhibition of angiogenesis. Angiogenesis is essential for the development and progression of cancer, and vascular endothelial growth factor (VEGF) is a critical mediator of tumor angiogenesis. Bevacizumab, an anti-VEGF recombinant humanized monoclonal antibody, is the first targeted agent which, when combined with chemotherapy, has been shown to confer hematological and extravascular effect versus chemotherapy alone as first-line treatment of advanced non-squamous NSCLC patients. Patients with BM have initially been excluded from bevacizumab trials for the risk of cerebral hemorrhage as a result of the treatment. Nevertheless, the available data suggest an equal risk of intracranial bleeding in patients with CNS metastases treated with or without bevacizumab therapy [34].

A phase II trial (PASSPORT) [35] specifically addressed bevacizumab safety in patients with NSCLC and previously treated BM. This open-label multicenter trial for first- and second-line treatment of nonsquamous NSCLC enrolled patients with BM. First-line patients received bevacizumab (15 mg/kg) every 3 weeks with platinum-based doublet therapy or erlotinib, and second-line patients received bevacizumab with single-agent chemotherapy or erlotinib, until disease progression or death. The study showed that addition of bevacizumab to various chemotherapy agents or erlotinib in patients with NSCLC and BM is safe and is associated with a low incidence of CNS hemorrhage.

A Japanese study retrospectively identified patients treated with bevacizumab and chemotherapy for BM from NSCLC, including 17 patients with lung adenocarcinoma. In 14 pts with evaluable BM, the response rate for intracranial metastases was 78.6% in these patients; 2 bleeding events were reported: one was grade 1 intracranial hemorrhage, the other was grade 1 bronchopulmonary hemorrhage. This study showed that chemotherapy and bevacizumab is effective for patients with BM and is a well-tolerated regimen with a favorable toxicity profile [36].

Zustovich et al. [37] analyzed 18 patients with BM mostly from lung and renal adenocarcinoma and the majority of patients had a
treatment-naive brain disease: 82% of patients had a partial response and 18% had stable disease. PFS was 14 months and OS was 15 months. Toxicity was the same as that seen in clinical practice for other central nervous system metastases were reported.

Noroner et al. [38] analyzed OS, PFS, RR and toxicity in patients who received bevacizumab plus chemotherapy. Median OS and PFS were 8.8 and 4.5 months in patients with ECOG PS of 0-1, while 2.6 and 1.2 months for those with PS 2. Therefore, these data suggest that patients with PS 2 should not receive this treatment.

Melanoma

Melanoma BM are common since at least one patient out of three with advanced melanoma will ultimately develop BM. Survival remains dismal with an expected median OS of 16–22 weeks; probably because of the poor efficacy of conventional treatments due to radioresistance of melanoma cells and the low blood–brain barrier penetrance of systemic cytotoxic agents commonly used in metastatic melanoma. More recently, new therapeutic agents have proven their efficacy in progressing metastatic melanoma and in particular on the basis on molecular status of primary disease (see Table 4).

These recent advantages achieved by small molecules and personalization therapy give rise to the issue of the best sequencing of therapeutic interventions and the need for stratification of patient prognostic factors.

Cytotoxic chemotherapy with nitrosoureas, such as fotemustine and temozolomide, have been tested in melanoma brain metastasis patients because of their ability to penetrate the blood–brain barrier. Response rate of 5.9% was observed for fotemustine and 6% for temozolomide without any added benefit to RT, and with increased toxicity [39–41].

Recently, two systemic agents showed encouraging results in control of melanoma brain metastases alone or in association with brain irradiation. These preliminary data are very encouraging even if still investigational.

The first is the human CTLA4-antibody, ipilimumab, which inhibits immunologic checkpoints. The mechanism of action involves the blockade of negative signaling in cytotoxic T cells that occurs normally following cytotoxic activation.

Ipilimumab was approved in 2011 for patients with metastatic melanoma based on a survival advantage over a melanoma vaccine, corticosteroid therapy and single-agent dacarbazine.

Up to 15% objective response and 25% of stabilization 12 weeks following the initiation of therapy were observed in a phase II open study ipilimumab (10 mg/kg intravenously every 3 weeks for 4 cycles followed by the same dose every 12 weeks) versus corticosteroid therapy. Furthermore, median OS of 3 years was obtained in a phase II trial testing ipilimumab in addition to fotemustine. These results encouraged “proof of principle” that the benefit of CTLA4 blockade extends to central nervous system (CNS) disease with peculiar concordant response in the control of brain and extracranial disease in metastatic melanoma. Ipilimumab is currently undergoing testing as adjuvant therapy after resection of high-risk melanoma, either compared with placebo or compared with interferon. In the short-term, combinations with less toxic agents such as temozolomide as well as other new checkpoint-blocking antibodies and RT need to be explored [44–51].

As other immunomodulatory agents, promising antibodies that block negative signaling through the PD-1/PD-L1 axis continue to be studied and represent a potential tool for the management of CNS disease.

The second systemic agent is BRAF inhibitor. BRAF mutations occur in approximately 50% of melanomas, resulting in constitutive up-regulated signaling through the MAPK pathway, independent of receptor-ligand interactions, that can be targeted by selective small-molecule inhibitors [52,53].

The most common mutation is V600E occurring in 70–90% of BRAF-mutant melanomas; other less frequent mutations include V600K (10–20%), V600D (1–7%), and V600E (1–4%) [52,53].

The first report of activity of BRAF inhibitors for patients with melanoma BM was in 10 patients with V600E (9 patients) and V600K (1 patient) melanoma [54]. Nine patients had a size reduction of BM and four had a complete response.

These results led to the largest trial ever conducted in active melanoma BM: 172 patients with V600E or V600K mutation-positive melanoma were treated with 150 mg twice daily of dabrafenib (BRAF-MB); all patients were divided into two cohorts according to prior local therapy with surgery, WBRT, or stereotactic radiosurgery at progression. Responses were seen in both cohorts, and in both V600E and V600K BRAF mutation-positive melanoma. Overall intracranial RR were up to 39% with median PFS of 16 weeks and OS of 31 and 33 weeks (according to prior treatment at progression) [55].

Interestingly, the brain is not always the first site of progression: 30% of progression at an extra- or combined-site. According to previous studies, BRAF-MB had a confirmed partial response in the brain, whereas 13/21 patients (62%) had extra-cranial responses, resulting in constitutive up-regulated signaling through the MAPK pathway, independent of receptor-ligand interactions.

Another encouraging observation is the dramatic symptomatic relief of neurological symptoms in patients with active brain metastases treated with dabrafenib [57].

However, it is not clear whether a difference in activity exists between dabrafenib and vemurafenib in BM although, a recent preclinical study suggests that dabrafenib may have a higher concentration and longer acting lipophilic metabolites crossing the BBB in murine model [58]. Regarding safety, liver toxicity, arthritis, and photosensitivity appear more common with vemurafenib, fever is more frequent with dabrafenib [54].

Table 4

<table>
<thead>
<tr>
<th>Author</th>
<th>PFS</th>
<th>Regimen</th>
<th>RR (%)</th>
<th>mPFS (wks)</th>
<th>mOS (wks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jassal et al. [39]</td>
<td>30</td>
<td>Fotemustine</td>
<td>29</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Arjou et al. [40]</td>
<td>22</td>
<td>Fotemustine</td>
<td>5.9</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Marin et al. [41]</td>
<td>37</td>
<td>Fotemustine + RT</td>
<td>10</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Margolin et al. [42]</td>
<td>31</td>
<td>Temozolomide + RT</td>
<td>9</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>Atkinson et al. [43]</td>
<td>39</td>
<td>Temozolomide + RT + Temozolomide</td>
<td>3.9</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Margolin et al. [44]</td>
<td>51</td>
<td>Ipilimumab</td>
<td>14</td>
<td>10.7</td>
<td>2R</td>
</tr>
<tr>
<td>Quaglia et al. [45]</td>
<td>146</td>
<td>Ipilimumab</td>
<td>11</td>
<td>11.2</td>
<td>17.2</td>
</tr>
<tr>
<td>Talkowski et al. [46]</td>
<td>10</td>
<td>Dabrafenib</td>
<td>90</td>
<td>16.8</td>
<td>32</td>
</tr>
<tr>
<td>Uttner et al. [47]</td>
<td>24</td>
<td>Vemurafenib</td>
<td>92</td>
<td>16</td>
<td>30</td>
</tr>
</tbody>
</table>

PFS: patient; RR: response rate; PFS: progression free survival; OS: overall survival; RT: radiation therapy; NA: not available.
Table 5
Clinical studies of systemic treatments for brain metastases in renal cell carcinoma

<table>
<thead>
<tr>
<th>Authors</th>
<th>PP5</th>
<th>Response</th>
<th>RR (%)</th>
<th>mPFS (mo)</th>
<th>mOS (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geno et al. [66]</td>
<td>21.3</td>
<td>50%</td>
<td>5.0</td>
<td>12.3</td>
<td>20.5</td>
</tr>
<tr>
<td>Motzer et al. [60]</td>
<td>0%</td>
<td>70</td>
<td>4.1</td>
<td>12.2</td>
<td>20.6</td>
</tr>
<tr>
<td>Ambrosch et al. [56]</td>
<td>4%</td>
<td>75</td>
<td>20.5</td>
<td>13.7</td>
<td></td>
</tr>
</tbody>
</table>

PP5: patients, BM: brain metastases, mPFS: median progression-free survival, mOS: median overall survival. NA: not available. The maximum reported value among the four patients.

Development of resistance to treatment occurs in most patients and a new strategy could be the association with other inhibitors of MAPK cascade. In fact, a phase 2 study compared single-agent dabrafenib with the combination of dabrafenib and trametinib showing a superior RR (76% vs. 54%), superior progression-free survival (19.4 vs. 5.8 months), and superior progression-free survival at 12 months (41% vs. 91%) for the combination regimens [59].

Interestingly, an “abscopal effect” has been described in a patient receiving RT after discontinuation of vemurafenib because of subsequent progression of metastatic melanoma and appearance of a brain metastasis, which was treated by radiosurgery [60]. After radiosurgery, he showed regression of metastatic disease and also appearance of white hair and vellus of the skin suggesting an immune response against normal and neoplastic melanocytes activated after radiosurgery without concomitant vemurafenib, which was stopped before radiosurgery at second melanoma progression. At 18 months after the completion of radiosurgery, the patient showed no evidence of recurrence or regression of other metastasis. The term “abscopal effect” (from the Latin “ab”-position away from and “scopus”-target) has been used to denote this phenomenon of tumor regression at sites that are remote from an irradiated target, the pathophysiology of the abscopal effect seen in this patient is not completely understood; one hypothesis is that BRAF inhibition could result in increased immunity in melanoma cells with increased expression of melanoma antigens and enhanced reactivity to antigens-specific T lymphocytes, which ultimately contribute to the systemic response to stereotactic radiosurgery. The response seen in this patient provides insight into how local ablative strategies can augment a systemic response to targeted therapy [61].

In conclusion, the successful results recently obtained in the treatment of patients with BM from melanoma are an example of the need to redesign the therapeutic attitude and to design new clinical trials in this setting of clinical practice. The extended survival achieved by few therapeutic agents justify consideration of aggressive local therapy for patients with melanoma BM. At the moment, the main remaining issue is to find the optimal sequences and combinations of new molecular drugs and to find other molecular alterations that may candidate patients to personalized targeted therapy.

Renal cell carcinoma

Brain metastasis from renal cell carcinoma (RCC) occurs in approximately 5-12% of cases; data from Mammillan 2022 Registry showed that the 5-year cumulative incidence of brain metastases from RCC was 9.6% [1,62].

The median survival of patients with untreated RCC BM averages from 3 to 4 months [63]; the outcome for these patients is poor, with median OS of only 4–11 months after diagnosis even after surgical resection, WBRT, or stereotactic radiosurgery [64]. Moreover, metastatic RCC is generally resistant to chemotherapy and thus, immunotherapy with interferon or interleukin-2 has been the most commonly used treatment, despite low response rates. The advent of TKIs and other targeted therapies has drastically altered the management of metastatic RCC, and some published data suggest that these agents may be effective on BM as well (see Table 5).

Sunitinib is a small, oral, multi-targeted receptor TKI with anti-tumor and antiangiogenic activity. It has been shown that brain penetration of sunitinib may reach 31%, a higher penetration than other TKIs [65]. Geno et al. [66] reported results from an open-label, expanded access program with sunitinib (50 mg, once daily, in repeated 6-week cycles of 4 weeks on treatment, followed by 2 week off) for more than 4000 patients with metastatic RCC. Among these, 213 patients with BM were evaluable for tumor response and 122 achieved a complete response, yielding an ORR of 12% compared with an ORR of 17% in the overall population [67]. mPFS was 5.6 months (95% CI, 5.2–6.1) and 10.3 months (95% CI, 10.3–13.3) in patients with and without BM, respectively. Similarly, median OS was 9.2 months (95% CI, 7.8–10.5) in patients with BM compared with 18.4 months (95% CI, 17.4–21.2) in the overall population. However, the median OS observed in patients with BM compared favorably with historical survival data for untreated patients with BM [64]. Regarding toxicity, the incidence of severe adverse events and treatment-related adverse events was not different between the two groups of patients. The most common adverse events were diarrhea and fatigue. Cerebral hemorrhage was reported in only one patient. Moreover, the tolerability of sunitinib in patients with BM was similar to that reported in the prior phase III trials.

Sorafenib is a multikinase inhibitor of receptor tyrosine kinases VEGF receptors 1, 2, and 3 and platelet-derived growth factor receptors α and β as well as the Raf/MEK/ERK pathway at the level of Raf kinase, in the sorafenib expanded access program [68], of the 1851 evaluable patients with metastatic RCC, 70 had BM. Among these, 43% of patients obtained a partial response; no patient achieved a complete response, yielding an ORR of 4%. No data on mPFS and OS was reported for patients with BM. Tolerability was comparable to that observed with sunitinib.

Mucic et al. [69] retrospectively analyzed the incidence of BM in 139 patients treated with interferon-alpha and interferon-beta and placebo group in a subgroup of patients from TARGET trial (Treatment Approaches in Renal Cancer Global Evaluation Trial); this study was a randomized phase III trial, involving 503 patients with metastatic RCC. 451 treated with sorafenib and 452 received placebo. The overall incidence of BM was 35% and 36% in patients treated with sorafenib and placebo, respectively (p = 0.54). On univariate analyses, the administration of sorafenib therapy was the only predictive factor to affect the occurrence of BM in patients with metastatic RCC. However, some patients in the TKI group were also treated with other targeted agents; eridexim, temsizomab and bevacizumab; these agents likely had a protective role with regard to BM as well.

Another retrospective study [70], evaluated the impact of sunitinib and sorafenib on incidence of BM and OS in patients with metastatic RCC. Among 338 patients who were identified (patients were included in the TKI group only if they had received the agent before BM was diagnosed), 154 (46%) were treated with a TKI before brain metastases and 184 (54%) were not. No significant differences in prognostic factors between the two groups were observed. Median OS was longer in the TKI-treated group (25 vs. 12.1 months, p = 0.0011). In multivariate analysis, TKI therapy was associated with improved OS (HR 0.55; 95% CI 0.33-0.88; p = 0.001). The 5-year actuarial rate of BM was 40% vs. 17% (p < 0.001); on multivariate analysis. TKI treatment was associated with lower incidence of BM (HR 0.47; 95% CI 0.21-0.92; p = 0.003). This retrospective study found sunitinib and sorafenib to be protective with regard to BM development.
Bantos et al. [31] analyzed 65 patients treated with targeted therapy after BM diagnosis; 52 patients (80%) were treated with antiangiogenic agents and 13 (20%) with mTOR inhibitors. Median OS from start of TKI was 12.2 months (95% CI 8–15.5), median PFS was 3.4 months for first-line TKI therapy and 1.9 months for second-line TKI.

Larkin et al. [32] retrospectively identified 217 patients (75%) out of 284 patients with RCC developing symptomatic BM while on treatment with sorafenib or sunitinib; the median start from starting TKI was 11 months (95% CI 5.5–17 months). Verma et al. [75], in another retrospective study, confirmed that the development of symptomatic metastasis is rare but a significant problem in advanced RCC during therapy with sorafenib or sunitinib; in fact, they analyzed 81 patients with BM; 41 patients never received TKI and the remaining 40 received TKI therapy; the median OS from BM diagnosis was 5.4 months for the whole group: 4.4 vs. 6.7 months (p < 0.037) in the TKI versus TKI groups, respectively. However, patients who received TKI therapy prior to BM development had a median OS of 23.6 months vs. 2.08 and 4.41 months for the patients who received TKI pre-BM or nev-TKI, respectively (p < 0.0001).

A few case reports described the efficacy of other targeted agents on BM from RCC, pazopanib, a potent and selective multi-targeted receptor tyrosine kinase inhibitor of VEGFR-1, VEGFR-2, PDGFR-β, and c-kit, demonstrated efficacy in a patient who developed more than 20 brain metastases plus multiple bone, lymph node, and soft tissue metastases, and who survived 23 months [76].

Vickers et al. [75], in a retrospective study, analyzed prognostic factors of survival for patients with BM from RCC treated with targeted therapy; they studied 106 patients: 77 treated with sunitinib, 30 with bevacizumab and 1 patient with temsirolimus. On multivariate analysis, Kamolzky performance status (HR 2.15; 95% CI 1.02–4.56), RCC diagnosis to targeted therapy time (HR 1.12; 95% CI 1.01–1.23), and higher number (4) of BMs (HR 1.4; 95% CI 1.13–1.75) were associated with worse survival from the time of BM diagnosis. Moreover, patients treated with targeted therapy after BM diagnosis had survived longer than patients who developed BM while receiving targeted therapy.

Finally, Zanetta et al. [76] described 4 cases of RCC patients with BM treated with bevacizumab with or without α-interferon. They reported a maximum PFS of 26.5 months and a maximum OS of 33.2 months from start of bevacizumab treatment.

Conclusions
In recent years, the frequency of metastatic brain tumors has been increasing and primitive lung cancer is the most common cause. Radiation therapy and surgery can be used in selected patients but can be responsible for acute or delayed neurological deficits. Recently, new targeted drugs have been developed and employed either on established brain metastases or in a preventive setting. Interestingly, these new molecular drugs reported interesting activity and safety in selected cases and in retrospective or prospective studies, with or without radiation therapy in BM from common solid tumors in adults. However, a multidisciplinary collaboration is always required to obtain the appropriate treatment that balances a good quality of life with the prolongation of survival in these patients.

Conflict of interest
None.

Acknowledgment
We thank Ms. Christina Drace for English support.

References


Imputation and subset-based association analysis across different cancer types identifies multiple independent risk loci in the TERT-CLPTM1L region on chromosome 5p15.33

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Genome-wide association studies (GWAS) have mapped risk alleles for at least 10 distinct cancers to a small region of 63 000 bp on chromosome 1p15.33. This region harbors the TERT and CLPTM1L genes; the former encodes the catalytic subunit of telomerase reverse transcriptase and the latter may play a role in apoptosis.

To investigate further the genetic architecture of common susceptibility alleles in this region, we conducted an epistatic subset-based meta-analysis (association analysis based on subsets) across six distinct cancers in 34248 cases and 45 036 controls. Based on sequential conditional analysis, we identified as many as six independent risk loci marked by common single-nucleotide polymorphisms: five in the TERT gene (Region 1: rs777792G, P = 2.10 x 10^{-18}; Region 2: rs82836773, P = 3.30 x 10^{-18} and P_{conditional} = 2.36 x 10^{-7}; Region 4: rs1376898, P = 3.87 x 10^{-11}; P_{conditional} = 5.91 x 10^{-1}; Region 5: rs13762201, P = 9.04 x 10^{-17} and P_{conditional} = 2.04 x 10^{-7}; and Region 6: rs10096990, P = 7.49 x 10^{-15} and P_{conditional} = 5.35 x 10^{-7}) and one in the neighboring CLPTM1L gene (Region 2: rs451360; P = 1.90 x 10^{-13} and P_{conditional} = 7.06 x 10^{-7}). Between three and five cancers mapped to each independent locus with both risk-enhancing and protective effects. Allele-specific effects on DNA methylation were seen for a subset of risk loci, indicating that methylation and subsequent effects on gene expression may contribute to the biology of risk variants on 1p15.33. Our results provide strong support for extensive pleiotropy across this region of 1p15.33, to an extent not previously observed in other cancer susceptibility loci.

INTRODUCTION

Genome-wide association studies (GWAS) have identified independent susceptibility loci in a region on chromosome 1p15.33 that are associated with at least 10 distinct cancers. The published findings include bladder (1), estrogen-negative breast (2), glioma (3), lung (4-7), ovary (8), melanoma (9), nonsmall cell lung cancer (10-12), pancreas (12a), prostate (13) and testicular germ cell cancer (13). This degree of pleiotropy for common susceptibility alleles suggests that the region harbors an important set of elements that could influence multiple cancers. It has been observed previously that one allele may be protective for one cancer while conferring susceptibility to another (13). These independent loci map to ~63,000 bp of 1p15.33 that harbors two plausible candidate genes: TERT, which encodes the catalytic subunit of telomerase reverse transcriptase (16) and CLPTM1L, which encodes the clef lip and palate-associated transmembrane 1-like protein (also called cliastral resistance
related protein, CBX9). CLPTM1L appears to play a role in apoptosis and cytokinesis, is overexpressed in both breast and pancreatic cancer and is required for KRAS-driven lung cancer (17–21). Germline mutations in TERT can cause dyskeratosis congenita (DC), a cancer-prone inherited bone marrow failure syndrome caused by aberrant telomere biology (22). Clinically related telomere biology disorders, including idiopathic pulmonary fibrosis and acquired aplastic anemia, can also be caused by germline TERT mutations (reviewed in 23).

To investigate the genetic architecture of common susceptibility alleles across this region of 5p15.33 in multiple cancer sites, we utilized a recently developed method called association analysis based on subsets (ASSET) that combines association signals for an SNP across multiple traits by exploring subsets of studies for true association signals in the same, or the opposite direction, while accounting for the multiple testing required (24). The method has been shown to be more powerful than the standard meta-analysis in the presence of heterogeneity, where the effect of a specific SNP might be restricted to only a subset of traits or and may have different directions of association for different traits (24).

RESULTS
In this study, we conducted a cross-cancer fine-mapping analysis of a region on chromosome 5p15.33 known to be associated with multiple cancer sites. We imputed each dataset across a 2 Mb window (chr5: 250,000–2,250,000 bp) using the 1000 Genomes (1000G) and DCG reference datasets (25,26) and applied a subset-based meta-analysis method (ASSET) (24) to combine results across six cancers (11 studies) (see Materials and Methods for details). This method has been shown to improve power and interpretation when compared with other traditional methods for the analysis of heterogeneous traits (24).

In this analysis, we focused on six distinct cancer sites in which 5p15.33 had previously been reported and had a nominal P-value in our dataset ("Tier-I" analysis, see Materials and Methods). In the second analysis, we examined the regions identified above in eight cancers in which 5p15.33 had not been reported in the literature (NHEK Catalog of Published GWAS studies: http://www.genome.gov/pwastudies), or did not show a nominal P-value in our dataset ("Tier-II" analysis).

Application of ASSET by sequential conditioning of associated SNPs revealed up to six independent loci on 5p15.33, each influencing risk of multiple cancers (Fig. 1, Table S1). In the primary analysis of all subjects, we performed the ASSET meta-analysis based on unconditional association results from each of the six cancer scans (11 studies). This identified rs7726159 with the lowest P-value (P = 2.10 × 10^{-10}), thus marking Region 1. The next four SNPs, ranked by P-values, were highly correlated with the index SNP based on 1000G CEU data: rs7726128 (P = 2.98 × 10^{-10}, pairwise r^2 = 0.90), rs4495853 (P = 3.37 × 10^{-10}, pairwise r^2 = 0.91), rs7055526 (P = 1.00 × 10^{-10}, pairwise r^2 = 0.74) and rs4975538 (P = 4.11 × 10^{-12}, pairwise r^2 = 0.76). These five SNPs reside in the second and third intron of the TERT gene and are common, with effect allele frequencies ranging between 0.18 and 0.43 in African (AFR), 0.35–0.37 in Asian (ASN) and 0.32–0.38 in European (EUR) populations, each estimated in the 1000G (Supplementary Material, Table S2). A search for surrogates using an r^2 threshold of 0.7 across a 1 Mb window centered on the index SNP did not identify additional highly correlated SNPs. The effect allele (A) of rs7726159 was positively associated with glioma (Odds Ratio (OR) = 1.47, 95% CI = 1.38–1.56), but negatively associated with testicular cancer (OR = 0.85, 95% CI = 0.80–0.91) (Fig. 2A).

The most significant SNP after conditioning on rs7726159 was rs451360 (P = 1.90 × 10^{-10}, P_{conditional} = 7.06 × 10^{-11}), residing in intron 13 of CLPTM1L and marking Region 2 (Fig. 1, Table S1). Six SNPs were correlated with rs451360 with r^2 > 0.7, all located within 500 kb of this SNP and spanning the entire length of CLPTM1L: rs380145, rs379044, rs379064, rs36151365, rs59593391 and rs7446601. This effect allele (A) was positively associated with pancreatic cancer (OR = 1.47, 95% CI = 1.38–1.56), but negatively associated with lung cancer (African American Lung Cancer (AALC) OR = 0.48 × 10^{-10}, OR_{conditional} = 0.85, 95% CI = 0.69–0.99) (Fig. 2B). Although large differences were seen in the effect allele frequencies across the 1000G continental populations, 0.02–0.03 in EUR, 0.12 in ASN and 0.17–0.24 in EUR (Supplementary Material, Table S2), we were still sufficiently strong to be detected, particularly in African and Asian lung studies, suggesting its importance in lung cancer etiology.

In our sequential analysis across all 355 SNPs (located in the first intron of TERT) was the most significant SNP after conditioning on both rs7726159 and rs451360, marking Region 3 (P = 3.30 × 10^{-12}, P_{conditional} = 2.36 × 10^{-12}) (Fig. 1, Table S1). No additional SNPs with an r^2 > 0.7 were located within 500 kb of this SNP, which has relatively low LD with both rs7726159 (r^2 = 0.13) and rs451360 (r^2 = 0.12) in 1000G CEU data. Region 3 (rs2835677-A) was positively associated with testicular cancer (TGCN) and pancreatic cancer (PanScan and ChiPAC) (P = 1.36 × 10^{-10}, OR_{conditional} = 1.22, 95% CI = 1.13–1.31), but negatively associated with lung cancer (African American Lung Cancer (AALC) OR = 2.79 × 10^{-10}, OR_{conditional} = 0.73, 95% CI = 0.70–0.77) (Fig. 2C). The effect allele frequency for rs2835677 was consistent across the three continental 1000G populations corresponding to the studies included in this analysis: 0.60 in EUR, 0.67 in ASN and 0.71 in AFR (Supplementary Material, Table S2). A conditional analysis based on the three SNPs above rs7726159, rs451360 and rs2835677 yielded Region 4, marked by rs2736096 (P = 3.87 × 10^{-12}, P_{conditional} = 5.19 × 10^{-10}), a synonymous variant (A305A) in the second exon of TERT (Fig. 1, Table S1). Three additional SNPs with an
Figure 1: Sequential conditional analyses and ASSET meta-analyses identified top two independent signals for the TERT-CLPTM1L region on chromosome 5p15.33. SNPs mapping each region are plotted in the upper panel with two P-values (solid diamonds correspond to an unconditional test and open diamonds correspond to a conditional test) on a negative log scale (left y-axis) against genomic coordinates (x-axis, log10). Cancers from different GWAS scans (assays detailed in box in top panel) that are associated within each region in the subset meta-analyses are listed (red, positively associated; green, negatively associated) from the unconditional ASSET meta-analysis. Effect alleles are shown next to SNP identifiers. Replication haplotypes (curved lines, top panel) were inferred from three populations from the DECODE Imputation Reference Set version 1 (red: CEU, green: ASW, blue: YRI) as the likelihood ratio statistic (right y-axis). Also shown are the gene structures for TERT, HIRA/F7, and CLPTM1L (middle panel) and LD heat map based on r² using the 1000 Genomes EUR population (lower panel). Results are shown for the ALL analysis except the region marked by rs8000690 (top panel) is labeled with a *** that was identified in the European ancestry only analysis (EUR).
Table 1. Association results for SNPs on chromosome 5p15.33 with the risk of cancer

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene</th>
<th>Region</th>
<th>Position</th>
<th>Unconditional OR (95% CI)</th>
<th>Unconditional P-value</th>
<th>Significant associations</th>
<th>Condition OR (95% CI)</th>
<th>Condition P-value</th>
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<tr>
<td>A1L</td>
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<tr>
<td>n2756021</td>
<td>ISG7</td>
<td>1</td>
<td>12058319</td>
<td>1.15 (1.00 - 1.31)</td>
<td>0.05 (0.00 - 0.95)</td>
<td>2.01 x 10^-4</td>
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<td>96856</td>
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The results from the haplotypic association trend ASSYR were analyzed to show that the SNP A1L was significantly associated with a higher risk of cancer in the group of patients with the SNP in the high-risk haplotype. This association was significant at the 5% level and remained robust even after adjustment for potential confounders. The effect size was estimated to be 2.01 x 10^-4, indicating a strong association between the SNP and the risk of cancer. Further studies are needed to confirm these findings and to explore the biological mechanisms underlying the association.

*Note: SNP = single nucleotide polymorphism; OR = odds ratio; CI = confidence interval.*
Figure 2. (A–F) Forest plots for individual risk loci on chromosome 5,33 for the unconditional ASSET meta-analysis. For each causal GWAS scan, OR and 95% CI were listed and plotted along each line as per the unconditional association analysis. A vertical line of OR = 1 indicated the red. Two summary lines list ORs for the positively or negatively associated subsets as estimated by the ASSET program. (A) rs7372615, (B) rs431600, (C) rs2835071, (D) rs377600, (E) rs1417250, and (F) rs10469500 in the analysis of European-ancestry studies only. Forest plots for the conditional analysis are shown in Supplementary Material, Figure S1A–E.
\( r^2 > 0.7 \) were located within 500 kb of this SNP: rs2853669, rs2736168 and rs2730617, all in the promoter of TERT, from -200 to 2700 bp upstream of the transcriptional start site. This region (rs270659-T) was positively associated with lung cancer (Eur Lung and AA Lung), prostate cancer (Pegas) and bladder cancer (Bladder NCI) \((P = 2.58 \times 10^{-18}, \text{OR}_{\text{controll}} = 1.15; 95\% \text{CI} = 1.10-1.21)\), and negatively associated with testicular cancer (TGCT NCI) and pancreatic cancer (PanScan) \((P = 4.83 \times 10^{-7}, \text{OR}_{\text{controll}} = 0.81; 95\% \text{CI} = 0.74-0.90)\) (Fig. 2D). The effect allele frequencies displayed a wide range across the three continental populations in 1000G, interestingly with the lowest frequency in the most ancient populations, 0.06-0.08 (AFR), whereas the other two populations were comparably high: 0.23-0.29 (EUR) and 0.22-0.33 (ASN) (Supplementary Material, Table S2).

An additional suggestive region (Region 5) marked by rs13172201 \((P = 0.05; \text{OR}_{\text{controll}} = 1.31 \times 10^{-1})\) was determined by our sequential conditional analyses (Fig. 1, Table 1), unmasked mainly due to conditioning on rs726159 (Region 1). The risk alleles for rs13172201 and rs726159 were negatively correlated \((r = -0.27, \text{based on 1000G CEU data})\) and, in an exploratory analysis of rs13172201 in the Eur Lung scan, this SNP appeared to have a stronger association in rs726159 CC carriers \((P = 7.0 \times 10^{-4}, \text{OR} = 1.21; 95\% \text{CI} = 1.08-1.35)\) when compared with rs726159 AC/AA carriers \((P = 0.10, \text{OR} = 1.12; 95\% \text{CI} = 0.98-1.27)\). Region 5 (rs13172204-C) was positively associated with lung cancer (Eur Lung and AA Lung), prostate cancer (Pegas) and pancreatic cancer (PanScan) and negatively associated with testicular cancer (TGCT NCI) and glioma (Glioma scan) (Fig. 2E). The effect allele for rs13172201, the sentinel SNP in Region 5, was the minor allele in European (0.26 in EUR) and African (0.39 in AFR) populations, while it has become the major allele in Asians (0.35 in ASN).

In an analysis restricted to studies of European ancestry (EUR scans), we found strong associations for Regions 1, 2, 4, 5, 7 and 8 (Table 1) but not Region 3 (marked by rs2853677). The conditional \(P\)-value for Region 5, suggestive in the analysis based on all ethnic groups, improved in this subset and surpassed the threshold of \(1 \times 10^{-12}\) \((rs13172201: P = 0.04; \text{OR}_{\text{controll}} = 2.04 \times 10^{-12}; P = 5.35 \times 10^{-12})\) in intron 4 of TERT was identified in the European ancestry-only analysis (Fig. 1, Table 1). The significance for this region did not reach Bonferroni-corrected \(P\)-value threshold in the analysis of all studies \((P = 5.4 \times 10^{-10} \times 4 = 2.16 \times 10^{-10})\). As Regions 3 and 6 were located between the same two recombination hotspots (Fig. 1), we assessed correlation in 1000G CEU subjects and noted virtually no LD for rs1006960 and rs2853677 \((r^2 = 0.0052)\), thus supporting the notion that they are independent signals. Low LD existed for these two SNPs in the 1000G YRI \((r^2 = 0.098)\) and ChB/JPT \((r^2 = 0.045)\) populations (Supplementary Material, Table S3). Region 6 (rs10069600-T) was positively associated with glioma (Glioma scan) \((P = 4.67 \times 10^{-10}, \text{OR}_{\text{controll}} = 1.48; 95\% \text{CI} = 1.31-1.67)\) and negatively associated with testicular (TGCT NCI), prostate (Pegas) and Ad/PCaA, bladder (Bladder NCI) and pancreatic cancer (PanScan) \((P = 4.95 \times 10^{-7}, \text{OR}_{\text{controll}} = 0.87; 95\% \text{CI} = 0.83-0.92)\) (Fig. 2F). Highly correlated SNPs \((r^2 > 0.7)\) were not observed within 500 kb of rs10069600. Notably, the \(P\)-value for rs10069600 in the Advanced Prostate cancer scan improved from \(1.64 \times 10^{-10} \times 3.05 \times 10^{-10}\) after conditioning on Region 1. The correlation between rs10069600 and rs726159 (Region 1) is \(r^2 = 0.13\) in the 1000G CEU, \(r^2 = 0.30\) in YRI and \(r^2 = 0.42\) in ChB/JPT populations (Supplementary Material, Table S3). SNP rs10069600 was nominally significant in the other two prostate cancer scans with unconditional \(P\)-values of 0.003 (Pegas) and 0.02 (CGEMS ProCa) but was not significant after conditioning on the first region in these scans \((P = 0.36\) in Pegas, \(P = 0.078\) in CGEMS ProCa). For the six signals noted, Regions 1, 3 and 6 are flanked by two recombination hotspots that separate them from Region 5 on the telomeric side and from Region 4 on the centromeric side. Recombination hotspots also separate Regions 2 and 4 (Fig. 1).

The LD between SNPs in loci 1, 2 and 6 was low to moderate \((r^2 = 0.0092, 0.131, 0.449\) in 1000G CEU, \(r^2 = 0.098, 0.298, 0.0765\) in YRI and \(r^2 = 0.0848, 0.415, 0.341\) in ChB/JPT), however, the conditional analyses supported the presence of three signals bounded by strong recombination hotspots on either side. Region 5 is the most telomeric one and separated from the rest by a strong recombination hotspot. Supplementary Material, Table S1 shows \(P\)-values for the six regions along each step of the sequential conditional analysis to reflect the change in significance in the analysis. We also assessed the associations for each of the regions in the ‘10×10’ studies comprising nine GWAS datasets across eight cancers, including 11,385 cases and 18,322 controls. None of the regions showed significant association (data not shown).

In addition to characterizing independent signals in the TERT-DEPTML region, we have fine-mapped previously reported signals. For pancreatic cancer, the reported GWAS SNP rs681681 had a \(P\)-value of \(3.7 \times 10^{-19}\) and an \(r^2 = 0.19\) (12). After imputation, an improved \(P\)-value was seen for rs451369 (marking Region 2) \(P = 2.07 \times 10^{-8}\) (OR = 1.29). After conditioning on rs451369, the \(P\)-value of rs681681 was no longer significant \((P = 0.1)\). The LD between these two SNPs is moderate \((r^2 = 0.36)\). For glioma, the GWAS SNP rs2736100 had a \(P\)-value of 8.49 \times 10^{-10} and an \(r^2 = 0.10\) in the Glioma scan (27). The best imputed SNP rs495833 \((r^2 = 1)\) with rs726159, marking Region 1, showed a much improved \(P\)-value of \(4.1 \times 10^{-12}\) with OR = 1.56, and the \(P\)-value of rs2736100 was no longer significant after conditioning on rs495833 \((P = 0.64)\). The LD between these two SNPs was moderate \((r^2 = 0.39)\).

Bioinformatic analyses using public data bases (ENCODEx and TCGA) were performed to investigate the possible function of SNPs that mark each of the six regions as regulators of expression of TERT or DEPTML, as well as other genes. Based on ENCODE data, the strongest evidence for putative regulatory functions was seen for SNPs in Regions 1 (rs7725318 and rs975538), 2 (rs6113563 and rs980455), 4 (rs2736108 and rs2853669) and 5 (rs1317201) with evidence of an open chromatin conformation, regulatory histone modification marks and transcription factor binding in multiple cell types such as prostate, pancreas, breast, lung and brain (Supplementary Material, Table S2).

We next examined the TCGA datasets for expression (eQTL) and methylation (meQTL) quantitative trait loci for lung adenocarcinoma (LUAD), prostate adenocarcinoma (PRAD) and...
glioblastoma multiforme (GBM). We did not observe significant eQTLs (P > 0.4; data not shown) but noted multiple meQTLs in LIAD and PRAD tumor samples (Supplementary Material; Tables S5 and S6). Methylation at a subset of CpGs probes with meQTLs correlated with expression of TERT and/or CLPTM1L, including two for Region 4 in TGCA LIAD samples (cg26209169: \( \beta = -0.57; P = 1.18 \times 10^{-3} \); cg16286080: \( \beta = -0.36; P = 0.001 \)). These CpGs are located ~1800 bp downstream of CLPTM1L (227 bp apart), overlap with long transcripton factor binding sites (e.g., TCF1, TCF4, INP3A, MAX, RUNX3, AM1, ATF-2, and USF1/USF2), and show negative correlation with expression of TERT and CLPTM1L (Supplementary Material, Table S5 and Fig. S2). Replication was seen in normal lung samples (cg26209169 and Region 4, \( \beta = -0.650; P = 5.17 \times 10^{-2} \); cg16286080 and Region 4, \( \beta = -0.493; P = 0.00274 \) from EAGLE (28). The most significant meQTLs in TGCA PRAD samples were seen for Region 1 (cg06935339: \( \beta = -1.06; P = 8.67 \times 10^{-11} \); cg06531176: \( \beta = -1.18; P = 2.61 \times 10^{-1} \)). These replicated in EAGLE (\( \beta = 5.93 \times 10^{-2} \) and \( P = 0.002 \), respectively), did not correlate with expression of TERT or CLPTM1L, and were both located within exon 3 of TERT (Supplementary Material, Table S6).

Analysis of TGCA data also revealed increased expression of TERT and CLPTM1L in tumors compared with normal tissues for lung and prostate cancer (on average 1.29- to 2.02-fold change for paired samples). Copy number differences were more evident in lung tumors (average number of copies was 2.03 in normal and 2.54 in tumors for 51 paired samples, \( P = 1.10 \times 10^{-3} \); Supplementary Material, Fig. S3).

**DISCUSSION**

Chrm15:33 harbors a unique cancer susceptibility region that contains at least two plausible candidate genes: TERT and CLPTM1L. Through a subset-based meta-analysis of GWAS data on human cancers from three continental populations, we have characterized up to six independent, common, susceptibility alleles, all with evidence of both risk-enhancing and protective effects, differing by cancer type. TERT encodes the catalytic subunit of the telomerase reverse transcriptase, which, in combination with an RNA template (TERC), adds nucleotide repeats to chromosome ends (29). Although telomerase is active in germline cells and in early development, it remains repressed in most adult tissues. Telomeres shorten with each cell division and when they reach a critically short length, cellular senescence or apoptosis is triggered. Cancer cells can continue to divide despite critically short telomeres, by upregulating telomerase or by alternative lengthening of telomeres (16,30,31). While studies investigating the relationship between surrogate tissue (e.g., buccal or blood cell DNA) telomere length and cancer risk have been contradictory, larger prospective studies have not reported an association for risk but only survival time (32–33). Heritability estimates of telomere length in twins studies suggest a significant genetic contribution, between 53% and 78% (36,37). GWAS SNPs on 5p15.33 have been associated with telomere length implying that TERT may indeed be the gene targeted by at least some neurotrophic variant in this region (28–40). In addition, germline TERT promoter mutations have been identified in familial melanoma as well as somatic mutations in multiple cancers (41,42).

The most commonly reported SNP in the TERT gene, rs237361, was first reported in several GWAS: glioma (3.43), lung cancer in Europeans and Asians (7.44–46) and testicular cancer (14). We have fine-mapped this locus (Region 1) to a set of five correlated SNPs in the second and third intron of TERT (marked by rs7726159). In addition to the cancers listed above, we noted novel contributions to this locus by prostate and pancreatic cancer. Fine-mapping efforts in lung (47) and ovarian cancer (48) have reported the same SNP. Region 3 (rs2853677), located in the first intron of TERT, has been associated with glioma in Chinese subjects (49) and lung cancer in Japanese subjects (50), in agreement with the strong contribution to this region seen in our analysis by means performed in individuals of Asian ancestry. In addition to lung cancer and glioma, we noted novel associations for Region 3 with pancreatic and testicular cancer; Region 4 was marked by a synonymous SNP (rs7364608) located in the second exon of TERT, with three additional highly correlated SNPs in the promoter region. This region has been reported via fine-mapping in lung, bladder, prostate, ovarian and breast cancer, and shown to influence TERT promoter activity (9). Novel contributions to Region 4 were noted for pancreatic and testicular cancer.

In our analysis, we uncovered a new susceptibility locus, Region 5 (marked by rs3172521, Fig. 1), which surpassed the Bonferroni threshold in European studies. We found evidence for a negative correlation between this SNP and rs7270159 (Region 1), indicating a possible interaction. This locus is not significant at a GWAS threshold and requires confirmation in independent regions. Region 6 (marked by rs6969690) has previously been associated with estrogen- and progesterone receptor-negative breast cancer in populations of European and African ancestry (2,51); our analysis adds five cancers to this list: glioma, prostate, testicular germ cell, lung, and urinary bladder.

The gene adjacent to TERT, namely CLPTM1L, encodes a protein that is differentially expressed in lung and pancreatic cancer, promotes growth and survival, and is required for KRAS-driven lung cancer, indicating that it is a plausible candidate gene in this region (17–21). The locus in CLPTM1L (Region 2) has previously been associated with risk of cancer in multiple GWAS, marked by rs401681 or rs902730 in pancreatic, lung, and bladder cancer as well as in melanoma (1,4,12,52). Our subset-based approach has fine-mapped this signal to a set of seven correlated SNPs that span the entire length of CLPTM1L.

Two recent papers from the Collaborative Oncology Gene-Environment Study (COGS) fine-mapped 5p15.33 in prostate, breast and ovarian cancer and identified four of the six loci noted in the current study (53,54). In prostate cancer, COGS identified three regions that corresponded to our Region 1 (COGS Region 1, rs7725210), Region 3 (COGS Region 2, rs2853676, \( r^2 = 0.32 \) with rs2853677) and Region 4 (COGS Region 3, rs2853669, \( r^2 = 0.54 \)). Interestingly, COGS reported protective alleles in Region 1 associated with increased TERT expression in benign prostate tissue samples. The fourth COGS prostate cancer locus, marked by rs13190087, was significant in our study (\( P = 0.008 \), possibly due to a more specific effect for prostate cancer for this locus where our study had less power.)
In breast and ovarian cancer, COGs identified three regions corresponding to our Region 1 (COGs Region 2), r7705572, associated with risk of ovarian cancer with low malignant potential, telomere length and promoter activity); Region 4 (COGs Region 1, r273610, associated with risk of ER-negative and BRCA1 mutation carrier breast cancer, telomere length and altered promoter activity) and Region 6 (COGs Region 3, r8006090, associated with risk of ER-negative breast cancer, breast cancer in BRCA1 carriers and invasive ovarian cancer) (57). Regions 2 (in CLPTM1L) and 5 (in TERT) were not observed in the COGs reports, perhaps due to the choice of SNPs by COGs for fine-mapping as well as the more comprehensive reference set for 1000 Genomes used to conduct our imputation, or because of cancer-specific effects for these loci.

It is becoming increasingly clear that DNA methylation is under genetic control. Regions of variable methylation exist across tissues and individuals, tend to be located in intergenic regions, overlapping known regulatory elements. Notably, these are enriched for disease-associated SNPs (28-55,56). Analysis of TCGA data, while not uncovering significant eQTLs, indicated that DNA methylation could play a role in the underlying biology at 5p15.33. Methylation in a small region downstream of CLPTM1L, with features suggesting an active regulatory function, was consistent with lower methylation levels in carriers of risk alleles for lung cancer (Region 4) and higher expression of TERT and CLPTM1L. Increased expression of both genes is consistent with a pro-tumorigenic role in lung cancer (19,21,31). For prostate cancer, the most notable eQTLs were located within exam 3 of TERT with increased rates of methylation for carriers of risk alleles in Regions 1 and 6. Although gene-body methylation has been observed to positively correlate with gene expression (57), we did see evidence to support this for this particular set of CpGs. As a large fraction of the eQTLs does not overlap with eQTLs (55), they may influence molecular phenotypes other than gene expression such as alternative promoter usage, splicing and even mutability (58-60). It is intriguing that methylation QTLs observed in TCGA data differ to some degree between lung and prostate cancer, whereas they were observed in glioblastoma. This indicates that the TERT-CLPTM1L region may harbor multiple elements that have the capacity to influence molecular phenotypes that in turn impact cancer development. However, only a subset of these elements may be active in each organ, thus leading to different mechanistic avenues for risk modification in different tissues. It is possible that the interplay between risk variants, multiple biological mechanisms and associated genes, in addition to environmental and lifestyle factors that differentially influence various cancers may eventually come to explain how the same alleles at this complex locus can mediate opposing cancer risk in different organs.

In summary, we report up to six independent loci on chr5p15.33, each influencing the risk of multiple cancers. We observed pleiotropy for common susceptibility alleles in this region, defined as the phenomenon wherein a single genetic locus affects multiple phenotypes (61). These alleles could influence multiple cancers distinctly, perhaps in response to environmental factors or in interactions with other genes. Our cardinal observations underscore the complexity of the alleles and suggest the importance of tissue-specific factors that contribute to cancer susceptibility. Further laboratory analysis is needed to validate our findings using TCGA data, and investigate the optimal functional variants in each of the six independent loci in order to provide a clearer understanding of each of the loci in this multi-cancer susceptibility region.

**MATERIALS AND METHODS**

**Study participants**

Participants were drawn from a total of 26 previous GWAS scans of 13 distinct cancer types: bladder, breast, endometrial, esophagogastric, gastric, glioma, lung, larynx, ovarian, pancreatic, prostate, renal cancer and testicular germ cell tumors. We first assessed a set of 11 GWAS representing six distinct cancers (Tiers I studies) in which 5p15.33 had previously been implicated (NHGRI Catalog of Published GWAS studies: http://www.genome.gov/gwasstudies/). The GWAS scans and their acronyms were: Asian lung cancer scan (Asian Lung), European lung cancer scan (Eur Lung), African American lung (AA Lung), ProstateScan, China pancreatic cancer scan (ChinaPC), Testicular germ cell tumor (TGCT NC1) scan, glioma scan (Bladder NC1 scan, Prostate scan), Prostate cancer scan (Prostate), CGEMS prostate cancer scan (CGEMS PrCa) and Advanced prostate cancer scan (Adv PrCa) (see case and control counts in Supplementary Material, Tables S4A–D). In a second analysis, we separately assessed a set of nine GWAS scans representing eight cancers (Tiers II studies) in which 5p15.33 had not been previously reported in the literature (NHGRI Catalog of Published GWAS studies: http://www.genome.gov/gwasstudies/). These studies were: Asian esophageal scan (Asian EsocCa), Asian gastric cancer scan (Asian GastrCa), CGEMS breast cancer scan (CGEMS Breast), Endometrial cancer scan (EndometCa), E3 negative breast cancer scan (ERneg BPC3 Bca), China prostate cancer scan (Ghana PrCa), Osteosarcoma scan (OS), Ovarian cancer scan (OvarCa) and Renal cancer scan (Renal Ca) (see case and control counts in Supplementary Material, Tables S4E–H). Studies were conducted in individuals of European background (EUR scans) but we did include studies in populations of Asian ancestry (i.e., esophageal squamous, gastric, non-smoking lung and pancreatic cancers) and African ancestry (i.e., lung and prostate cancer) (ALL scans). Study characteristics, genotyping and quality control have been previously published for all studies listed by cancer type and GWAS scan acronyms: Bladder cancer/Bladder NC1 (1.62), breast cancer/CGEMS BcCa (63), breast cancer/ERneg BPC3 Bca (64), endometrial cancer/EndometCa (65), gastric cancer and esophageal squamous cell carcinoma/Asian UpperGI (66), glioma/Glioma scan (37), lung cancer in Europeans/Eur Lung (7), lung cancer in African Americans/AALung (67), lung cancer in non-smoking women from Asia/Asian Lung (68,69), osteosarcoma/OS (70), ovarian cancer/OvarCa (71), pancreatic cancer/ProstScan (12,72), pancreatic cancer in Asians/ChinaPC (73), prostate cancer/ProstateScan (unpublished data), prostate cancer/CGEMS PrCa (74), advanced prostate cancer/AdvPrCa (75), prostate cancer in Africans/OluPrCa (unpublished data), renal cancer/Renal US (76) and testicular germ cell tumors/TGCT NC1 (77).

Each participating study obtained informed consent from study participants and approval from its Institutional Review Board (IRB) including IRB certification permitting data
sharing in accordance with the National Institutes of Health (NIH) Policy for Sharing of Data Obtained in NIH-Supported or Conducted GWAS.

Genotyping

Arrays used for scanning included the Illumina HumanOmni series (317 + 248S, 550, 610 K, 660 W and 1 M), as well as the Illumina Omni series (OmniExpress, Omni1M, Omni2.5 and Omni5M). The majority of the studies were genotyped at the Cancer Genomics Research Laboratory (formerly Core Genotyping Facility) of the National Cancer Institute (NCI) of the NIH. The ChinaPC GWAS (Affymetrix 6.0) was genotyped at CapitalBio in Beijing, China. This necessitated imputation before the cross-cancer subset-based meta-analysis. We used a combination of public resources, 1000 Genomes (1000G) (25) and DCEG (26) reference datasets, to impute existing GWAS datasets (78) using IMPUTE2 (79).

In addition to the standard QC procedures previously applied in the primary GWAS publications, we further filtered SNPs as follows: (i) completion rate per locus < 90%, (ii) MAF < 0.01, (iii) Haney–Weinberg proportion P-value < 1 × 10^-6, (iv) exclusion of A/T or G/C SNPs.

Lift over the genomic coordinates to NCBI genome build 37 or hg19

Because the March 2012 release of the 1000 Genomes Project data is based on NCBI genome build 37 (hg19), we utilized the LiftOver tool (http://hgdownload.cse.ucsc.edu/) to convert genomic coordinates for scan data from build 16 to build 37. The tool re-maps only coordinates, but not SNP identifiers. We prepared the inference.hed file and then performed the lift over as follows:

```
$tools/liftover/liftover inference.hed $tools/liftover/hg18 ToHg19.over.chain.gz output.hed unified.hed
```

A small number of SNPs that failed LiftOver, mostly because they could not be unambiguously mapped to the genome by NCBI, were dropped from each imputation inference set.

Strand alignment with 1000 Genomes reference data set

Since A/T or G/C SNPs were excluded, strand alignment for the scan data required checking allele matches between the inference set and reference set loci by locus. If they did not match, alleles were complemented and checked again for matching. SNPs that failed both approaches were excluded from the inference data. Locus identifiers were normalized to those used in the 1000 Genomes data based on genomic coordinates, although the IMPUTE2 program uses only the chromosome/locus location to align each locus overlapping between the imputation inference and reference set.

Conversion of genotype files into WTCCC format

After LiftOver to genome build 37 and ensuring that alleles were reported on the forward strand, we converted the genotype data into IMPUTE2 format using GLU. We split the genotypes into one per chromosome and sorted SNPs in order of genomic location using the GLU transform module.

Imputation of a 2Mb window on chr5p15.33

We used both the 1000G data (March 2012 release) (25) and the DCEG imputation reference set (26) as reference datasets to improve overall imputation accuracy. The IMPUTE2 program (79) was used to impute a 2 Mb window on chr5p15.33 from 250 000 to 2250 000 (hg19) with a 250 kb buffer on either side as well as other recommended default settings. For the association analysis, we focused on a smaller region from chr5: 1250 000 - 1450 000 delineated by recombination hotspots (discussed below).

Post-imputation filtering and association analysis

We excluded imputed loci with INFO < 0.5 from subsequent analyses. SNPTEST (79) was used for the association analysis with covariate adjustment and score test of the log additive genetic effect. The same adjustments as used originally in each individual scan were used. Note that the per SNP imputation accuracy score (IMPUTE2’s INFO field) is calculated by both IMPUTE2 and SNPTEST. The two INFO metrics calculated during imputation by IMPUTE2 and during association testing by SNPTEST are strongly correlated, especially when the additive model is fitted (79). We chose the INFO metric calculated by SNPTEST for post-imputation SNP filtering.

Subset and conditional analyses

Association outputs from SNPTEST were re-formatted and subsequently analyzed using the ASSET program, an R package (http://www.bioconductor.org/packages/devel/bioc/html/ASSET.html; https://bioconductor.org/packages/devel/bioc/html/ASSET.html) for subset-based meta-analyses (24). ASSET is a suite of statistical tools specifically designed to be powerful for pooling association signals across multiple studies when true effects may exist only in a subset of the studies and could be in opposite directions across studies. The method explores all possible subset (or a restricted set if user specifies so) of studies and evaluates fixed-effect meta-analysis-type test-statistics for each subset. The final test-statistics is obtained by maximizing the subset-specific test-statistics over all possible subsets and then evaluating its significant after efficient adjustment for multiple testing, taking into account the correlation between test-statistics across different subtests due to overlapping subjects. The method not only returns a P-value for significance for the overall evidence of association of an SNP across studies, but also outputs the ‘best subset’ containing the studies that contributed to the overall association signal. For detection of SNP association signals with effects in opposite directions, ASSET allows subset search separately for positively and negatively associated studies and then combines association signals from two directions using a chi-square test-statistics. The method can take into account correlation due to overlapping subject across studies (e.g., share controls). More details about these and other features of the method can be found elsewhere (22).

For our current study, the matrices of the overlapping counts for cases–controls across datasets, which are utilized by ASSET to adjust for possible correlation across studies, were constructed and passed into the ASSET program (Supplementary Tables S4A−H). We used a two-sided test P-value, which
can combine association signals in opposite directions, to assess the overall significance of whether an SNP was associated with the cancers under study. For detection of independent susceptibility SNPs, we performed sequential conditional analysis in which in each step the ASSET analysis is repeated by conditioning on SNPs that have been detected to be most significant in previous steps. The process was repeated until the P-value for the most significant SNP for a step remained $< 1.3 \times 10^{-3}$, a conservative threshold that corresponds to Bonferroni adjustment for the 1924 SNPs used in the analysis for an alpha level of 0.05 and the two analyses performed (for the ALL vs. the EUR scans).

In the primary analysis, we included all GWAS scans in which one or more susceptibility alleles on 5p15.33 had been previously noted at genome-wide significant threshold (“Tier-I studies”). We further required a nominal signal in euraxis ($P < 0.05$). This yielded 11 GWAS across six distinct cancer sites and includes 34,248 cases and 45,036 cancer-free controls (Supplementary Material, Tables S4A–D). In a secondary analysis, we assessed the associations for each of the six regions in scans in which 5p15.33 had not been previously reported in the literature (http://www.genome.gov/gwasstudies/), or did not show a nominal P-value in the GWAS datasets used in the current study (“Tier-II studies”). This yielded nine GWAS datasets across eight cancers, including a total of 11,385 cases and 18,322 controls (Supplementary Material, Tables S4E–H).

Recombination hotspot estimation

Recombination hotspots were identified in the region of 5p15.33 harboring TERT and CLPTM1L (1:246,968–1,360,487) using SequenceDloot (80), a program that uses the approximate marginal likelihood method (81) and calculates likelihood ratio statistics at a set of possible hotspots. We tested three sample sets from East Asians ($n = 88$), CEU ($n = 116$) and YRI ($n = 59$) from the 1000 Genomes Project Reference Set. The PHASE v2.1 program was used to calculate background recombination rates (82,83).

Validation of imputation accuracy

Imputation accuracy was assessed by direct TaqMan genotyping. TaqMan genotyping assays (ABI, Foster City, CA, USA) were optimized for six SNPs (rs7726159, rs455360, rs2855677, rs2736098, rs10069690 and rs13172201) in the independent regions. In an analysis of 2337 samples from the Glioma brain tumor study (Glioma BSS, 339 samples) (27), testicular germ cell tumor (TGCT STEED study, 865 samples) (77) and Pegasus (PLCO, 1132 samples) (unpublished data), the allele P’ (84) measured between imputed and assayed genotypes were 0.88, 0.98, 0.86, 0.85, 0.81 and 0.61 for the six SNPs listed in the same order as above.

Bioinformatic analysis of functional potential

HaploReg v2 (http://www.broadinstitute.org/ mammals/haploreg/haploreg.jsp) was used to annotate functional and regulatory potential of highly significant and highly correlated SNPs that mark each of the regions identified (using ENCODE data) (85). RegulomeDB (http://regulome.stanford.edu/) was used to assess and score regulatory potential of SNPs in each locus (86). eQTL effects were assessed using the Multiple Tissue Human Expression Resource database (http://www.sanger.ac.uk/resources/software/index.html) but significant findings at a $P < 1 \times 10^{-5}$ threshold were not noted (data not shown) (87). Predicted effects of SNP on splicing were assessed using NetGene2 (http://www.chb.dmu.edu/services/NetGene2) (88) but no effect were seen for any of the SNPs in the six regions (data not shown).

We carried out eQTL and methylation quantitative trait locus (meQTL) analyses to assess potential functional consequences of SNPs in the six regions identified in normal and tumor derived tissue samples from TCGA: LUAD (25403 normal/tumor samples for eQTL analysis; 26354 normal/tumor samples for meQTL analysis), PRAD (31133 normal/tumor for eQTL; 39158 normal/tumor for meQTL) and GBM (109 normal for eQTL; 83 tumor for meQTL; normal GBM samples were not available). Transcriptome (Illumina HiSeq 2000, level 3), methylation (Illumina Infinium Human DNA Methylation 450 platform, level 3), genotype data (Affymetrix Genome-Wide Human SNP Array 6.0 platform, level 2) and phenotypes were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). Methylation probes located on X/Y chromosomes, annotated in repetitive genomic regions (GEO GPL16304), with SNPs (Illumina dbSNP137 haplotype table v2) with MAF $> 1\%$ in the respective TCGA samples, with missing rate $< 5\%$, as well as 65 quality control probes on the 450 K array. We excluded transcripts on X/Y chromosomes and those with missing rate $> 5\%$. A principle component analysis was conducted on a genome-wide level in B using gene expression and methylation data (separately in normal and tumor tissues, and after excluding transcripts with variance $< 0.0001$ and methylation probes with variance $< 0.0001$). Genotype imputation was performed as described above. The 2 Mb window centered on TERT and CLPTM1L. For eQTL analysis, normalized transcript counts for CLPTM1L and TERT were normal quantile transformed and used as input. The regression model included age, gender (not for PRAD), stage (only for tumor samples), copy number, top five principal components (PCs) of imputed genotype dosage and top five PCs of transcript counts to account for possible measured or unmeasured confounders and to increase detection power. The meQTL analysis was conducted in a similar manner in TCGA LUAD, PRAD GBM samples; beta-values of methylation at 169 CpG probes in the region encompassing TERT and CLPTM1L were normal quantile transformed and regressed as described above with the exception of inclusion of the top five PCs of methylation instead of expression values. We report the estimate of regression coefficient of imputed dosage, its standard error and P-values, adjusted by the Benjamin–Hochberg procedure for controlling false discovery rate (89). Spearman’s rank-order correlation was calculated to assess the relationship between the methylation and gene expression for TCGA LUAD ($n = 486$), PRAD ($n = 186$) and GBM ($n = 126$) tumor samples. P-values were adjusted by the Benjamin–Hochberg procedure as described above. For the purpose of visualizing meQTLs, the most likely genotype was selected from the imputed genotype dosages.

Methylation QTLs were assessed in EAGLE normal lung tissue samples ($n = 215$) as previously described with the
addition of imputation of the 19 SNPs in the 6 regions under study here (28). SOURCER'S CONTRIBUTIONS
SUPPLEMENTARY MATERIAL
Supplementary Material is available at IHEG online.
ACKNOWLEDGEMENTS
The authors acknowledge the contribution of the staff of the Cancer Genomics Research Laboratory for their invaluable help throughout the project. Conflict of Interest statement: None declared.
FUNDING
This work was supported by the Intramural Research Program and by contract number N01-CP-00200 to the US National Institutes of Health (NIH), National Cancer Institute. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services nor does mention of trade names, commercial products or organizations imply endorsement by the US Government. Additional funding acknowledgements are listed in Supplementary Material. The funders had no role in study design, data collection, analysis, decision to publish or preparation of the manuscript.

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Human Molecular Genetics, 2014


Gliomas are a heterogeneous group of tumors developing from glial cells in the central nervous system. Gliomas are divided into two histopathological subgroups: low and high grade gliomas. High-grade gliomas, such as glioblastoma and anaplastic astrocytoma, are extremely aggressive lesions and represent the most common primary malignant brain tumors.

In the last years, there have been important developments about their biologic mechanism, their surgical and drug treatment, and their diagnosis and genetic mutations; indeed, the recent IDH gene mutation identification in gliomas has been an important contribution to the knowledge improvement of biological mechanism and prognosis of these tumors. Through the analysis of IDH gene mutation it is possible to add molecular characteristics to refine the WHO classification in order to define more homogeneous gliomas subgroups. X.-W. Wang et al. showed that IDH mutation is almost constant in low-grade tumors and they stratified low- and high-grade gliomas according to the codelletion of 1p/19q and IDH mutation to define three prognostic subgroups: 1p/19q and mutation, IDH mutation alone, and none of these alterations; they demonstrated that the presence of IDH mutation combined with other genomic markers can be used to refine the prognostic classification of gliomas, independently of tumor grade. Noteworthy, X.-W. Wang et al., in another work, showed that IDH-R132H mutation could be predictive of response to radiation therapy; indeed, they suggested that IDH mutation could increase radiosensitivity in hypoxic conditions, underlining the primordial IDH mutation determination whatever the diagnostic approach. Indeed, in a recent work, G. Lombardi et al. [1] reported the possibility to discriminate IDH mutation analyzing the concentration of 2-hydroxyglutarate in urinary and plasma samples.

As described by P. Gonzalez-Gomez et al., another signaling pathway such as bone morphogenetic proteins (BMPs) could present with both prognostic value and promising therapeutic tools for gliomas.

A very interesting study about the use of 5-aminolevulinic acid (5-ALA) fluorescence in high-grade gliomas surgery was reported by A. Della Puppa et al.; they analyzed 94 patients who underwent surgery guided by 5-ALA fluorescence and stratified data for recurrent surgery, tumor location, tumor size, and tumor grade; they concluded that this surgical approach enables a gross total resection in 100% of cases and recurrent surgery, location, size, and tumor grade can be predictor of surgical outcome. The role of salvage radiosurgery in patients with recurrent malignant gliomas was studied by M. Martinez-Carrillo et al.; retrospectively, they analyzed 87 patients with recurrent anaplastic astrocitoma.
and glioblastoma who underwent stereotactic radiosurgery; although the population was very heterogeneous and various prior studies showed conflicting results about the efficacy of reirradiation, they concluded that this treatment was safe and may be a potential treatment option in selected patients.

New technological instruments such as brain magnetic resonance imaging (MRI) with spectroscopy and perfusion can help in the right diagnosis for these tumors; in fact, A. L. Di Stefano et al., evaluating perfusion MRI in grades III and IV gliomas, showed that any significant difference in rCBV between grade III and grade IV is detectable in the contrast-enhancement area while areas of high perfusion on CBV maps appear capable of best characterizing the degree of neovascularization and should be considered as the reference areas to be targeted for gliomas grading. The role of diffusion tensor histogram analysis was studied in pediatric diffuse intrinsic pontine gliomas by E. A. Steffen-Smith et al. from National Institutes of Health in Bethesda; they evaluated tumor structure in children using histogram analyses of mean diffusivity, concluding that this method can show significant interpatient and intraintratumoral differences and quantifiable changes in tumor structure.

Finally, an Italian study by V. Vaccaro et al. analyzed the efficacy of bevacizumab in association with fotemustine in patients with recurrent malignant gliomas. Antiangiogenic treatments for glioma patients have been tested in numerous clinical trials, both retrospective and prospective studies, with conflicting results; indeed, recently, two randomized prospective phase III studies failed to demonstrate the bevacizumab efficacy when added to temozolomide and radiation therapy for new glioblastoma patients [2, 3]. The combination treatment with bevacizumab and fotemustine was previously studied by R. Soffietti et al. [4] in recurrent glioblastoma patients, although with a different dosage and schedule. In both studies, this regimen showed interesting results with good safety in these patients.

In conclusion, gliomas represent an important subject of study and in this special issue very interesting works on recent developments about diagnosis, molecular biology, surgical treatment, and new targeted therapies for gliomas were selected.

References


VEGFA SNP rs2010963 is associated with vascular toxicity in recurrent glioblastomas and longer response to bevacizumab

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Received: 15 May 2014 / Accepted: 30 November 2014
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Abstract Although anti-VEGF therapy is widely used in high-grade gliomas, no predictor of response or toxicity has been reported yet. We investigated here the association of the functional single nucleotide polymorphism (SNP) rs2010963, located in the 5′ untranslated terminal region of the VEGFA gene, with survival, response to bevacizumab (BVZ) and vascular toxicity. The rs2010963 was genotyped by TaqMan assay in blood DNA from 954 glioma patients with available survival data, including 225 glioblastoma (GBM) patients treated with BVZ. VEGFA plasma levels were assessed by ELISA in 87 patients before treatment. Thrombo-hemorrhagic adverse events were recorded during BVZ treatment or not, and in an independent population of 92 GBM patients treated with temozolomide. The CC genotype was associated with the occurrence of thrombo-hemorrhagic events (CC 25 versus CG 13.5 and GG 5.2 %; P = 0.0034) during BVZ. A similar but weaker and non significant trend was observed in patients not receiving BVZ. A CC genotype was associated with higher levels of plasma VEGFA at baseline (107.6 versus 57.50 pg/mL in heterozygotes (CG) and 52.75 pg/mL in GG patients, P = 0.035 and P = 0.028 respectively). The CC genotype tended to be associated to longer PFS when treated with BVZ (P = 0.05), but not

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Published online: 07 December 2014 © Springer
when treated with the temozolomide treatment. Our data suggest that the rs2010963 genotype is associated with longer PFS, higher risk of vascular events in recurrent GBM especially treated with BVZ, and higher plasma VEGFA concentration. It may help to identify patients at risk of vascular adverse events during BVZ treatment.

**Keywords** Glioblastoma - VEGF - Bevacizumab - SNP - rs2010963

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>VEGFA</td>
<td>(Vascular endothelial factor A)</td>
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<tr>
<td>OS</td>
<td>(Overall survival)</td>
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<td>PFS</td>
<td>(Progression free survival)</td>
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<td>WHO</td>
<td>(World Health Organization)</td>
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<td>KPS</td>
<td>(Karnofsky performance status)</td>
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<td>SNP</td>
<td>(Single nucleotide polymorphism)</td>
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**Introduction**

Glioblastoma (GBM), the most frequent and malignant glioma subtype, is characterized by both hyper vascularisation and endothelial proliferation[1]. VEGFA is the most important pro-angiogenic factor in GBM and VEGFA concentrations in gliomas correlate with vascularity [2]. A high response rate (30-50%) has been observed in recurrent GBMs treated with bevacizumab (BVZ), a human monoclonal anti-vascular endothelial growth factor-A (VEGFA) antibody, administered alone or in combination with irinotecan, with a 35–50% estimated 6-month progression-free survival (PFS) [3–5]. Unpredictably, some patients experience drug resistance, limited antitumor activity, or toxicity. The most frequent grades 3–4 adverse events include hypertension, asthenia, diarrhea and severe thrombo-hemorrhagic events in about 5% of patients [3, 6]. Therefore there is a need for markers predictive of response and toxicity.

The VEGFA gene, located at 6p21.1 [7], spans approximately 14 kilobases and encodes 8 exons [8, 9]. The 5' and 3' untranslated regions (UTR) contain key regulatory elements responsive to hypoxia [10], and contribute to a high variability in VEGF production among tissues [11]. SNP rs2010963 (G>405C, G>634C) in the 5'-UTR enhances VEGFA expression at both transcriptional and translational levels and may hypothetically influence tumor aggressiveness or the response to anti-angiogenic therapies [12, 13]. Moreover, the G>405C allele was found to be correlated with a higher risk of developing different solid cancers, such as non-small cell lung cancer (NSCLC) [14], prostate cancer [15] and glioma [16]. The rs2010963 SNP has also been associated to vascular disorders (Ref 20–24) and higher VEGF plasma levels (Ref 20). However, the

**Patients and methods**

**Populations**

Bevacizumab treated glioblastoma population

We retrospectively investigated response and tolerance to a BVZ-based regimen in patients with recurrent glioblastoma (GBM), according to the following criteria: histological diagnosis of primary GBM, initially treated with the Stupp regimen [17], Karnofsky Performance Status Score (KPS) > 40, receiving BVZ (10 mg/kg every 14 days) at recurrence in monotherapy or in association with chemotherapy for at least two administrations.

Clinical and radiological responses were assessed according to the RANO criteria [18]. Patients that discontinued BVZ before tumor progression because of toxicity were censored for survival analyses. Adverse events were graded according to CTCAE v4.0 (Common Terminology Criteria for Adverse Events: http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm).

Whole glioma population and independent data set

Patients were selected according to the following criteria: histological diagnosis of grade II to grade IV glioma, clinical data and follow-up available and written informed consent. Clinical data were retrieved from a prospectively maintained database; clinical data used included: age at surgery, sex, histology according to the 2007 World Health Organization Classification (WHO) [19].

Collection of blood samples and clinicopathological information was undertaken with informed consent and relevant ethical board approval in accordance with the tenets of the Declaration of Helsinki.

**Methods**

The VEGF SNP rs2010963 genotype was assessed from blood DNA using a Taqman SNP Genotyping Assay (assay ID: C_831161_10, Applied Biosystems). Plasma VEGFA
concentrations were assessed using Quantikine® Sandwich ELISA kits (R&D Systems) following manufacturer instructions at baseline before initiation of bevacizumab.

Statistical analysis

Statistical analyses were undertaken using the R software (www.r-project.org). The independence of alleles (Hardy–Weinberg equilibrium) was confirmed using the Chi-squared test at one degree of freedom for each polymorphism (chi-2 < 3.84; p value > 0.05). Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Patients who were recurrence-free at the last follow-up were considered as a censored event in the analysis. Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Patients who were still alive at the last follow-up were considered as a censored event in the analysis. Survival curves were calculated according to the Kaplan–Meier method and differences between curves were assessed using the log-rank test. The difference in distribution of categorical variables was analyzed using Fisher exact and the Chi square test for trend and logistic regression. The Mann–Whitney method was employed to test the distribution of the VEGF-A plasma level and the Spearman test was used to assess any correlation with age and body mass index (BMI). We considered a P value ≤ 0.05 (two-sided) to be statistically significant.

Results

Vascular events in GBM patients treated with bevacizumab at recurrence

A population of 225 glioblastoma patients treated with BVZ at recurrence was analyzed; characteristics are listed in Supplementary Table 1. Median PFS was 19.42 weeks. Median OS was 37.86 weeks. No significant difference in PFS and OS was observed between patients receiving BVZ in monotherapy, or in association with temozolomide and irinotecan.

Thirty-four patients out of 225 (15.1%) presented adverse events during BVZ treatment. Twenty-five patients (11.1%) had thrombo-hemorrhagic (TH) complications and 9 patients (4%) had other complications. Among them, two patients presented a sudden death. Characteristics, frequency and grading of adverse events is reported in Supplementary Table 4. Eleven out of the 25 patients with TH complications and 4 out of the 9 patients with other complications discontinued BVZ and shifted towards other chemotherapies.

Impact of rs2010963 on outcome and vascular events in GBM patients during bevacizumab treatment

VEGFA rs2010963 genotypes are shown in Table 1. Minor allele frequency was 0.34. The distribution of allele frequencies in our population met the H-W equilibrium (χ² = 0.16; P = 0.68). PFS was better in CC (median = 28.3 weeks) than CG (18.1 weeks) and GG patients (19.0 weeks; log-rank: P = 0.05) (Fig. 1a, Table 1, Supplementary Fig. 1a), but not overall survival (CC patients 40.6 weeks, CG 41.7 weeks, GG patients 36.28 weeks) (Fig. 1b, Table 1, Supplementary Fig. 1b). rs2010963 genotypes did not show significantly different response rates according to the RANO criteria.

Twenty-five patients experienced TH events during BVZ, 6 of 25 CC (24.0%), 14 of 104 CG (13.5%) and 5 of 96 GG (5.2%) (Fig. 2) (P = 0.0044; Hazard Ratio 2.44, P = 0.006). rs2010963 status and thrombo-hemorrhagic events in patients not receiving BVZ

To determine if vascular events were dependent on BVZ-based therapy, we analyzed available clinical files from our cohort of 141 genotyped GBM patients not receiving BVZ: thrombo-hemorrhagic events were reported in 2/14 CC patients (14.3%: one pulmonary embolism, one phlebitis); 5/66 CG (8%: two pulmonary embolism, three intracranial hematomas) and 7/61 GG (11%: two pulmonary embolism, three phlebitis, two intracranial hematoma), during the whole course of the disease (p = 0.3). We then

<table>
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<th>rs2010963 allele</th>
<th>VEGFA rs2010963 genotype</th>
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<td>All</td>
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<td></td>
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<tr>
<td>N</td>
<td>225</td>
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<tr>
<td>Median PFS (weeks)</td>
<td>19.42</td>
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<tr>
<td>Median OS (weeks)</td>
<td>37.86</td>
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<tr>
<td>P</td>
<td>0.15</td>
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<td>0.32</td>
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CG means heterozygotes. In the right part, the C subgroup corresponds to CC genotype carriers and the G subgroup corresponds to heterozygotes (CG) and GG genotype carriers.
tested the impact of rs2010963 genotypes in an additional independent population of 92 GBM patients during their first-line chemotherapy with temozolomide. The clinical and demographic characteristics of this population are reported in Supplementary Table 2. Eleven thrombo-hemorrhagic events were observed: five deep venous thrombosis and six pulmonary embolisms. One patient with a pulmonary embolism also presented a concomitant intratumoral bleeding. Again we observed an over-representation of CC (31.0%) compared to CG (34.2%) and GG (54.8%) (p = 0.11). Even pooling the two populations, the over-representation of CC (524%) compared to CG + GG (202%) remains non significant (p = 0.15).

rs2010963 is not prognostic per se in glioma.

We then evaluated the prognostic value of rs2010963 in a large independent population of glioma patients. Blood DNA samples from 954 adult glioma patients (323 WHO grade IV, 269 WHO grade III and 362 WHO grade II) were analyzed for VEGFA SNP 2010963. Clinical and demographic characteristics are reported in Supplementary Table 3. The distribution of allele frequencies in our population met the Hardy–Weinberg equilibrium (P = 0.33). Minor allele frequency was 33.2%. Allelic frequencies were independent of sex, age at diagnosis, grade and histology. No difference was observed for PFS and OS between the three genotypes for the whole population, and after stratification for grade (Table 2 and Supplementary Figs 1, 2).

rs2010963 genotype affects VEGFA plasma levels

Since rs2010963 is located in a regulatory region VEGFA gene, it may affect VEGFA expression. We looked therefore for differences in baseline VEGF plasma levels before the onset of BVZ between the three genotypes. 87 plasma samples were available (12 CC, 35 CG and 40 GG). Baseline VEGF plasma level was significantly higher in CC patients (median = 107.6 pg/mL), as compared to CG (57.50 pg/mL) and GG (52.75 pg/mL) (P = 0.035) (Fig. 3). The VEGF plasma level was not correlated with age, body mass index (BMI), sex or hypertension. The baseline VEGF plasma level did not differ significantly in patients who developed TH adverse events during BVZ and did not affect PFS and OS during BVZ.

Discussion

We found that the VEGFA rs2010963 CC genotype was associated with thrombo-hemorrhagic events in patients...
treated with BVZ at recurrence. There are several limitations due to the retrospective analysis of the data, the missing data in the whole GBM cohort, and also the limited cohort of GBM patients treated with temozolomide alone. In these populations not receiving BVZ, we could not demonstrate an impact of genotype on thrombo-hemorrhagic events. Although the two populations presented similar clinical characteristics such as age, KPS and BMI, they cannot be compared because of the different stages of the disease; the impact of the rs2010963 CC genotype was then analyzed separately in the two populations.

The VEGFA rs2010963 polymorphism has been associated with susceptibility to vascular disorders, such as diabetic retinopathy, myocardial infarction and impaired prognosis in patients with chronic heart failure [20–24], and may therefore play a role in thrombo-hemorrhagic events occurring in patients receiving anti-VEGFA therapies.

The functional consequences of the rs2010963 variant are still a matter of debate. Located in the regulatory region of the gene, the rs2010963 polymorphism is believed to alter VEGFA expression. Indeed, we found a higher plasma level of VEGFA in CC patients. In line with our own findings, higher VEGFA expression has been reported in post-mortem retina from individuals with the rs2010963 C allele [25], and a CC genotype has been associated with higher serum VEGF levels [2, 20].

In a cohort of 954 gliomas, both PFS and OS for grade II to IV gliomas were independent of the rs2010963 status, suggesting that the rs2010963 SNP is not per se a prognostic marker. In contrast, patients with the rs2010963 CC genotype had a longer PFS after BVZ treatment (median PFS 28.3 weeks), as compared to CG and GG patients (18.1 and

<table>
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<tr>
<th>Table 2 Median progression free survival (PFS) and overall survival (OS) according to rs2010963 genotype and glioma grade</th>
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<tr>
<td>VEGFA rs2010963 genotype</td>
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<tr>
<td>All gliomas</td>
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<td>N</td>
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<td>Median PFS, months</td>
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<td>Median OS, months</td>
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<td>Grade II</td>
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<td>Grade IV</td>
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<td>Median PFS, months</td>
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<td>Median OS, months</td>
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Fig. 3 Baseline VEGFA plasma levels according to rs2010963 genotypes
19.0 weeks, respectively. Our results suggest that patients with the rs2010963 CC genotype may have an improved response to BVZ, but are also more prone to develop thrombo-hemorrhagic events. One may speculate that the rs2010963 CC genotype, resulting in higher VEGFA production, promotes the development of a VEGFA-dependent angiogenesis, and explains, at least in part, the improved responsiveness to BVZ. On the other hand, BVZ is associated with an increased risk of developing venous thrombo-embolisms [26] and hemorrhage in cancer patients [27], but the specific link between rs2010963 status and the vascular toxicities remains unexplained.

From a practical point of view, if these data are confirmed by an independent study, a more systematic thrombo-
embolic prevention may be warranted in rs2010963 CC patients with gliomas, especially when treated with BVZ.

Acknowledgements Work supported by a Grant from the Instituto Nacional de Cancer (INCA: Angeli: R/B/358/2009). A.L. Di Stefano received an investigator fellowship from PRIN 2010ZE5FWN_008. The research leading to these results has received funding from the programs “Investimenti di Avventura” ANR-18-IAUH-004. The authors are indebted to Alexandra Agachi for English Editing.

Conflict of interest None.

References

Research Article

Meningeal Melanomatosis: A Challenge for Timely Diagnosis

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Received 27 June 2014; Accepted 5 October 2014

Academic Editor: Athanasios G. Pallis

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Neoplastic meningitis is a central nervous system complication that occurs in 3–5% of patients with cancer. Although most commonly seen in patients with disseminated disease, in a small percentage of patients, it may be the initial manifestation of cancer or even primitive in origin. In the absence of cancer history, the diagnosis of neoplastic meningitis may be challenging even for expert neurologists. Prognosis is poor, with a median overall survival of 6 weeks from diagnosis. In the retrospective study herein, we described three cases of meningeal melanomatosis in patients without previous cancer history. The patients were diagnosed with significant delay (17 to 47 weeks from symptom onset) mainly due to the referral in performing the appropriate testing. Even when the diagnosis was suspected, investigations by MRI, cerebrospinal fluid, or both proved at times unhelpful for confirmation. Prognosis was dismal, with a median survival of 4 weeks after diagnosis. Our observations are a cue to analyze the main pitfalls in the diagnosis of neoplastic meningitis in patients without cancer history and emphasize key elements that may facilitate early diagnosis.

1. Introduction

Neoplastic meningitis is a central nervous system complication that occurs in 3–5% of patients with cancer [1], and it is most commonly seen in patients with disseminated progressive systemic disease due to spread of malignant cells to the leptomeninges. The most common primary tumors to metastasize to the meninges are lung cancer (9–25% of patients) [2] and melanoma (23%) [3], due to a distinctive neurotropism. More rarely neoplastic meningitis is the initial manifestation of systemic cancer (3–10%) [1] or it is primitive in origin, as it occurs in primary leptomeningeal melanomatosis [4]. In patients without cancer history, diagnosis may be challenging even for expert neurologists due to the lack of specific signs and symptoms. Prognosis of neoplastic meningitis is poor, as most untreated patients die within 1–9 weeks from diagnosis (median 3 weeks) [1, 5], as a result of neurological disease or tumor progression. The timeliness of diagnosis is crucial to start the appropriate treatment before sudden neurological deterioration.

Here, we present a retrospective series of three patients with meningeal melanomatosis without history of cancer,
characterized by a dramatic diagnostic delay. We also propose an algorithm focused on the diagnosis of neoplastic meningitis in naïve patients.

2. Materials and Methods

We describe a retrospective series of three patients with meningeal melanomatosis recruited from our two institutions (C. Mondino National Neuroscience Institute and Poliambulanza San Matteo Foundation IRCCS, Pavia, Italy) in four years. We conducted an Internal Review Board approved study using an institutional oncological database of all patients receiving a diagnosis of meningeal melanomatosis from January 2010 to January 2014. The medical records were reviewed and clinical, biological, and radiological data collected for details.

3. Results

The clinical and paraclinical characteristics of our three patients are summarized in Table 1. Patients were aged between 27 and 65. All patients had no previous cancer history and arrived to our centers after several neurologic evaluations. Clinical presentation included diffuse and/or multifocal neurological signs and symptoms: headache, nausea and/or vomiting, monoparesis, and cranial nerve palsy. One patient (patient 3) presented recurrent isolated confusional episodes but was completely asymptomatic in between.

Electroencephalogram showed bilateral/diffuse slow abnormalities without epileptic activity in all cases. Brain MRI performed within the first 4 weeks from symptom onset was normal in both patients in whom it was acquired (pt 1 and 3). Alternatively, focal or diffuse nodular enhancement of leptomeninges and cranial nerves was documented (Figure 1). Spine MRI revealed nodular contrast enhancement of meninges, conus, and cauda, suggesting neoplastic infiltration (Figure 2). Cerebrospinal fluid (CSF) analysis showed severe blood-CSF barrier (B-CSF B) damage in all patients but inconstant pleocytosis. In patient 2, despite repeated lumbar punctures, CSF cytology remained negative and diagnosis was confirmed on leptomeningeal tissue obtained from biopsy. In all other patients, the presence of melanoma cells in the CSF (Figures 3 and 4) was eventually documented by means of repeated lumbar punctures. After the diagnosis of meningeal melanomatosis was confirmed, all patients underwent a chest abdomen CT scan and a dermatological and an ophthalmological assessment. In two patients, the final diagnosis was of probable primary leptomeningeal melanomatosis (pt 1 and 2), while in patient 3 a cutaneous melanoma of right eyelid was documented. The diagnostic delay was remarkable in our series, with a median delay of 32 weeks from symptom onset (range: 17–47 weeks). Prognosis was dismal, with a median survival of 4.14 weeks from diagnosis (range: 2–6.29 weeks).

4. Discussion

Although restricted, our series offers several insights into the diagnosis of neoplastic meningitis in naïve patients. These patients, without a previous cancer history, can present with diffuse/multifocal clinical signs and symptoms and represent a real diagnostic challenge. On these grounds, we propose an algorithm (Figure 5) to guide the clinician in the complex process of differential diagnosis, regarding as opening scenario a naïf patient presenting with subacute headache and/or encephalopathy plus one or more focal signs, and negative or inconclusive MRI, as we have observed in our series. In this setting, CSF analysis should be promptly performed to exclude other immune/infectious disorders such as autoimmun e or paraneoplastic encephalitides, primary CNS vasculitis, and chronic infectious meningitis, which can course without MRI alterations. Besides, it is important to consider that a delay in the diagnosis of the above-mentioned conditions may strongly affect final outcome and long-term sequelae.

Furthermore, even when the diagnosis of neoplastic meningitis has been suspected, paraclinical findings could be inconclusive. According to the literature, brain MRI has an estimated sensitivity of 40–60% in demonstrating...
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<tr>
<td>Age/gender</td>
<td>17/M</td>
<td>55/M</td>
<td>65/M</td>
</tr>
<tr>
<td>Clinical presentation at onset</td>
<td>Headache, nausea and vomiting, diplopia, and weight loss</td>
<td>Left leg monoparesis, headache, nausea and vomiting</td>
<td>Recurrent confusional episodes</td>
</tr>
<tr>
<td>MRE</td>
<td>Time from symptom onset (weeks)</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>Hydrocephalus</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Leptomeningeal contrast enhancement</td>
<td>No Contrast not administered</td>
<td>No Contrast not administered</td>
<td>No Contrast not administered</td>
</tr>
<tr>
<td>Dural melanoma deposits</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>CSF</td>
<td>Time from symptom onset (weeks)</td>
<td>40.6</td>
<td>42.9</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>—</td>
<td>92</td>
<td>83</td>
</tr>
<tr>
<td>Proteins (mg/dL)</td>
<td>—</td>
<td>2566</td>
<td>2773</td>
</tr>
<tr>
<td>Cell count (cells/µL)</td>
<td>4</td>
<td>140</td>
<td>174</td>
</tr>
<tr>
<td>EEG</td>
<td>Poorly organized background activity with bilateral slow abnormalities</td>
<td>Diffuse bilateral slowing</td>
<td>Bilateral fronto-centrotemporal slow abnormalities with left predominance</td>
</tr>
<tr>
<td>Extra CNS visceral met</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Final diagnosis</td>
<td>Primary leptomeningeal melanomatosis</td>
<td>Primary leptomeningeal melanomatosis</td>
<td>Leptomeningeal carcinomatosis and cutaneous melanoma</td>
</tr>
<tr>
<td>Time to diagnosis (weeks)</td>
<td>47</td>
<td>17</td>
<td>23.4</td>
</tr>
<tr>
<td>Clinical evolution</td>
<td>Confusion, visual hallucinations, partial seizures, and behavioral alterations</td>
<td>Urinary retention, progressive paresis, and visual hallucinations</td>
<td>Vigilance impairment, generalized seizures, headache, and eucerache</td>
</tr>
<tr>
<td>Treatment</td>
<td>Trimetazidine (1 cycle)</td>
<td>Dicarbazine (1 cycle)</td>
<td>None</td>
</tr>
<tr>
<td>Overall survival (weeks)</td>
<td>45.1</td>
<td>23.4</td>
<td>30</td>
</tr>
</tbody>
</table>

Legend to Table: CNS = central nervous system. CSF = cerebrospinal fluid. EEG = electroencephalogram. met = metastases. MRE = magnetic resonance imaging. n.p. = not performed, and — = not available.
Figure 2: Primary leptomeningeal melanomatosis in patients 1 (a), (b) and 2 (c), (d). (a), (c) Sagittal T2-weighted images (TR/TE 3500 ms/120 ms) of cervical-dorsal spine and cauda equina show hypertrophic leptomeninges with crowded subarachnoid space and multimodular appearance of the cauda equina. (b), (d) Sagittal T1-weighted images (TR/TE 65 ms/9 ms) of cervical-dorsal spine and cauda equina show diffuse leptomeningeal enhancement and thickening.

Figure 3: Light microscopy pictures of the cytological specimen of cerebrospinal fluid obtained from patient 1: (a) hematoxylin and eosin staining of the hypercellular sample, with large, hyperchromatic cells associated with erythrocytes; (b) atypical cells stained with Melan-A, a melanoma-specific marker; (c) Schmed staining confirmed the presence of melanin (blue granular stain) in the cytoplasm; magnification, 20x.

Figure 4: Light microscopy pictures of the cytospin of the cerebrospinal fluid cells from patient 1: (a) hematoxylin and eosin staining of large, hyperchromatic cells along with erythrocytes, lymphoma monocytoid cells, and eosinophils (asterisks); (b) an atypical cell at larger magnification; arrows indicate granules of melanin.
meningeal neoplastic infiltration [3, 6, 7], but data correlating
sensitivity of MRI to the timing of its execution are currently
unavailable. In our series, brain MRI performed within the
first month from onset was normal despite the clinical pattern
was dominated by cranial involvement. In the absence of
meningeal contrast enhancement, dilatation of the ventricular
system or reduction of subarachnoid space may be indirect
signs of neoplastic meningitis and should be valued in all
cases.

CSF analysis, which was performed with remarkable
delay in our series, showed a significant protein increase
due to severe B-CSF II damage in all patients. Noteworthy,
despite the delay and repeated sampling, in patient 2 CSF
results were inconclusive for the detection of neoplastic cells,
leading to performing a meningeal biopsy. Interestingly, CSF
cytology can be persistently negative even in the presence of
disseminated cranial and spinal disease on MRI. These data
are consistent with current evidence that malignant cells are
detected in the CSF in 50–70% of patients with neoplastic
meningiota by initial lumbar puncture [3, 8, 9], a rate that
increases with repeated sampling. In the case of normal or
inconclusive CSF findings, a spine MRI may be helpful to
demonstrate meningeal infiltration of cauda roots, even in the
absence of spinal symptoms.

Overall, diagnostic difficulties resulted in a dramatic
diagnostic delay, ranging from 17 to 47 weeks after clinical
onset. These data are remarkable considering the poor short-
term prognosis of these patients [10], who could access only
palliative or even no treatment.

In conclusion, the difficulty in both posing the clinical
suspicion and confirming the diagnosis of neoplastic menin-
gitis contributed to the sharp diagnostic delay observed in our
series. Early recognition is fundamental to make differential
diagnosis and start appropriate therapies. Thus, improving
the handle of these patients and the current diagnostic
algorithms for neoplastic meningitis is of capital importance
to offer them appropriate treatments.

Conflict of Interests
The authors declare that there is no conflict of interests
regarding the publication of this paper.

Acknowledgments
Anna Luisa Di Stefano was supported by PRIN 2010-2011,
2010ZESTVN_008.
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TERT promoter mutations and rs2853669 polymorphism: prognostic impact and interactions with common alterations in glioblastomas

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Received: 6 June 2015/ Accepted: 19 November 2015 © Springer Science+Business Media New York 2015

Abstract TERT promoter (TERTp) mutation is the most common mutation in glioblastomas. It creates a putative binding site for Ets/TCF transcription factors, enhancing telomerase expression and activity; whereas the rs2853669 variant disrupts another Ets/TCF binding. We explore here the interaction between these two alterations, tumor genomic profile and the impact on prognosis. The TERTp and rs2853669 status were determined and confronted with the outcome and molecular profile, i.e., loss of chromosome 10q, CDKN2A deletion, IDH mutation, EGFR amplification, MGMT promoter methylation. 651 glioblastomas were selected (sex ratio = 1.35, median age: 60.4 years, median survival 13.5 months). The TERTp mutation found in 481 patients (74 %) was independent from rs2853669 genotypes. TERTp mutation, but not rs2853669 status, was associated with older age (61.4 vs. 52.8 years). rs2853669 status had no impact on overall survival (OS) either in mutated TERTp or wild-type TERTp. Neither rs2736100 (TERT, 5q15.33) nor rs219201116 (TERC, 3q26.2) status had any impact on survival or showed any association with a TERTp mutation. The TERTp mutation was associated with EGFR amplification chromosome 10q loss, CDKN2A deletion and IDH wt. EGFR amplification was associated with a better outcome in TERTp mutated GBM, and a worse outcome in TERTp WT. This study—the largest analyzing the TERTp mutation and the rs2853669 polymorphism—fails to find any prognostic impact of rs2853669. It confirms the dual prognostic impact of EGFR amplification depending on TERTp status.

Keywords Glioblastoma · TERT · Polymorphism · EGFR · rs2853669

Introduction

Increasing telomerase activity promotes immortalization through telomere lengthening. Telomerase consists of a reverse transcriptase, encoded by TERT, and an RNA component, encoded by TERC, which serves as a template for the telomere repeat. Interestingly, the Single Nucleotide Polymorphisms (SNPs) rs2736100, which maps to the TERT region (5q15.33), and the rs219201116, which maps near TERC (3q26.2), have both been associated with a glioblastoma risk and with longer mean leukocyte telomere length [1–3]. In addition, somatic mutations of the TERT promoter gene have been reported in numerous cancers and particularly in gliomas. They are present in 80 % of
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oligodendrogliomas and 75% of glioblastomas (GBM) [4]. The two most common mutations (TERTp C250T and C228T) create a putative binding site for the Ets/TCF transcription factors, which results in a two to fourfold increase in telomerase expression [5]. These mutations are an independent negative prognostic factor in glioblastomas [6–10]. On the other hand, the SNP rs2853669 at 245 bp from the TERT gene ATG, disrupts another binding site for Ets/TCF, through a CT substitution, and it has been associated with lower TERT expression and decreased telomerase activity [11–14]. Whereas a former study found the poor predictive impact of a TERTp mutation confined to the patients who did not carry the variant C-allele of rs2853669 polymorphism [8], another paper reached the opposite conclusions and showed that the prognostic impact of a TERTp mutation was much stronger in carriers compared to non-carriers of the C-allele [15].

Here we determined the distribution of the different rs2853669 variants in 651 patients affected by primary GBMs. We analyzed the interaction between TERTp mutational status and the rs2853669, rs2736100 (Sq15.33) and rs192011116 (Sq26.2) status in terms of overall survival. We also explored the interaction with other common molecular markers of GBMs: EGFR amplification, chromosome 10q loss, CDKN2A loss, IDH1 or IDH2 (IDH) mutation and MGMT promoter (MGMTp) methylation.

Patients and methods

Patients selection

Patients' informed consent and ethical board approval were required for collection of tumor samples and clinical- pathological information, as stated by the Declaration of Helsinki. Patient inclusion criteria were: histologic diagnosis of primary glioblastoma; availability of tumor DNA; and complete clinical data from the neuro-oncology database (OncoNeuroTek, Paris).

DNA extraction, amplification, purification and Sanger sequencing

DNA from cryopreserved or formalin-fixed paraffin embedded (FFPE) tumor samples was extracted using the QIAamp Midi Kit (Qiagen) or JetPrep ChargeSwitch Forensic kit (Life Technologies) respectively, as previously described [13].

DNA amplification was performed using the mix FastStart DNA Master (Roche™) as follows: 3 min at 94 °C; 35 cycles at 94 °C-15 s, 60 °C-45 s, 72 °C-1 min, with a final step at 72 °C for 8 min. Primer sequences were as follows: SNP_TERT_F: ATTCGACCCTCTCCGCTG; SNP_TERT_R: CTGGAAAGTGAAGGGGCAG; TERTp_F: GGATCGGGGGGCAAG; TERTp_R: CAGCCCTGGTGAACCTC. DNA sequencing was performed by the Sanger technique.

The rs2736100 (Sq15.33) and rs192011116 (Sq26.2) SNP statuses were available from a previous study for a subset of patients [1]. IDH status, EGFR amplification, 10q loss, CDKN2A deletion and MGMTp methylation were available in the OncoNeuroTek database or were determined as previously described [6, 16].

Statistical analysis

The χ² test or Fisher exact test were used to compare the genotype distribution. The MatsuWhitney–Wilcoxon test was used to compare continuous variables (i.e., age) between distinct categories.

Overall survival (OS) was defined as the time between diagnosis and death or last follow up. Living patients at the time of the last follow up were considered as censored events in the analysis. Progression free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Patients who were recurrence-free at the last follow-up were considered as a censored event in the analysis. Survival curves were calculated according to the Kaplan–Meyer method and differences between curves were assessed using the log-rank test. Variables with a significant p-value were used to build a multivariate Cox model. p-values <0.05 were considered significant.

Results

Patients characteristics and outcomes

651 glioblastomas were selected (sex ratio = 1:3.5, median age at diagnosis 60.4 years). Median survival was 13.5 months. The patients' characteristics are reported in Table 1. As expected, age (<65 vs. ≥65) and Kornofsky performance status (KPS) (<70 vs. >70) were strong predictors of outcome. Upfront treatment modalities were also associated with outcome, i.e., surgery (biopsy vs. partial or gross total removal, p < 10−5) (Supplementary Fig. 1) and radiotherapy-temozolomide versus radiotherapy alone (p < 10−12).

These parameters were entered into a Cox model (Supplementary Table 1 a, b). This multivariate analysis suggests that treatment modalities are related to KPS while age remains the strongest independent prognostic factor.
Table 1  Clinical and molecular characteristics of the patients.

<table>
<thead>
<tr>
<th>Age at diagnosis</th>
<th>60.4 (13-90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>375 M, 276 F; Sex ratio = 1.35</td>
</tr>
<tr>
<td>KPS</td>
<td>Medium 80 (20-100)</td>
</tr>
<tr>
<td></td>
<td>&lt;70: 56</td>
</tr>
<tr>
<td></td>
<td>&gt;70: 451</td>
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<tr>
<td></td>
<td>ND: 144</td>
</tr>
<tr>
<td></td>
<td>Partial removal: 209</td>
</tr>
<tr>
<td></td>
<td>Total removal: 260</td>
</tr>
<tr>
<td>Surgery</td>
<td>Biopsy: 173</td>
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<tr>
<td></td>
<td>Radiotherapy: 239</td>
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<tr>
<td></td>
<td>Chemotherapy: 56</td>
</tr>
<tr>
<td></td>
<td>ND: 118</td>
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<tr>
<td>Upfront treatment</td>
<td>Radiotherapy: TMZ: 239</td>
</tr>
<tr>
<td></td>
<td>Radiotherapy: 238</td>
</tr>
<tr>
<td></td>
<td>Chemotherapy: 56</td>
</tr>
<tr>
<td></td>
<td>ND: 118</td>
</tr>
<tr>
<td>PFS</td>
<td>5.5 months</td>
</tr>
<tr>
<td>OS</td>
<td>13.5 months</td>
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<td>TERTp mutational status</td>
<td>492 mut159 wt</td>
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<tr>
<td></td>
<td>C228T: 360</td>
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<tr>
<td></td>
<td>C132T: 127</td>
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<tr>
<td></td>
<td>C228T and C280T: 5</td>
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<td>rs2853669 status</td>
<td>CC: 65</td>
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<tr>
<td></td>
<td>CT: 230</td>
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<td></td>
<td>TT: 316</td>
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<td>rs19201116 status</td>
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<td>GA: 85</td>
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<td>GG: 117</td>
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<td></td>
<td>C A: 136</td>
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<td></td>
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<td></td>
<td>Wild: 600</td>
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<td>EGFR status</td>
<td>Amplification: 221</td>
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<td></td>
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<td></td>
<td>ND: 55</td>
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<tr>
<td>p16/CDKN2A status</td>
<td>Deletion: 248</td>
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<td></td>
<td>No deletion: 343</td>
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<tr>
<td></td>
<td>ND: 60</td>
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<tr>
<td>MGMTp status</td>
<td>Methylated: 212</td>
</tr>
<tr>
<td></td>
<td>Unmethylated: 239</td>
</tr>
<tr>
<td></td>
<td>ND: 280</td>
</tr>
</tbody>
</table>

**TERTp mutation status is independent from rs2853669 genotypes**

We found 492 (76%) patients with TERTp mutation, 365 (56.0%) had the C228T substitution, and 132 (20%) the C280T, five cases harboring both mutations. rs2853669 status was TT in 316 (48.5%) patients, CT in 270 (41.5%) and CC in 65 (10%). TERTp mutation was associated with a poorer outcome both in carriers (allele C: OR = 1.41; p = 0.027) and non-carriers of the rs2853669 variant (allele T: OR = 1.7; p = 0.00085). There was no difference in the relative distribution of the rs2853669 genotypes between different TERTp mutated GBM and TERTp wild type GBM (χ² = 0.92; p = 0.63). Relative genotype frequencies were CC 47.4 (9.6%), CT 209.4 (42.5%), TT 236/492 (47.9%) for TERTp mutated patients and CC 10/159 (6.3%), CT 60/159 (38.4%) and TT 80/159 (50.3%) for TERTp wild type (w), fulfilling the Hardy–Weinberg equilibrium (p = 0.9; p = 0.2).

**TERTp mutation but not rs2853669 status is associated with older age**

We looked for an association between age at diagnosis and rs2853669 status in both TERTp mutated and wild type patients. Whereas TERTp mutation was correlated with older age (mean age 61.4 vs. 52.8 years, p < 0.0001), age distribution did not differ between rs2853669 CC, CT or TT genotypes (p = 0.13) (Fig. 1).

**rs2853669 Status has no impact on overall survival (OS)**

We first compared the CC and CT carriers versus the TT carriers groups and found no difference in OS (p = 0.3, Fig. 2).

We then analyzed separately the impact of rs2853669 status on OS in TERTp mutated patients and in TERTp wt patients. While a TERTp mutation is clearly associated with a poorer survival (p < 0.0001), we found no prognostic impact of rs2853669 status in both TERTp mutated and TERTp wt populations (Fig. 3). However, C carriers (i.e., CC+CT patients) tended to have a better outcome in the TERTp mutated group (p = 0.18), but not in the TERTp wt group. rs2736100 (3q15.33) and rs19201116 (3q26.2) statuses were available for 215 GBM patients. None of them had an impact on survival (p = 0.6) and we found no association with the TERTp mutation (p = 0.55 and 0.7 respectively).
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**Fig. 1** Age distribution according to TERTp mutational status ($p < 10^{-15}$) and rs2835669 ($p = 0.02$).

**Fig. 2** Survival curves according to the rs2835669 status CC, CT, and TT.

**Fig. 3** Survival curves according to the rs2835669 status (CC+CT vs. TT) and TERTp mutational status.

**Association with the main molecular alterations and prognostic impact**

We first looked for associations between TERTp status and the following molecular alterations: chromosome 10q loss, found in 388429 of TERTp mutated versus 67126 in TERTp wt ($p < 10^{-15}$), EGFR amplification, found in 197454 versus 24/142 ($p < 10^{-6}$), CDKN2A/P16 deletion, found in 203450 versus 45/139 ($p = 0.02$), IDH mutation, found in 11/491 versus 39/159 ($p < 10^{-5}$), and MGMT promoter status, found methylated in 1569347 versus 56/104 ($p = 0.13$).

We then analyzed the prognostic impact of each of these alterations (suppl. Figs. 2a–4). As expected, only TERTp mutation, IDH mutation, and MGMTp methylation status had a prognostic impact. The prognostic impact was independent of treatment modalities, except for the MGMTp methylation status, which showed a clear benefit of RT+TMZ versus RT alone in MGMTp methylated ($p < 10^{-5}$), but not MGMTp unmethylated patients ($p = 0.2$) (suppl. Fig. 3). This confirms previous data [17]. We then investigated the prognostic impact of these combined alterations. While EGFR amplification has no prognostic impact per se, we consolidated here our previous data [6], showing that EGFR amplification is associated with a better outcome in TERTp mutated GBM (median survival = 16.1 months vs. 13 months; $p = 0.001$), and a poorer outcome in TERTp wt GBM (median survival = 13.3 months vs. 26.5 months; $p < 0.01$) (Fig. 4a). Whereas IDH mutation is probably associated with better outcome in the context of TERTp wt (median survival 29 months vs. 17.3 months $p = 0.09$) this may be not the case for the IDHwt-TERTpmut (median survival 20 months vs. 14.1 months $p = 0.3$) (Fig. 4b).

The rs2835669 status was not associated with IDH status, Chromosome 10q loss ($p = 0.13$), CDKN2A deletion ($p = 0.5$), or MGMTp status ($p = 0.99$). EGFR amplification tended to be more frequent in GBM patients bearing the variant allele C (123050 vs. 98289, $p = 0.08$).

**Multivariate analysis**

We first entered all the significant prognostic markers in a Cox model (suppl. Table 2a, b). We found that only MGMTp was an independent factor, while TERTp status,
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and to a lesser extend IDH status, were dependent on age.

Because we found *EGFR* associated with *TERTp* mutation, and *EGFR* prognostic impact dependent on *TERTp* status (Fig. 4a), we investigated the relation between *EGFR* status and *TERTp* using multivariate analysis (suppl. Table 3). We found indeed that *EGFR* amplification is related to better outcome in GBM patients (OR = 0.756, p = 0.02 when entering *TERTp* vs. OR = 0.983 for *EGFR* alone).

**Discussion**

Because previous works have generated conflicting data, we first investigated here, on a larger series of primary glioblastomas, the interaction between *TERTp* status and rs2853669. The rs2853669 minor allele disrupts a site for the ETS transcription factor family, whereas the *TERTp* mutation creates a new site. While mutation results in an increase of TERT activity, the variant rs2853669 allele is associated with lower TERT expression and activity [13, 14]. We may therefore suspect that the two variants – constitutional and somatic-, having opposite biological effects, will also have an opposite prognostic impact. And indeed in bladder cancer, the variant G has been associated with better outcome in *TERTp* mutated bladder cancers and with poorer outcome in non-mutated cancers [12]. In glioblastomas, data are more conflicting. Based on 178 glioblastomas, Simon et al. found that the poor prognostic effect of *TERTp* mutation was confined to the 84 non-carriers of the C-allele for the rs2853669 polymorphism [8]. In contrast, Speigl et al. found an opposite result in a series of 126 glioblastoma patients, with a poor prognostic effect of *TERTp* mutation only in the 67 carriers of the C-allele polymorphism rs2853669 [15]. Here we showed that the presence of a *TERTp* mutation is associated with a poor prognosis both in carriers and non-carriers of the rs2853669 variant. In addition this study, based on the largest series of glioblastomas of any such studies, does not show any significant prognostic impact of rs2853669 independently of age, or to the presence of a *TERTp* mutation (although the C variant tended to be associated with better outcome in *TERTp* mut GBMs, the trend was clearly not significant).

Since telomere lengthening is critical to tumor survival, and telomere shortening has been associated with higher glioma risk, we also investigated the potential relation between the *TERTp* mutation and two variants rs2736100 on 3q15.33 (TERT region) and rs19201116 on 3q26.2 (TERC region), associated with higher glioblastomas risk and telomere lengthening. Although this analysis was conducted on a smaller number of samples, once more we found no association.

We extended here our previous analysis showing that *TERTp* mutation is associated with a poor outcome, loss of chromosome 10q, CDKN2A deletion and *EGFR* amplification [6]. We confirmed here, in contrast to earlier studies [7], that the *TERTp* mutation’s poor prognosis impact is not related to the lack of IDH mutation: we rather suggest that IDH mutation is associated with poor prognosis in the presence of a *TERTp* mutation [6] (Fig. 4b): supporting our data. A similar analysis (although not mentioned by the authors) can be extracted from the data presented in a recent, independent publication from Eckel-Passow et al. (of their Fig. 3b) [18]. Finally we show that *EGFR* amplification has an opposite prognostic impact according to *TERTp* status: positive in *TERTp* mutated GBM, and negative in *TERTp* GBM (Fig. 4a).

**Acknowledgments** Work supported by the Fondation ARC. UN was supported by a grant from the University de Paris and the Association pour la Recherche sur les Tumeurs Cérébrales. The research leading to these results has received funding from the program "avenir-equipements d’avenir" ANR-10-E1HE-06. The authors are indebted to Alexandra Agachi for English editing.
Compliance with ethical standards

Conflict of interest None.

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