Clinical Cancer Research

Fluorescein-Guided Surgery for Resection of High-Grade Gliomas: A Multicentric Prospective Phase II Study (FLUOGLIO)

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Abstract

Purpose: Sodium fluorescein is a dye that, intravenously injected, selectively accumulates in high-grade glioma (HGG) tissue through a damaged blood–brain barrier. In this article, the final results of a multicentric prospective phase II trial (FLUOGLIO) on fluorescein-guided HGG resection through a dedicated filter on the surgical microscope were reported.

Methods: Patients with suspected HGGs considered suitable for removal were eligible to participate in this trial. Fluorescein was intravenously injected at a dose of 5 to 10 mg/kg. The primary endpoint was the percentage of patients with histologically confirmed HGGs, without contrast-enhancing tumor at the immediate postoperative MRI. Secondary endpoints were PFS, residual tumor on postoperative MRI, overall survival, neurologic deficits, and fluorescein-related toxicity. The sensitivity and specificity of fluorescein in identifying tumor tissue were estimated by fluorescent and nonfluorescent biopsies at the tumor margin. The study was registered on the European Regulatory Authorities website (EudraCT 2011-002527-18).

Results: Fifty-seven patients aged 45 to 75 years were screened for participation, and 46 were considered for primary and secondary endpoints. Mean preoperative tumor volume was 28.75 cm^3 (range, 1.3–87.8 cm^3). Thirty-eight patients (82.6%) underwent a complete tumor removal. Median follow-up was 11 months. PFS-6 and PFS-12 were 56.6% and 15.2%. Median survival was 12 months. No adverse reaction related to SF administration was recorded. The sensitivity and specificity of fluorescein in identifying tumor tissue were respectively 80.8% and 79.1%.

Conclusions: Fluorescein-guided technique with a dedicated filter on the surgical microscope is safe and enables a high percentage of contrast-enhancing tumor in patients with HGGs.
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Introduction

High-grade gliomas (HGGs) are aggressive tumors of the central nervous system (CNS) with poor prognosis despite the use of surgery, chemotherapy, and radiotherapy (1). There is strong evidence supporting the extent of resection (EOR) as a significant predictor of better survival in patients with HGGs (2–7). However, complete removal of the whole enhancing mass can be obtained only in a low percentage of cases (2, 3) due to both glioma infiltration into eloquent cortical and subcortical regions (7) and difficulty in distinguishing intraoperatively the viable tumor tissue at the margin of the resection even in noneloquent areas (8).

Therefore, several techniques were proposed with the aim of improving HGG visualization and EOR, such as neuronavigation (9), intraoperative MRI (iMRI; refs. 10–12), and ultrasound (13, 14). Photodynamic detection, that is the use of a photosensitive drug or dye that enhances tumor visualization by fluorescence, has been also proposed during removal of HHG. In particular, 5-aminolevulinic acid (5-ALA), a natural biochemical precursor of hemoglobin that provokes the synthesis and accumulation of fluorescent porphyrins in different tumors, has been shown to increase the rate of complete resection and the 6 months PFS in a randomized trial (15), and it has been recently approved also in the USA by FDA as an optical imaging agent in HGGs (https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm562645.htm). The sodium salt of fluorescein (SF) represents an alternative to 5-ALA. SF presents a major blue excitation wavelength peak ranging from 460 to 500 nm and a major green emission peak fluorescent radiation in the region ranging from 540 to 690 nm. When intravenously injected, SF has the capability to selectively accumulate in brain areas with altered blood–brain barrier

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

This is the first prospective multicentric phase II trial on fluorescein-guided removal of high-grade gliomas by the use of a dedicated filter on the surgical microscope. The results of this prospective trial showed the easy applicability of this technique for high-grade tumor removal, the absence of side effects, and a high rate of complete resection. These data represent the prerequisite to build up a prospective randomized controlled phase III trial to definitely confirm the advantage of this technique over a control cohort of patients. The use of fluorescein to enhance intraoperative tumor visualization and increase extent of resection could significantly improve survival of patients with high-grade gliomas.

(BBB), related to the presence of high-density HGG tumor cells (16). It has been shown that SF improves tumor visualization, either with white light illumination and, more precisely, by the aid of a specific filter in the surgical microscope (17–21). In 2011, our group started the first prospective phase II trial (FLUOGLIO) with minimax Simon 2-stage design, to evaluate the safety and efficacy of fluorescein-guided resection of HGGs by the use of a dedicated fluorescence filter in the surgical microscope. This article describes the final result of the study, including short-term and long-term outcome.

Materials and Methods

Patients

Patients from 18 to 75 years old, with suspected, newly diagnosed, untreated HGGs, considered suitable for a surgical removal were eligible for trial participation. Exclusion criteria were tumors starting from the midline, basal ganglia, cerebellum, or brain stem; multifocal tumors; the presence of a large, noncontrast-enhancing area, suggesting low-grade gliomas with malignant transformation; medical reasons precluding MRI (e.g., pacemaker); inability to give consent due to cognitive dysfunction or language barrier; Karnofsky performance scale (KPS) 60 or less; renal or hepatic insufficiency; history of active malignant tumors at any body site; known allergy to contrast agents and/or history of previous anaphylactic shocks; lactating or pregnant women.

All patients gave written informed consent, and the study was approved by the Ethical Committee of the Foundation IRCCS Neurological Institute Carlo Besta of Milan (Italy) and the Regensburg University Hospital (Germany) and registered on the European Regulatory Authorities website (EudraCT No. 2011-002527-18). The protocol and preliminary data on extent of resection, as derived from study design, were reported previously (18, 19).

Pre- and postoperative management

General physical performance was evaluated with the KPS. Neurologic status was assessed by the "National Institute of Health Stroke Scale" (NIHSS). Preoperative clinical evaluation was performed up to 14 days before surgery, whereas immediate postoperative evaluation was completed within 72 hours after surgery.

All patients were submitted to MRI (1.5 T, Siemens), including 3D volumetric sequences with and without contrast (T1 weighted), within 4 days before surgery, and within 72 hours after surgery. Tumor volume was calculated by segmentation of the whole contrast enhancement. The lesions were classified into three different functional grades with respect to their relationship with eloquent brain areas, as described in a previous article (22). All tumors located in noneloquent brain areas are considered as grade I. Grade III lesions include tumors involving eloquent brain areas (e.g., motor/sensory cortex, visual area, speech center, internal capsule, basal ganglia, hypothalamus/thalamus, brainstem, dentate nucleus), whereas grade II lesions were considered those located near eloquent brain areas or in the corpus callosum.

The calculation of the residual tumor volume (RV) was made as follows: the volume of hyperintense tissue on postoperative volumetric T1 images without contrast was subtracted from the volume of hyperintense tissue in postcontrast volumetric T1 images, to avoid inclusion of hyperintense blood or blood product (23).

Surgery was to be followed by standard conformational radiotherapy (60 Gy in 30 fractions for a total of 6 weeks) and concurrent chemotherapy with temozolomide (75 mg/m^2/die), followed by 4 weeks of interval and six cycles of maintenance chemotherapy with temozolomide (5 days every 28 at a dose of 150 mg/m^2/die for the first cycle and 200 mg/m^2/die for the subsequent in case of absence of serious adverse events), according to Stupp protocol (1). No restrictions were imposed on treatment after disease progression.

The first clinical and radiologic evaluation following the postoperative one was performed 4 weeks after the end of radiotherapy and concomitant temozolomide. Subsequently, follow-up was performed every 3 months for grade III gliomas and every 2 months for grade IV. Clinical and radiologic assessment included the same evaluation performed in the preoperative phase. When unable to reach the hospital, the patient was allowed to undergo MRI examinations elsewhere and send MRI images to the study physician for follow-up; in this case, clinical examinations could be substituted by a phone contact.

All MR images were centrally analyzed at Besta Institute by an independent radiologist (C. Boffano), using an open-source software, already validated in clinical setting, including brain tumors (refs. 24, 25; Osirix for Macintosh, www.osirix-viewer.com).

Once progression was detected, neuroimaging parameters were no longer registered for the purposes of the study, and the normal radiologic follow-up could be performed at other radiologic centers. In these cases, clinical follow-up could be carried out by telephone interview.

Surgical protocol

SF was intravenously administered, at a dose of 5 to 10 mg/kg (Monico SpA in Italy, Alcon Pharma GmbH in Germany), by a central venous line in the operating room, immediately upon completion of the induction of general anesthesia or anesthetic procedures in cases of surgery while the patient was awake.

A dedicated surgical microscope (Pentero, Carl Zeiss Meditec) was used for the surgical procedure. In the first seven cases, the BLU 400 filter, originally designed for 5-ALA, was used. Since January 2012, the YELLOW 560 filter, specifically designed for fluorescein (excitation wavelength range, 460–500 nm; observation wavelength range, 540–690 nm), was employed. Since then, the fluorescein dose was reduced to 5 mg/kg. Neuronavigation was allowed only for surgical planning, initial tumor localization,

and orientation during tumor removal, but not for judgment regarding EOR. Other technical adjuncts (US, neurophysiologic monitoring, etc.) were available when needed. During resection, the microscope could be switched alternatively from fluorescent to white-light illumination, as desired by the surgeon (18, 19). In cases classified as grade II or III, intraoperative neurophysiologic and clinical monitoring was used, as described previously (26). Tumor was removed in an inside-out fashion until all fluorescent tissue was removed, as considered feasible by the surgeon.

Endpoints and assessment of response

Only patients with a histologically confirmed HGGs and a total removal of the fluorescent tissue, as judged intraoperatively by the individual study surgeon, were considered for the evaluation of the primary and secondary endpoints (apart from toxicity). Otherwise, patients were included only in the analysis of toxicity.

The primary endpoint was the proportion of patients with a complete removal of the contrast-enhanced tumor. EOR was calculated as follows: (preoperative tumor volume − postoperative residual tumor volume)/preoperative tumor volume.

Secondary endpoints were (i) PFS at 6 and 12 months; (ii) postsurgical RV; (iii) overall survival (OS); (iv) presence and severity of neurologic deficits; and (iv) toxicity related to SF.

Disease progression was defined as evidence of a new measurable enhancing lesion for patients with total removal or an increase of the residual tumor volume for patients with incomplete removal, according to the RANO criteria (27). The PFS-6 and PFS-12 were defined as the proportion of patients with no progression respectively at 6 and 12 months after diagnosis. Patients who died for any cause before documented progression were counted as an event for this endpoint. The RV was calculated on early postoperative MRI. The OS was defined as the number of patients who had not died from any cause.

Patients were clinically examined by assessing the KPS and the eventual appearance of new neurologic deficits or worsening of preoperative deficit within 72 hours after surgery and then during follow-up visits until progression of disease.

Adverse events were registered during the follow-up period in the context of the FLUOGLIO phase II study, according to Common Terminology Criteria for Adverse Event (CTCAE), version 4.0, published by the US Department of Health and Human Services. To evaluate any possible correlation of adverse events with the administration of fluorescein, its known safety profile was considered.

Histologic analysis, O-6-methylguanine-DNA methyltransferase methylation, and IDH status

Histologic analysis of the tumor was performed with standard procedures and classified on the basis of the 2007 WHO classification by an independent neuropathologist (B. Pollo; refs. 1, 28). As part of an ancillary study, for patients with lesions in noneloquent areas, and giving their specific written informed consent, biopsies at the tumor margin were performed to evaluate the ability of fluorescein to discriminate between clear tumor tissue and peritumor area. Specifically, for each patient, four biopsy samples were performed at the tumor margin in areas located distant one from the other, two in the fluorescent and two in the nonfluorescent tissue, and analyzed in blinded fashion, in order to calculate sensitivity and specificity.

Methylation patterns in the CpG islands of O-6-methylguanine-DNA methyltransferase (MGMT) promoter were determined as previously described (29).

Isocitrate dehydrogenase gene 1 (IDH1) mutation was evaluated by PCR sequencing in the first 14 patients and by immunohistochemistry in the remaining 32 cases.

Statistical analysis

The sample size and stopping rules were determined according to a minimax Simon 2-stage design, with 80% power and $\alpha = 5\%$, in which a proportion of tumor complete resections of $\geq 50\%$ was considered promising and a response rate of $\leq 30\%$ was unacceptable. In the first stage, it was foreseen that 15 patients should be enrolled and, in case five or fewer responses were observed, further study should be terminated due to lack of treatment efficacy. Otherwise, an additional 31 patients should be enrolled for a total of 46. At the end of the second stage, if 18 or fewer responses were observed the treatment should be rejected, whereas if 19 or more responses were recorded the treatment could be considered sufficiently active to warrant further study.

The sample was described by means of the usual descriptive statistics: mean, median, and SD for continuous variables and proportions for categorical ones. ANOVA and Tukey multiple comparison tests were used to compare pre- and postoperative (immediate and 3 months postop) NIHSS and KPS.

PFS and OS were estimated by the Kaplan–Meier method. Sensitivity and specificity of fluorescein in identification of tumor tissue were calculated as follows: sensitivity as the number of fluorescent samples true positive (with histologically confirmed HGGs)/all samples with confirmed HGGs; specificity as the number of nonfluorescent samples true negative (histologically confirmed not neoplastic)/all samples histologically confirmed not neoplastic. Positive predictive value (PPV) and negative predictive value (NPV) were calculated as follows: PPV as the number of fluorescent samples true positive (with histologically confirmed HGGs)/all fluorescent samples; NPV as the number of nonfluorescent samples true negative (histologically confirmed not neoplastic)/all nonfluorescent samples.

Results

The first patient was enrolled in September 2011. Fifty-seven patients were screened for participation in this prospective trial (Fig. 1). There were 36 males and 21 females, ranging from 45 to 75 years old (median age, 65). One patient was excluded during screening, due to multifocal localization. Ten patients were excluded from analysis of primary and secondary endpoints apart from toxicity: (i) eight due to a histologic diagnosis different from HGG (three metastasis of carcinoma, two of melanoma, one of adenocarcinoma, one primitive neuroectodermal tumor, one lymphoma); (ii) two for the persistence of intraoperatively fluorescent tissue in the surgical cavity, considered not resectable by individual study surgeon (in one case, because the residual tissue was adherent to mesencephalon and posterior cerebral artery (case No. 25/57), in the other case because we had positive responses from the monopolar subcortical stimulation in fluorescent tissue, corresponding to corticospinal tract (case No. 24/57).

Finally, 46 patients, as expected from statistical analysis, were considered for primary endpoint. Median preoperative tumor volume was 22.4 cm^3 (range, 1.3–87.8 cm^3). There were 44

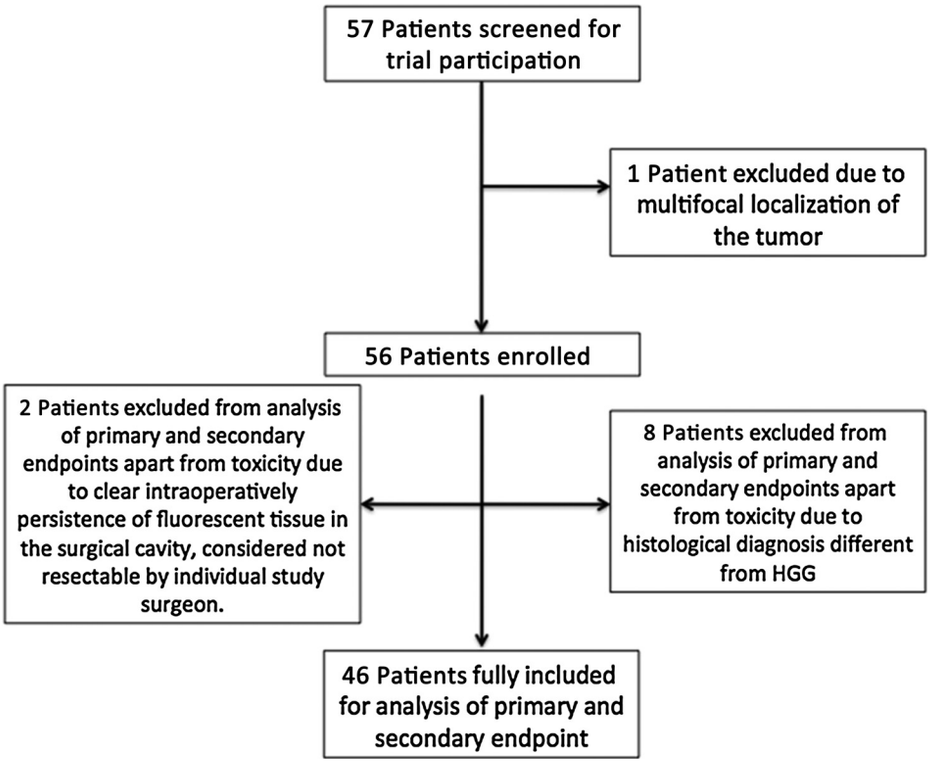

Figure 1.
Trial scheme of the FluoGlio study.

glioblastomas (GBMs) and one gliosarcoma (grade IV WHO) and one anaplastic astrocytoma (grade III WHO). Eleven out of 46 patients had the MGMT promoter methylated. All patients were IDH wild-type. According to the classification proposed by Sawaya and colleagues (22), 20 patients presented with grade III lesions, 10 patients with grade II lesions, and 16 with grade I lesions. Thirty-three patients were administered with dexamethasone in the preoperative period (up to 30 days before surgery in one case, most of them within 1 week pre-op) with an average daily-dose of 6.88 mg. Patient No. 21 decided to participate to another experimental trial after completion of immediate post-operative follow-up, and was then excluded from long-term analysis of PFS and survival. Characteristic of the patients were described in Tables 1 and 2.

Intraoperative fluorescence characteristics

There was no technical issue during surgery, related to filter activation. All tumors included in this study showed an intense fluorescent yellow-green signal that was clearly distinguished by normal peritumoral brain parenchyma, depicted as pinkish tissue, when the specific filter on the surgical microscope was activated (Fig. 2). Occasionally, the intraoperative fluorescence appeared more heterogeneous due to the presence of darker necrotic areas that were always located into the central tumor core, and never at the tumor margin. In addition, in HGGs with cystic components, the cystic fluid was brightly fluorescent as well. No differences were reported in fluorescence characteristics between patients with or without corticosteroid therapy.

There are some occasions in which fluorescent tissue could be visible also in intracranial areas not related to the tumor mass. For instance, dura mater is always fluorescent due to the absence of BBB. For the same reason, circumventricular organs (CVOs) could also appear intensively fluorescent (30). Fluorescein uptake could be visible in choroid plexus, in which, similarly to CVOs, the endothelial cells do not form a barrier and are fenestrated (31). We have never found fluorescein uptake during surgical manipulation of cortical or subcortical healthy tissue in peritumoral

Table 1. Patient characteristics

Variables	Value
No. of patients[a]	46
Age	
Median	65
Range	40–75
Preoperative KPS	
Median	90
Range	70–100
Pre-op NIHSS (mean ±SD)	1.043 ± 1.414
CE-PTV[b] (cm^3)	
Mean	28.75
Median	22.40
Range	1.3–87.8
EOR	
Median	100%
Range	82.8%–100%
OS (months)	
Median	12
Range	2–60
PFS (months)	
Median	*7*
PFS 6	56,6%
PFS 12	15.2%
Eloquence (see text)	
Grade I (non-eloquent)	16
Grade II (near eloquent)	10
Grade III (eloquent)	20

[a]Cases considered for primary and secondary endpoints.
[b]CE-PTV: contrast-enhanced preoperative tumor volume.

Table 2. Description of the 46 patients considered for primary and secondary endpoints in the FLUOGLIO study

Patient	Tumor location[a]	Eloquence grade	Tumor size (cm^3)	Histology	MGMT status	IDH1 status	% of resection	RV cm^3 (%)
1	L paratrigonal	III	28.0	GBM	Unmethylated	Wild-type	100%	0 (0%)
2	L temporal	III	12.8	GBM	Methylated	Wild-type	100%	0 (0%)
3	R parietal	III	49.8	GBM	Unmethylated	Wild-type	100%	0 (0%)
4	R parietal	III	87.8	GBM	Methylated	Wild-type	99.9%	0.09 (0.1%)
5	L frontal	III	12.2	GBM	Unmethylated	Wild-type	100%	0 (0%)
6	L occipital	II	41.0	GBM	Unmethylated	Wild-type	100%	0 (0%)
7	L temporo-insular	III	31.8	GBM	Unmethylated	Wild-type	88.6%	3.94(12.4 %)
8	L fronto-insular	III	34.5	GBM	Unmethylated	Wild-type	100%	0 (0%)
9	R frontal	III	9.6	GBM	Unmethylated	Wild-type	82.8%	1.65 (17.2%)
10	L temporal	III	2.4	AA	Methylated	Wild-type	100%	0 (0%)
11	R Frontal	I	20.0	GBM	Unmethylated	Wild-type	100%	0 (0%)
12	R frontal	I	83.8	GBM	Unmethylated	Wild-type	100%	0 (0%)
13	R temporal	I	86.1	GBM	Unmethylated	Wild-type	100%	0 (0%)
14	R parietal	III	12.5	GBM	Unmethylated	Wild-type	100%	0 (0%)
15	R frontal	I	69.7	GBM	Unmethylated	Wild-type	100%	0 (0%)
16	L frontal	III	8.3	GBM	Unmethylated	Wild-type	100%	0 (0%)
17	R frontal	II	31.8	GBM	Unmethylated	Wild-type	100%	0 (0%)
18	R frontal	I	8.9	GBM	Unmethylated	Wild-type	100%	0 (0%)
19	L temporal	III	28.8	GBM	Unmethylated	Wild-type	100%	0 (0%)
20	R parietal	III	38.9	GBM	Unmethylated	Wild-type	99.3%	0.3 (0.7%)
21	L temporo-parietal	III	1.3	GBM	Unmethylated	Wild-type	100%	0 (0%)
22	R parietal	I	1.9	GBM	Unmethylated	Wild-type	100%	0 (0%)
23	L frontal	III	18.8	GBM	Unmethylaed	Wild-type	100%	0 (0%)
24	R parietal	II	73.5	GBM	Unmethylated	Wild-type	100%	0 (0%)
25	L Frontal	III	9.2	GBM	Unmethyleted	Wild-type	100%	0 (0%)
26	L parietal	II	6.0	GBM	Unmethyleted	Wild-type	100%	0 (0%)
27	L temporal	III	38.6	GBM	Methylated	Wild-type	90.3%	3.74 (9.7)
28	L frontal	II	3.7	GBM	Methylated	Wild-type	100%	0 (0%)
29	R temporal	I	16.1	GLIOSARCOMA	Unmethylated	Wild-type	100%	0 (0%)
30	R temporal	I	33.2	GBM	Methylated	Wild-type	100%	0 (0%)
31	R frontal	II	20.8	GBM	Unmethylated	Wild-type	100%	0 (0%)
32	R temporal	I	11.7	GBM	Unmethylated	Wild-type	100%	0 (0%)
33	R parietal	II	3.8	GBM	Methylated	Wild-type	100%	0 (0%)
34	L temporal	III	7.9	GBM	Unmethylated	Wild-type	100%	0 (0%)
35	R temporal	I	81.3	GBM	Methylated	Wild-type	100%	0 (0%)
36	L parietal	II	42.1	GBM	Unmethylated	Wild-type	100%	0 (0%)
37	L frontal	III	44.85	GBM	Unmethylated	Wild-type	99.9%	0.04 (0.1%)
38	L temporoparietal	II	24	GBM	Methylated	Wild-type	100%	0 (0%)
39	L frontal	II	24	GBM	Unmethylated	Wild-type	100%	0 (0%)
40	L temporal	III	5.76	GBM	Methylated	Wild-type	99.5%	0.03 (0.5%)
41	R temporoparietal	I	44.68	GBM	Methylated	Wild-type	99.9%	0.04 (0.1%)
42	R frontal	I	59.77	GBM	Unmethylated	Wild-type	100%	0 (0%)
43	R temporal	I	6.4	GBM	Unmethylated	Wild-type	100%	0 (0%)
44	R parietal	I	6.04	GBM	Unmethylated	Wild-type	100%	0 (0%)
45	R occipital	I	2.51	GBM	Unmethylated	Wild-type	100%	0 (0%)
46	R occipital	II	35.8	GBM	Unmethylated	Wild-type	100%	0 (0%)

[a]R, right; L, left.

areas, because at that time, the amount of circulating fluorescein is very limited due to our strict protocol of injection timing and dosage. However, it is possible to find some examples of fluorescein uptake in cortical areas far from the tumor tissue, due to minimal contusion caused sometimes by burr hole during craniotomy, because that damage to the cortex has been performed closer to the injection time and the amount of circulating fluorescein is higher.

Extent of resection

Median EOR was 100% with a range from 82.8% to 100%. Thirty-eight patients (82.6%) were submitted to a complete tumor removal. The remaining eight patients with a noncomplete tumor resection had a median RV of 0.1945 cm^3 (1.229 $cm^3 \pm 1.701$ cm^3) or 0.58% of the initial tumor mass (4.97% ± 6.791%). Five of eight cases (62.5%) of incomplete removal had a >98% EOR (Tables 1 and 2).

Sensitivity and specificity of fluorescein in tumor identification at the tumor margin

A total of 50 biopsies (26 in fluorescent tissue and 24 in nonfluorescent tissue) were performed at the tumor margin in 13 patients. Twenty-one out of the 26 biopsies in fluorescent area showed clear HGG tissue. Nineteen out of the 24 biopsies in nonfluorescent area showed the absence of HGG tissue. These data were confirmed by the IHC analysis, as already showed in a previous report (18). The resulted sensitivity and a specificity of fluorescein in identifying tumor tissue were, respectively, 80.8% and 79.1%. The PPV and NPV were respectively 80.8% and 79.1%.

Adverse reactions to fluorescein, postoperative neurologic status, and adverse events during study period

Forty serious adverse events occurred in 56 patients during the study period, and none of them was considered related to fluorescein administration (see Supplementary Material).

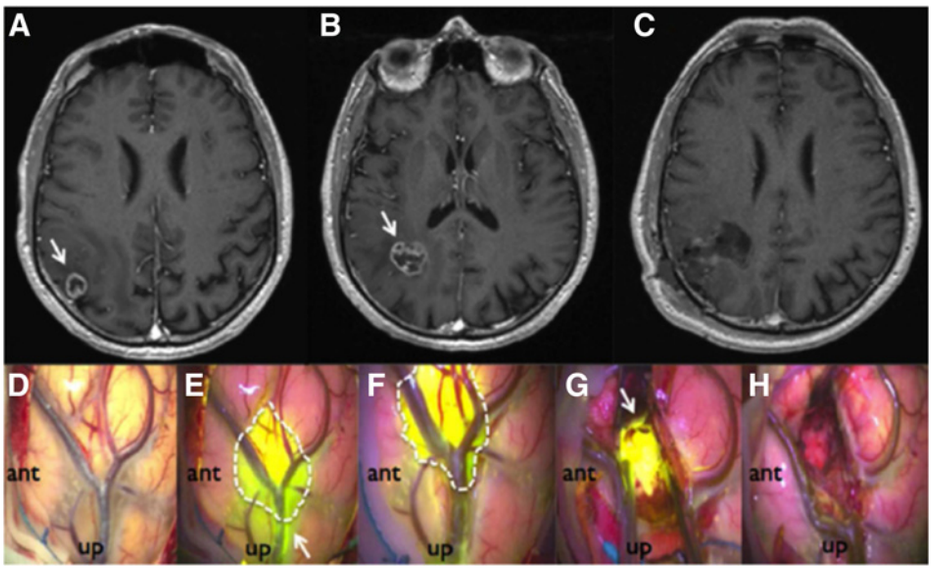

Figure 2.

Preoperative, postoperative MR images, and intraoperative pictures of a patient submitted to surgical resection of a right parietal multifocal glioblastoma with fluorescein-guided technique (5 mg/kg injected immediately after patient intubation). For orientation: ANT is toward the nose, and UP is toward the vertex. **A, B**, T1 axial preoperative MR with contrast, showing (white arrows in **A** and **B**) the presence of a tumor with irregular contrast enhancement in the right parietal area, compatible with suspected HGG. **C**, T1 axial postoperative MR with contrast, confirmed total removal of the lesion. **D**, Microscopic visualization under white light, after dural opening, with no insight about tumor localization. **E**, The same area after activation of YELLOW560 filter (Pentero miscoscope, Carl Zeiss Meditec, Germany), allowed to identify the superficial margin of the tumor (dotted line), appearing as brightly yellowish fluorescent tissue; some fluorescence is visualized also around the cortical vein (white arrow), due to extravasation of fluorescent liquid in the subarachnoid space. **F**, After arachnoid sharp dissection, and aspiration of some amount of fluorescent fluid, also the superior margin of the superficial part of the tumor can be appreciated (dotted line). **G**, The tumor (white arrow) can be removed in an inside-out fashion under YELLOW560 visualization. **H**, Appreciation of surgical cavity under YELLOW560 filter, after removal of all fluorescent tissue; peritumoral area can be identified as a pinkish tissue, clearly different from fluorescent tumoral area.

There was a significant difference in NIHSS and KPS evaluation between pre- and immediate postoperative follow-up in grade II and III lesions, that returned to baseline level at 3 months postoperatively (NIHSS pre-op 1.258 $\pm$ 0.2895 vs. immediate post-op 3.097 $\pm$ 0.6498 vs. 3 months post-op 1.706 $\pm$ 1.829, $P <$ 0.0001; KPS pre-op 88.06 $\pm$ 10.14 vs. immediate post-op 77.74 $\pm$ 16.06 vs. 3 months post-op 82.78 $\pm$ 12.27, $P = 0.0113$). No significant differences were found in grade I lesions (NIHSS pre-op 0.6667 $\pm$ 0.7237 vs. immediate post-op 1.533 $\pm$ 2.696 vs. 3 months post-op 1.167 $\pm$1.329, $P = 0.4616$; KPS pre-op 92 $\pm$ 10.82 vs. immediate post-op 84 $\pm$ 18.44 vs. 3 months post-op 81.67 $\pm$ 9.832, $P = 0.211$; Fig. 3).

PFS and overall survival

Forty-five patients were considered for PFS and survival. Nine of them (20%) completed the total Stupp protocol. Twenty-nine patients (64.4%) performed an incomplete Stupp protocol, including concomitant radiotherapy and temozolomide, but not all the six cycles after the completion of radiotherapy. One patient (2.2%) stopped the Stupp protocol 1 month after the beginning due to acute hydrocephalus needing surgical treatment. One patient (2.2%) underwent hypofractionated radiotherapy (30 Gy) and concomitant temozolomide due to postoperative hemiparesis. One patient (2.2%) underwent only adjuvant radiotherapy. Four patients (9%) did not undergo any postoperative treatment, due to early exitus or significant clinical deterioration. After a median follow-up of 11 months (range, 2–60 months), the median PFS was 7 months, with a PFS-6 and PFS-12 of 56.6% and 15.2%, respectively. During the study period, 35 patients died due to tumor progression or other causes. Median OS was estimated in 12 months. Survival at 6, 12, and 24 months was 90.7%, 49.9%, and 19.1%, respectively. (Fig. 4). Because of the presence of only eight cases with incomplete resection, we could not perform a comparison of survival curves between the subgroups of patients with complete and incomplete resection.

Discussion

Our prospective phase II trial showed that fluorescein-guided resection of HGGs is feasible, safe, and allows for a high rate of complete resection at early postoperative MRI.

In all cases, the fluorescence highlighted by the use of a dedicated filter on the surgical microscope was useful in tumor identification and discrimination from the normal brain parenchyma. Fluorescence visualization was not apparently altered by the use of preoperative corticosteroid therapy.

Intravenous fluorescein injection has been used for many years in ophthalmology with a very low rate of adverse events (32). In our cohort of patients, no specific adverse event related to fluorescein was registered. This could be related both to the fact that fluorescein was mainly administered to intubated patients under general anesthesia and to the use of low-dosage compared with that used in ophthalmology or in the first neurosurgic experiences

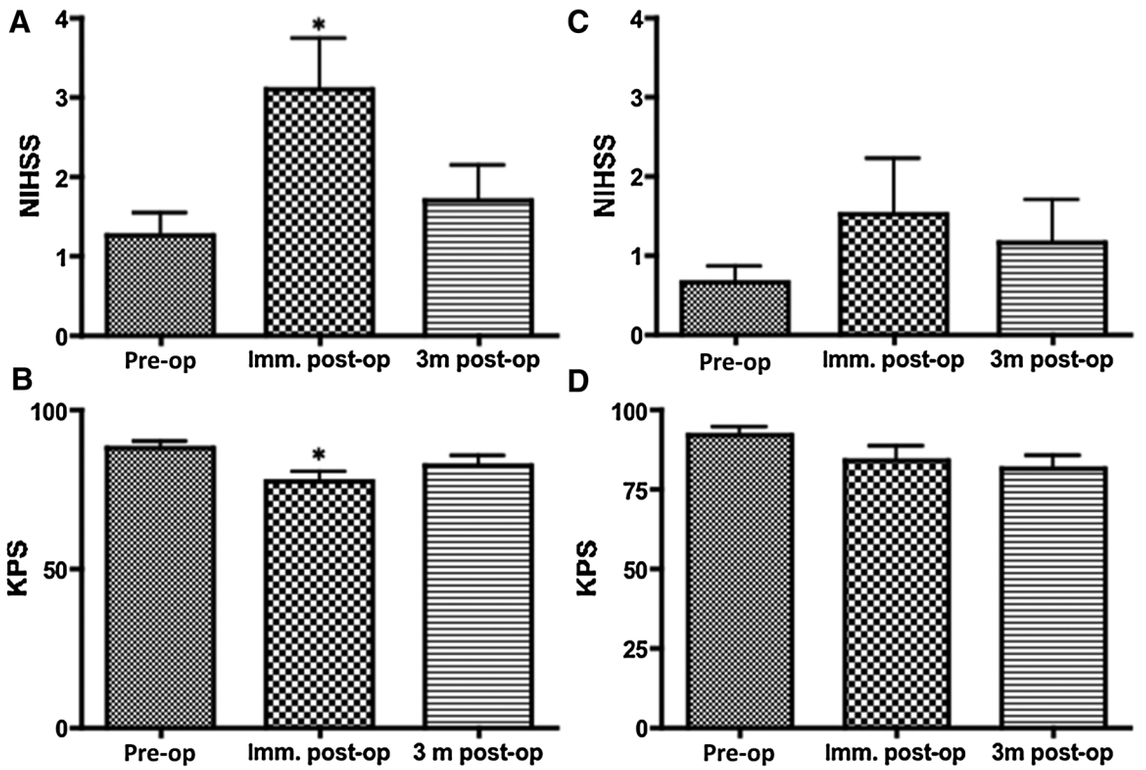

Figure 3.

Preoperative, immediate, and 3 months postoperative KPS and NIHSS, according to tumor location. **A** and **B,** Patients with grade II and III lesions. *, Represents statistical significant difference. **C** and **D,** Patients with grade I lesions.

without a specific filter on the surgical microscope. In fact, only two cases of anaphylactic reactions in neurosurgical application were reported in the scientific literature, all of them with a dosage of 20 mg/kg (33–34).

Our data on primary endpoints showed a complete resection in 82.6% of the cases, with a median RV in the remaining cases of 0.1945 cm^3. EOR of >98% was obtained in 93.4% of the cases. Thus, fluorescein-guided technique seems to be able to assure a high-rate of complete or near-complete resection in HGGs. There are multiple retrospective studies that showed a significant effect of EOR on survival of patients harboring GBMs (2, 6, 23, 35). In particular, a retrospective analysis of 416 patients showed a benefit in survival only when >98% EOR was achieved (2). However, more recent studies suggested a survival advantage with as little as 70% to 78% EOR, with a possible widening of benefit obtained with higher EOR (6, 35). Furthermore, a mathematical survival model based on data from 721 GBMs confirmed an incremental survival benefit associated with more aggressive surgical resection, taking into consideration patient characteristics such as age, KPS, and the use of adjuvant treatment (36). In addition, survival was also found to be dependent on RV, with advantages obtained with <2 or < 5 cm^3 (2, 23, 35). It should be noted that all of our cases of incomplete removal showed a RV that was <5 cm^3, with 75% of them (six of eight cases) being <2 cm^3.

Even if in our study there is no control group as a comparison, the obtained rate of EOR if much higher than the one that could be expected from historical series of patients operated on with the use of white light illumination. For instance, in a series of patients with GBM operated on in the period 1996 to 2007, that excluded locations precluding a complete resection, the total removal was possible only in 39% of the cases (3). These results are similar to those obtained in the control group of patients of the 5-ALA study [100% EOR in 36% of the cases (15), in the study by Lacroix and colleagues (46% of >98% EOR; ref. 2) and that of Grabowski and colleagues (ref. 23; 28.9% of >98% EOR)]. Moreover, the cohort of patients in FLUOGLIO study included 30 cases located in eloquent or near-eloquent areas (65% of the total cohort), and with a median tumor volume of 22.4 cm^3 (Table 1). Thus, it seems reasonable to consider the EOR in our series not related to the inclusion of only small tumors in noneloquent areas. In addition, our data confirmed previously published series on the use of fluorescein for HGGs, showing a percentage of resection ranging from 75% to 100% (17–19, 21, 37–41). In some of these experiences, the fluorescein use was associated with a significant improvement of resection rate as compared with the use of white light illumination (21, 37, 38). It is also important to recognize that, even with fluorescein-guided technique and under specific filter visualization, a complete removal of favorable HGGs is not always possible. As for other types of fluorescence tools (8, 42, 43), it is possible to visualize the pathologic tissue only if it is properly exposed during the surgical approach; thus, some residual may be left behind, especially when small corticectomy has been performed and the fluorescent tissue is in the blind corner of

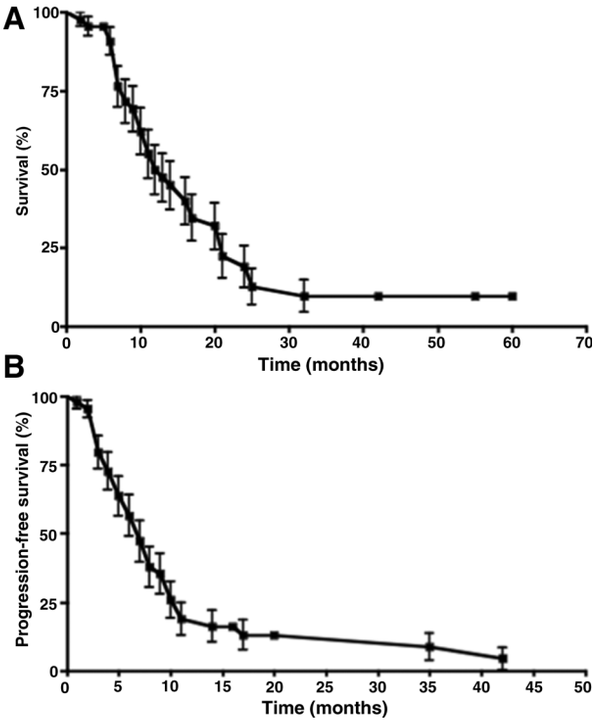

Figure 4.
Kaplan–Meier curve of PFS (**A**) and OS (**B**).

the approach. In addition, sometimes, in tumors located in close proximity to eloquent areas, a portion of fluorescent tissue could be left at the tumor margin if cortical or subcortical brain mapping shows positive responses.

HGG growth relies on the formation of new blood vessels that are characterized by increased permeability, with a consequent damage in the BBB (44–46). Fluorescein accumulation in the extracellular space of HGG tissue is related to the passage through this damaged BBB, due to the presence of high-density tumor cells (16). Thus, its accumulation in tumor tissue is not related to a specific uptake by tumor cells. This nonspecific mechanism of action could theoretically lead to a reduced accuracy in terms of tumor identification, as hypothesized by some authors (47). Nevertheless, we performed 50 biopsies (26 in fluorescent tissue and 24 in nonfluorescent tissue) at the tumor margin in 13 patients, obtaining a sensitivity and specificity in tumor identification of respectively 80.8% and 79.1%, and a PPV and NPV of, respectively, 80.8% and 79.1%. These data are similar to that obtained by Murray and colleagues, who systematically studied SF accuracy in tumor identification, performing 186 biopsies in 23 patients with brain malignancies, and obtaining a sensitivity of 96% and a specificity of 81% (48). Furthermore, it has been recently demonstrated that intraoperative fluorescein staining correlated with histologic alteration both in contrast enhanced and in noncontrast enhanced regions, with a sensitivity of 75.6% and a specificity of 75% (41). We are aware that sensitivity, specificity, PPV, and NPV measured in our samples, and in other similar studies, are biased by the conditionality of surgical samples, especially in the peritumoral "healthy" tissue. In our cohort of patients, fluorescein-negative biopsies were only included in patients with tumor located in non-eloquent areas, and all biopsies were performed at the tumor margin after main tumor debulking. However, our data, in combination with similar already published results, seems to confirm that fluorescein, independently from its nonspecific mechanism of action, is a reliable marker of glioma pathology. This could be also dependent on the strict protocol of SF injection applied in our study, with low dosage (5 mg/kg when Y560 filter was available) injected about 1 hour before dural opening. This allows accumulation of the molecule into the tumor mass and a good discrimination with brain parenchyma. The acute SF injection (in bolus or immediately before dural opening), used by some authors (47), is always associated with an intense fluorescence in the normal brain tissue due to perfusion of small capillary vessels, making any discrimination between tumoral and peritumoral tissue very difficult (49). The accuracy in tumor tissue identification is further strengthened by the absence of statistically significant deterioration in NIHSS and KPS for lesions located in non-eloquent areas and by the slight decline in the same scales observed in the immediate postoperative period for grade II–III lesions, that returned to baseline level at 3 months post-op, similarly to what can be observed in other series of patients with tumors in eloquent areas, without the use of fluorescein-guided technique (26, 50).

Patients enrolled in the FLUOGLIO study were followed up for a median of 11 months, with a median PFS of 7 months and a median survival of 12 months. Data about survival should be evaluated in the context of the fact that our study was neither designed nor powered to show any increase in long-term endopoints such as PFS and survival. Furthermore, it should be considered that only 20% of the patients were able to complete the full Stupp protocol. Moreover, only 24% of the patients had a methylated MGMT promoter, and none presented with mutated IDH1. Since MGMT methylation signature and IDH status have a relevant effect on disease outcome (51–53), their status in our cohort of patients could have been one of the most important determinant of survival. Unfortunately, no analysis could be performed on the effect of EOR on survival, because only a small subgroup of patients (eight cases) was submitted to incomplete resection.

Conclusions

The results of this phase II trial showed that fluorescein-guided removal of HGGs with a dedicated filter on the surgical microscope is feasible, safe, and is associated with a high percentage of complete resection. These data represent the prerequisite to build up a proper prospective randomized controlled phase III trial to definitely confirm the advantage of this technique in terms of EOR and possibly PFS and survival over a control cohort of patients.

Disclosure of Potential Conflicts of Interest

Francesco Acerbi and Karl-Michael Schebesch report receiving speakers bureau honoraria from Carl Zeiss Meditec. No potential conflicts of interest were disclosed by the other authors.

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