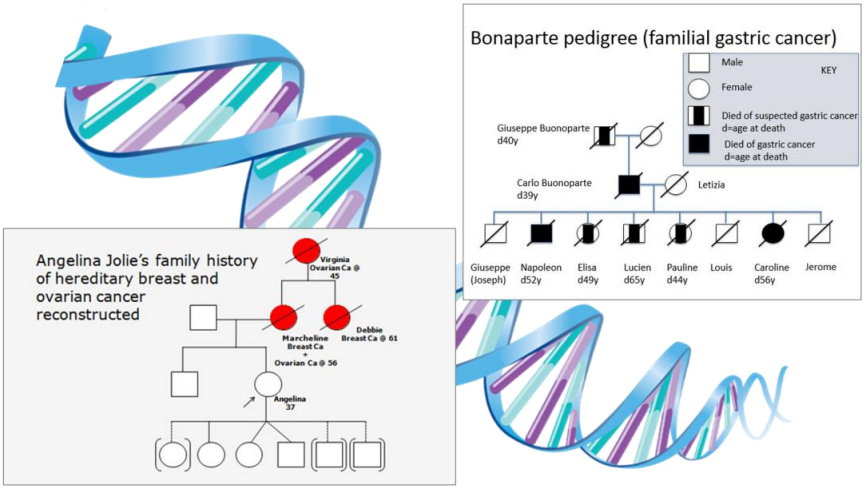
UNIVERSITA' DEGLI STUDI DI PAVIA

Dipartimento di Biologia e Biotecnologie

"L. Spallanzani"

A Next-Generation Sequencing approach for the study of hereditary tumors



Gianluca Tedaldi

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXI – A.A. 2015-2018

UNIVERSITA' DEGLI STUDI DI PAVIA

Dipartimento di Biologia e Biotecnologie

"L. Spallanzani"

A Next-Generation Sequencing approach for the study of hereditary tumors

Gianluca Tedaldi

Supervised by Prof. Guglielmina Nadia Ranzani

and Dr. Daniele Calistri

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXI – A.A. 2015-2018

Table of contents

Part one

Germline alterations in hereditary gastric cancer

Abstract

The main gene implicated in gastric cancer (GC) predisposition is *CDH1*, the variants of which have been linked to diffuse-type gastric cancer (DGC) and lobular breast cancer (LBC). *CDH1* only explains a fraction of patients with suspected hereditary cancers: detection rate of pathogenic variants ranges from 10% to 50% in different areas, with countries (like Italy) with medium-high incidence of GC showing the lowest values. In this scenario, multigene panels (MGPs) offer the most comprehensive testing to improve the identification of patients at risk of GC, as well as of other cancers.

We searched for germline pathogenic variants in GC or LBC patients with early-onset of the disease and/or family history of GC. In particular, we analyzed 94 genes associated with predisposition towards common and rare cancers in a cohort of 96 Italian patients mostly selected according to internationally established consensus criteria for the HDGC (Hereditary Diffuse Gastric Cancer) syndrome.

We found *CDH1* pathogenic variants in 10 out of 96 patients (10.4%). Eleven other patients (11.5%) proved to carry loss-of-function variants in a series of genes, including: *BRCA1*, *BRCA2*, *PALB2*, *ATM*, *MSH2*, *PMS2*, *BMPR1A*, *PRF1* and *BLM*. In 75 patients (78.1%), we did not find any variants with clear-cut clinical relevance: in 28/75 cases we identified rare missense variants (frequencies <1%) predicted to be probably-damaging by two bioinformatic tools.

Although additional studies are required to evaluate the penetrance of some identified variants and to confirm their role in GC risk, MGP testing allowed us to decrease the number of *CDH1* variant-negative patients that would've otherwise remained totally unexplained. In addition, MGP results indicate that, besides *CDH1*, rare variants associated with GC are distributed across many different cancer genes, including those that predispose to breast cancer, thus presenting a challenge for appropriate surveillance in variant carriers.

In addition to MGP testing, we performed a methylation analysis of *CDH1* promoter and enhancer sequences to deeply understand the mechanisms that regulate gene expression. The analysis we performed on cell lines derived from GC allowed us to identify specific sequences the methylation of which represents an important mechanism for *CDH1* regulation. This result provides the rationale for investigating promoter/enhancers methylation in patients with suspected genetic predisposition to GC, proven to be *CDH1*-mutation negative by DNA sequencing testing.

Abbreviations

ACMG: American College of Medical Genetics
BBC: bilateral breast cancer
BC: breast cancer
BQSR: Base Quality Score Recalibration
CA19-9: carbohydrate antigen 19-9
CEA: carcinoembryonic antigen
CIN: chromosome instability
CRC: colorectal cancer
CS: Cowden syndrome
D: damaging
DBC: ductal breast cancer
DGC: diffuse-type gastric cancer
EBV: Epstein-Barr virus
EC: extracellular domain
EGC: early gastric cancer
EGD: esophagogastroduodenoscopy
FAP: Familial Adenomatous Polyposis
FFPE: formalin-fixed paraffin-embedded
FGC: familial gastric cancer
FIGC: Familial Intestinal Gastric Cancer
GAPPS: Gastric Adenocarcinoma and Proximal Polyposis of the Stomach
GC: gastric cancer
GP: gastric polyposis
GS: genomically stable
HBOC: Hereditary Breast and Ovarian Cancer
HDGC: Hereditary Diffuse Gastric Cancer
HGC: hereditary gastric cancer
HNPCC: Hereditary nonpolyposis colorectal cancer
HR: homologous recombination
IARC: International Agency for Research on Cancer
IGC: intestinal-type gastric cancer
IGCLC: International Gastric Cancer Linkage Consortium
JPS: juvenile polyposis syndrome
LBC: lobular breast cancer
LFS: Li-Fraumeni syndrome
LOH: loss of heterozygosity
LS: Lynch syndrome
MAP: MUTYH-associated polyposis
MGP: Multi-gene panel

MLPA: Multiplex Ligation-dependent Probe Amplification
MMR: mismatch repair
MSI: microsatellite instability
NGS: Next-Generation Sequencing
OC: ovarian cancer
PCR: polymerase chain reaction
PDB: Protein Data Bank
PJS: Peutz-Jeghers syndrome
SGC: sporadic gastric cancer
SRCC: signet ring cell carcinoma
TCGA: The Cancer Genome Atlas
TNM: tumor, node, metastasis
TSS: transcription start site
VUS: variant of uncertain significance
WES: whole-exome sequencing
WGS: whole-genome sequencing
WHO: World Health Organization

1. Introduction

1.1 Epidemiology of gastric cancer

In 2016 gastric cancer (GC) globally ranked fifth for cancer incidence and second for cancer deaths, with 1.157.000 incident cases and 834.000 deaths having occurred [Global Burden of Disease Cancer Collaboration, 2018]. GC incidence is twice as high in men than in women and varies widely across countries (Fig. 1-2). Incidence rates are higher in Eastern Asia, Central and Eastern Europe, and South America and lower in Northern America and most parts of Africa [Torre LA *et al.* 2015].

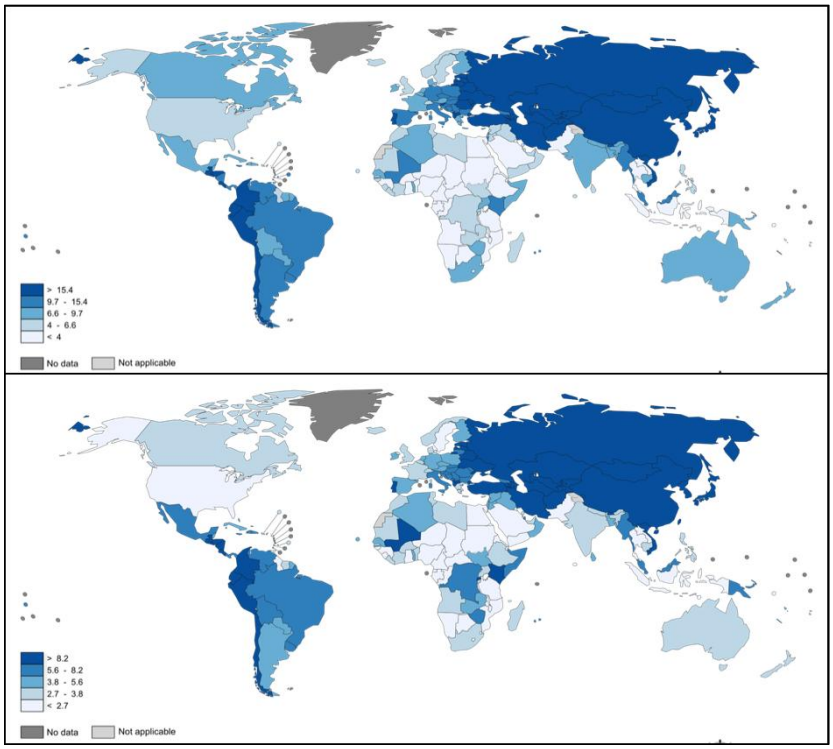


Fig. 1. Estimated GC incidence worldwide in 2012 among men (upper panel) and women (lower panel); the age-standardised rates are estimated per 100.000 individuals [Ferlay J *et al.* 2013]

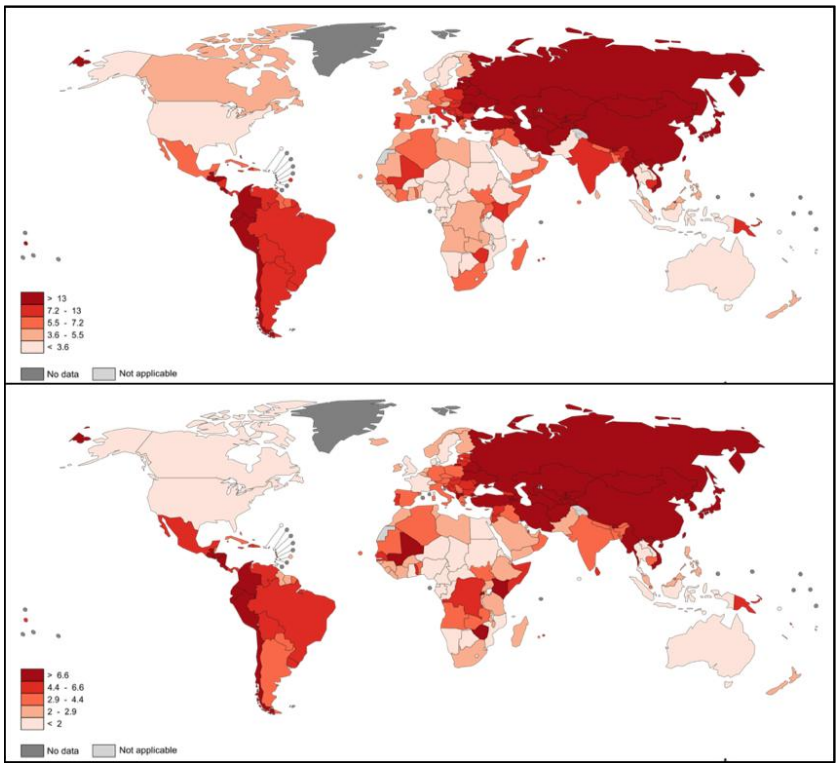


Fig. 2. Estimated GC mortality worldwide in 2012 among men (upper panel) and women (lower panel); the age-standardised rates are estimated per 100.000 individuals [Ferlay J *et al.* 2013]

In Europe, despite a steady decline in the incidence, 133.000 new GC cases have been estimated for 2018, with 102.000 deaths for the disease, placing GC at the eighth position for incidence and at the sixth position for mortality [Ferlay J *et al.* 2018].
In Italy, 12.800 new cases of GC have been estimated for 2017 and 9.557 deaths for the disease have occurred in 2014 [AIOM-AIRTUM 2017], placing GC as the ninth cancer for incidence, and the sixth for mortality. In Italy GC incidence and mortality are decreasing (-3% per year and -3.5% per year, respectively), but there is a high geographical variability. The regions of central Italy have a high GC incidence (22 cases/100.000 men and 13 cases/100.000 women), the regions of northern Italy have an intermediate GC incidence (22 cases/100.000 men and 11 cases/100.000 women), and the regions of southern Italy have a low GC incidence (16 cases/100.000 men and 7 cases/100.000 women). The mortality for GC in the different Italian regions is in accordance with the incidence rates.

1.2 Risk factors for gastric cancer

GC is a multifactorial disease associated with both genetic and environmental factors. The main risk factors include [Zali H *et al.* 2011; Ma K *et al.* 2017]:

- age: risk increases with age as a result of accumulation of DNA damages (somatic mutations) in proliferating epithelial cells;
- unhealthy diet: high consumption of smoked foods, salted fish, meat and pickled vegetables, and low consumption of fruit and fresh vegetables;
- Helicobacter pylori infection: chronic infection is the strongest identified risk factor, with about 90% of new cases of non-cardia GC worldwide attributed to this bacterium;
- obesity and lack of exercise: a sedentary lifestyle, often associated to overweight;
- tobacco smoking: cigarette smoke contains potent carcinogens and active and passive exposure to smoke increases the risk;
- alcohol consumption: alcohol drinking can increase the risk due to the primary metabolites, acetaldehydes, that have a local toxic effect;
- precancerous lesions, including atrophic gastritis, intestinal metaplasia and dysplasia;
- previous gastric surgeries: stomach surgery can induce a decreased acid production and a chronic inflammation due to reflux of bile in the gastric remnant;
- exposure to carcinogens, including ionizing radiations and specific substances, such as asbestos, chromium and inorganic lead compounds;
- genetic predisposition: about 10% of cases show familial aggregation and 1-3% of cases can be considered hereditary.

1.3 Classification of gastric cancers

The human stomach can be divided into four sections, Cardia, Fundus, Body and Pylorus (Fig. 3).

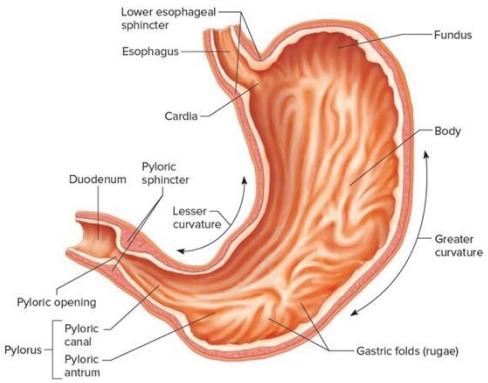


Fig. 3. Anatomy of the stomach

The gastric wall is composed by 4 layers, from the inside to outside (Fig. 4):

- **mucosa:** a mucous membrane layer of the stomach, which contains the glands and the gastric pits, constituted by a simple columnar epithelium, the lamina propria (thin layer of connective tissue) and the muscularis mucosae (thin layer of muscle);
- **submucosa:** a thin layer of dense irregular connective tissue that supports the mucosa;
- **muscularis externa:** a region of muscle adjacent to the submucosa;
- **serosa:** a smooth tissue membrane consisting of two layers of mesothelium, which secrete serous fluid and is separated from the muscularis externa by a thin layer of connective tissue (subserosa).

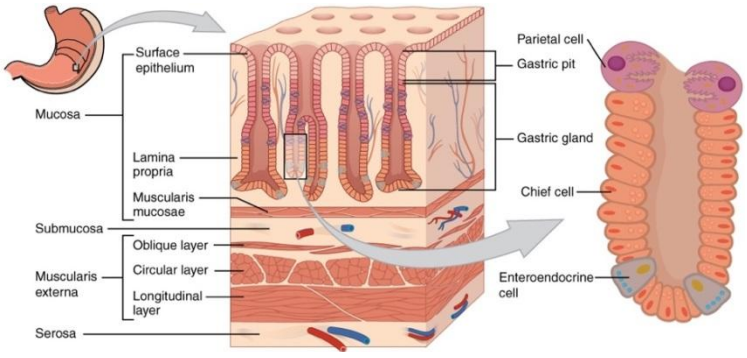


Fig. 4. Section of the gastric wall

There are different types of GC, depending on the tissue of origin:

- **adenocarcinomas (**90-95% of cases)**:** they originate from the glandular epithelium of the gastric mucosa;
- **lymphomas** (5% of cases): they originate from the lymphoid tissue;
- **stromal tumors** (2% of cases): they originate from the connective tissue;
- **carcinoid tumors** (1% of cases): they originate from the neuroendocrine system.

Given the rarity of lymphomas, stromal and carcinoid tumors, in this thesis gastric cancer (GC) refers to adenocarcinoma.

1.3.1 Histopathological classification of gastric cancers

The histopathological classification of GC has evolved over time generating partially homogeneous systems about which there is no general consensus [Hu B *et al.* 2012].
Regarding the macroscopic appearance of the tumor, the most commonly used system is the Borrmann classification [Borrmann R 1926] that divides GCs into 4 different types: I) polypoid; II) fungating, ulcerated with sharp raised margins; III) ulcerated with poorly defined infiltrative margins; IV) infiltrative, predominantly intramural lesion, poorly demarcated.
Regarding histology, the most common system is the Lauren classification [Lauren P 1965] which recognizes two main types of GC:

- **intestinal-type gastric cancer (IGC):** well-differentiated with cells describing irregular tubular structures with multiple lumens and reduced stroma (Fig. 5a);
- **diffuse-type gastric cancer (DGC):** poorly-differentiated with discohesive cells that produce mucin droplets which push the nucleus to the periphery, giving them a “signet ring” shape (Fig. 5b).

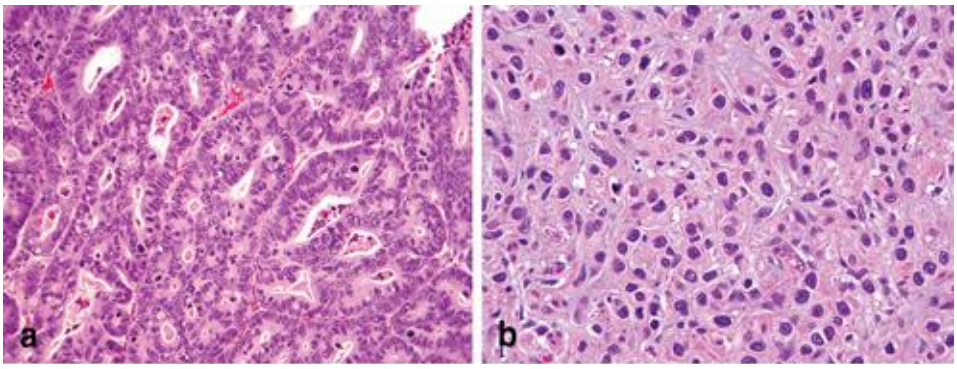


Fig. 5. a) Intestinal-type gastric cancer (IGC); b) Diffuse-type gastric cancer (DGC)

More recently, the World Health Organization (WHO) has developed a new classification [Bosman FT *et al.* 2010] to describe GC features in more detail:

- **tubular adenocarcinoma:** the cancer tends to form polypoid or fungating masses and is composed by fused or branching tubules of various sizes, often with intraluminal mucus, nuclear and inflammatory debris;
- **papillary adenocarcinoma:** the cancer grows outward from the stomach wall and is characterized by epithelial projections scaffolded by a central fibrovascular core;
- **mucinous adenocarcinoma:** the cancer cells form glandular architecture and irregular cell clusters and are characterized by extracellular mucinous pools in which occasional scattered signet ring cells are floating;
- **signet ring cell carcinoma and other poorly cohesive carcinomas:** these cancers are often composed of a mixture of signet ring cells and non-signet ring cells, that can form irregular microtrabeculae or lace-like abortive glands, often accompanied by marked desmoplasia in the gastric wall and with a depressed or ulcerated surface;
- **mixed carcinoma:** the cancer is a mix of different types of adenocarcinoma.

In addition to these subtypes, WHO classification also considers a series of rare histologic variants (Fig. 6).

WHO (2010)	Lauren (1965)
Papillary adenocarcinoma Tubular adenocarcinoma Mucinous adenocarcinoma	Intestinal type
Signet-ring cell carcinoma And other poorly cohesive carcinoma	Diffuse type
Mixed carcinoma	Indeterminate type
Adenosquamous carcinoma	
Squamous cell carcinoma	
Hepatoid adenocarcinoma	
Carcinoma with lymphoid stroma	
Choriocarcinoma	
Carcinosarcoma	
Parietal cell carcinoma	
Malignant rhabdoid tumor	
Mucoepidermoid carcinoma	
Paneth cell carcinoma	
Undifferentiated carcinoma	
Mixed adeno-neuroendocrine carcinoma	
Endodermal sinus tumor	
Embryonal carcinoma	
Pure gastric yolk sac tumor	
Oncocytic adenocarcinoma	

Fig. 6. WHO and Lauren classifications of GCs [Hu B *et al.* 2012]

1.3.2 Molecular classification of gastric cancers

During time, specific molecular approaches have been utilized to investigate genetic lesions in GC and different genes have been found to contribute to the carcinogenic process. More recently, comprehensive approaches have been applied to deeply understand this heterogeneous disease. In 2014 Wang and collaborators performed whole-genome sequencing in GC tumor and paired normal samples, along with DNA copy number, gene expression and methylation profiling, and identified subtype-specific genetic and epigenetic lesions and unique mutational signatures. The results of this study are summarized in Fig. 7 which shows Circos plots with specific genetic and epigenetic lesions characterizing the different GC molecular subtypes: GCs with chromosomal instability and without microsatellite instability (MSS) show extensive DNA demethylation; GCs with microsatellite instability (MSI) are chromosomal stable with extensive hypermethylation and demethylation and a large number of somatic single nucleotide variants (SNVs); Epstein Barr virus (EBV)-associated GCs are chromosomal stable with extensive hypermethylation.

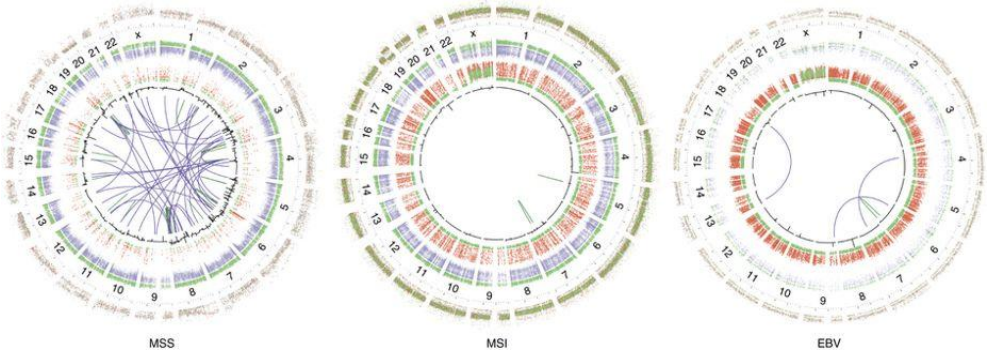


Fig.7. Genetic and epigenetic lesions in MSS, MSI and EBV GCs. The inner circle denotes somatic copy number change and chromosomal translocation; in the second circle, red dots denote the values of hypermethylated loci in tumors, and green dots denote the corresponding values in normal samples; in the third circle, blue dots denote the values of demethylated loci in tumors, and green dots denote the corresponding values in normal samples; and in the outer circle, each dot denotes one somatic SNV, colored according to the different mutation types [Wang K *et al.* 2014]

Moreover, Wang and collaborators found frequent mutations in previously known (*TP53*, *ARID1A* and *CDH1*) and in new (*MUC6*, *CTNNA2*, *GLI3*, *RNF43* and others) "driver" genes, being *RHOA* gene mutated in 14.3% of diffuse-type tumors but not in intestinal-type tumors ($P < 0.001$).
In 2014 "The Cancer Genome Atlas" (TCGA), a project to catalogue genetic mutations in cancer, published the molecular characterization of 295 primary gastric adenocarcinomas, performed with different approaches (whole exome sequencing, array-based somatic copy number analysis, array-based DNA methylation profiling, messenger RNA sequencing, microRNA sequencing and reverse-phase protein array) [Cancer Genome Atlas Research Network 2014]. This comprehensive work identified 4 major GC genomic subtypes (Fig. 8):

- **tumors with EBV-infection:** these tumors (9% of GCs) are characterized by high Epsterin-Barr virus (EBV) burden, extensive gene promoter hypermethylation in the context of a specific CpG island methylator phenotype (CIMP) and the presence of activating *PIK3CA* mutations;
- **tumors with microsatellite instability (MSI):** these tumors (22% of GCs) are characterized by microsatellite instability (MSI), high mutation rates and hypermethylation at the *MLH1* gene promoter in the context of a specific CIMP;

- **tumors with chromosomal instability (CIN):** these tumors (50% of GCs) are associated to the intestinal histology and are characterized by high degree of aneuploidy and chromosomal instability (CIN) and by high frequency of *TP53* gene mutations;
- **genomically stable (GS) tumors:** these tumors (20% of GCs) are associated to the diffuse histology and are characterized by the absence of aneuploidy, hypermethylation and hypermutation, and by the presence of somatic mutations in *CDH1* and *RHOA* genes.

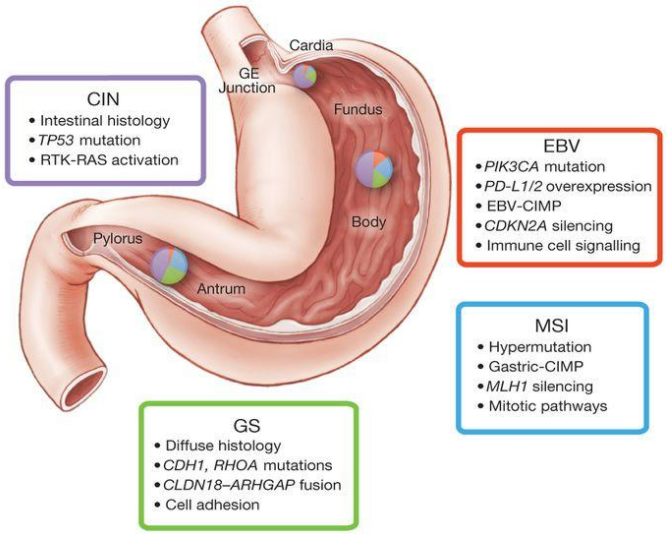


Fig. 8. Main features associated with each of the four molecular subtypes of GC; insert pie-charts show the relative distribution of molecular patterns with respect to cancer localization [Cancer Genome Atlas Research Network 2014]

Of relevance, heterogeneous molecular mechanisms leading to "driver" alterations have been found, including: gene mutations, somatic copy number alterations (sCNAs), structural variants, epigenetic changes, and transcriptional changes involving mRNAs and noncoding RNAs (ncRNAs) (Fig. 9) [Tan P and Yeoh KG 2015].

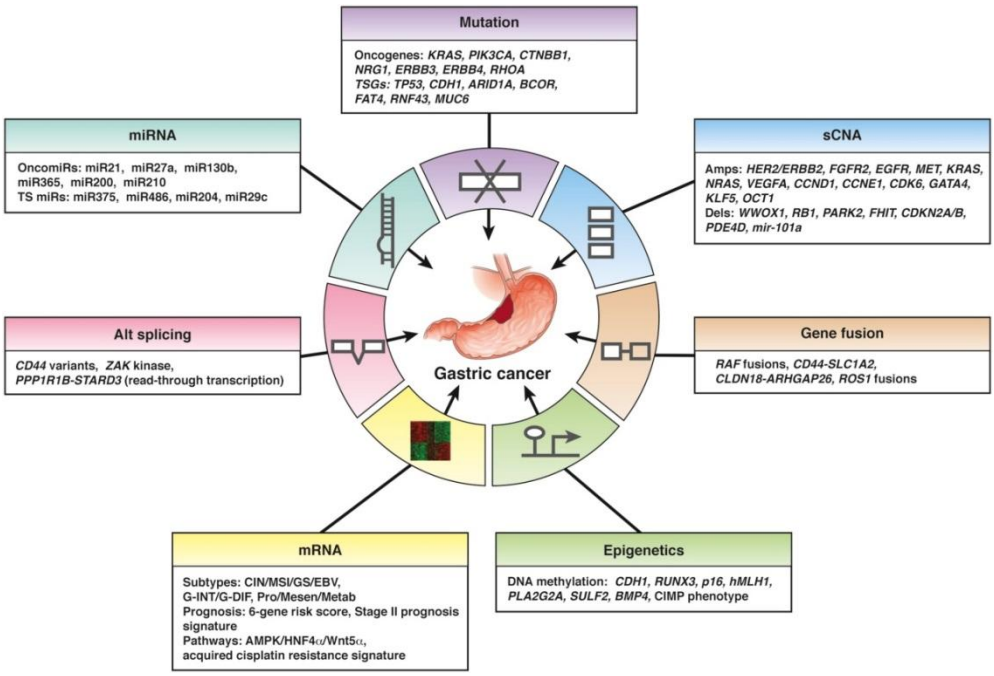


Fig. 9. Genetic and epigenetic mechanisms leading to "driver" alterations in GCs [Tan P and Yeoh KG 2015]

Although results derived from multi-omics approaches to GC have an intrinsic value and are of invaluable relevance for understanding GC development, at present they do not have impact on clinical practice. In particular, therapeutic strategies remain to be developed to target specific GC subtypes, based upon their "driver" alterations. The next years will likely see a further explosion of studies, which will be essential to beat this deadly disease.

1.4 Clinical manifestations and diagnosis of gastric cancer

In early stages, GC is often asymptomatic, or it manifests with symptoms consistent with gastritis or gastric ulcer such as nausea, vomiting, diarrhea, constipation, loss of appetite, and abdominal pain. If treatments for gastritis or ulcer have no positive effect, gastroscopy can confirm (or rule out) suspected conditions, including cancer. More severe symptoms, such as vomiting with blood or sudden weight loss, can occur when the disease has already reached an advanced stage. Advanced GCs can also generate symptoms of their spread to distant sites.

Clinical examination allows detection of some GC symptoms only, while esophagogastroduodenoscopy (EGD) is the gold standard diagnostic exam. EGD can detect cancerous lesions in the gastric mucosa, directly allowing their bioptic sampling; a conclusive diagnosis is then reached through histologic examination of the bioptic material. Moreover, endoscopic ultrasound can be performed to evaluate cancer penetration of the gastric wall. Computed tomography (CT) scan, positron-emission tomography (PET), magnetic resonance imaging (MRI) and radiological investigation of the abdomen are additional methods to evaluate the cancer spread in the nearby tissues and the possible presence of metastases.
Blood tests can be performed to detect tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), an increase of which is detectable in 50% and 20% of GC cases, respectively. Accordingly, these markers are not particularly useful in GC diagnosis; on the contrary, they acquire a more important role during follow-up, when their increase is highly predictive of disease relapse.

1.5 Evolution of gastric cancer

During development, GC tends to invade the gastric wall and then spread to regional lymph nodes, finally generating metastases in distant organs. The most common sites of GC metastases are liver (48%), peritoneum (32%), lung (15%), and bones (12%) [Riihimäki M *et al.* 2016].
The TNM (tumor, node, metastasis) tumors classification is a globally recognized standard to measure the extent of cancer spread [Brierley JD *et al.* 2017].
TNM is based on the combination of three factors:

- **T (tumor)** describes the size of the primary tumor and the invasion of nearby tissues (Fig. 10):
    - T0: no evidence of primary tumor;
    - Tis: cancer cells are growing in the epithelium without infiltrating the basement membrane (carcinoma *in situ*);
    - T1: the tumor invades the lamina propria or the muscularis mucosae (T1a) or invades the submucosa (T1b); it is commonly called early gastric cancer (EGC);
    - T2: the tumor invades the muscularis externa;
    - T3: the tumor invades the subserosa;
    - T4: the tumor invades the sierosa (T4a) or the nearby

structures (T4b);
- TX: size and invasion of the primary tumor cannot be assessed.

- **N (node)** describes the involvement of the regional lymph nodes:

  - N0: absence of metastases in the regional lymph nodes;
  - N1: presence of metastases in 1-2 regional lymph nodes;
  - N2: presence of metastases in 3-6 regional lymph nodes;
  - N3: presence of metastases in 7-15 regional lymph nodes (N3a) or in more than 15 regional lymph nodes (N3b);
  - NX: the presence of metastases in the regional lymph nodes cannot be assessed.

- **M (metastasis)** describes the presence of distant metastases:

  - M0: absence of distant metastases;
  - M1: presence of distant metastases;
  - MX: the presence of metastases cannot be assessed.

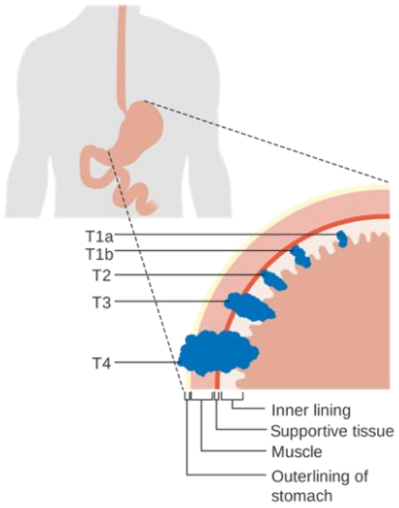


Fig. 10. GC classification according to size in the TNM system

Cancer staging is a GC classification based on size and spread of cancer:

- **stage 0:** there are abnormal cells in the stomach lining, but they're completely contained within the innermost layer of the lining (Tis, N0, M0);

- **stage IA:** the cancer has grown no further than the lining of the stomach; there is no cancer in the lymph nodes (T1, N0, M0);

- **stage IB:**
  - the cancer is still within the stomach lining, but there are cancer cells in 1 or 2 nearby lymph nodes (T1, N1, M0);
  - there are no cancer cells in the lymph nodes, but the cancer has grown into the muscle of the stomach wall (T2, N0, M0);

- **stage IIA:**
  - the cancer is still within the lining of the stomach, but between 3 and 6 nearby lymph nodes contain cancer cells (T1, N2, M0);
  - the cancer has grown into the muscle layer of the stomach wall and is also in 1 or 2 nearby lymph nodes (T2, N1, M0);
  - the cancer has grown into the outer layers of the stomach but there are no cancer cells in the lymph nodes (T3, N0, M0);

- **stage IIB:**
  - the cancer is within the lining of the stomach wall, but 7 or more lymph nodes contain cancer cells (T1, N3, M0);
  - the cancer has grown into the muscle layer of the stomach and 3 to 6 lymph nodes contain cancer cells (T2, N2, M0);
  - the cancer has grown into the outer layer of the stomach and is also in 1 or 2 nearby lymph nodes (T3, N1, M0);
  - the cancer has grown through the outer lining but there are no cancer cells in nearby lymph nodes (T4a, N0, M0);

- **stage IIIA:**
  - the cancer has either grown into the muscle layer of the stomach and 7 or more nearby lymph nodes contain cancer cells (T2, N3, M0);
  - the cancer has grown into the outer lining of the stomach and 3 to 6 nearby lymph nodes contain cancer cells (T3, N2, M0);

- the cancer has grown through the stomach wall and 1 to 2 nearby lymph nodes contain cancer cells (T4a, N1, M0);

- **stage IIIB:**
  - the cancer has grown into the outer lining of the stomach and more than 7 nearby lymph nodes contain cancer cells (T3, N3, M0);
  - the cancer has grown through the stomach wall and between 3 and 6 lymph nodes contain cancer (T4a, N2, M0);
  - the cancer has grown through the stomach wall into nearby tissues and organs, and the nearby lymph nodes either don't contain cancer, or up to 2 lymph nodes contain cancer (T4b, N0-N1, M0);

- **stage IIIC:**
  - the cancer has either grown through the stomach wall and more than 7 nearby lymph nodes contain cancer cells (T4a, N3, M0);
  - the cancer has grown through the stomach wall into nearby tissues and organs, and 3 to 7 lymph nodes contain cancer cells (T4b, N2-3, M0);

- **stage IV:** cancer is advanced and has spread to organs further away from the stomach, such as lungs, brain or bones (any T, any N, M1).

The correspondence between Cancer Staging and TNM classification is reported in the following table:

STAGE	TNM
Stage 0	Tis N0 M0
Stage IA	T1 N0 M0
Stage IB	T2 N0 M0 T1 N1 M0
Stage IIA	T3 N0 M0 T2 N1 M0 T1 N2 M0
Stage IIB	T4a N0 M0 T3 N1 M0 T2 N2 M0 T1 N3 M0

Stage IIIA	T4a N1 M0 T3 N2 M0 T2 N3 M0
Stage IIIB	T4b N0-1 M0 T4a N2 M0 T3 N3 M0
Stage IIIC	T4a N3 M0 T4b N2-3 M0
Stage IV	any T any N M1

Tumor grading, also called Broder's classification, is a measure of cell differentiation degree. Accordingly, it is a measure of tumor aggressiveness, a parameter which is different from staging, a measure of tumor spread.
The most used system includes 4 possible grades:

- **G1:** well differentiated (low grade) with less than 25% undifferentiated cells;
- **G2:** moderately differentiated (intermediate grade) with less than 50% undifferentiated cells;
- **G3:** poorly differentiated (high grade) with 50-75% undifferentiated cells;
- **G4:** undifferentiated (anaplastic) with more than 75% undifferentiated cells;
- **GX:** the grade cannot be assessed.

Tumor grading, together with cancer staging, are used to evaluate each cancer patient, in order to optimize the treatment strategy and predict prognosis.

1.6 Treatment of gastric cancer

The choice of treatments depends on tumor stage and on general conditions of patients. When feasible, surgical treatment is the preferred option and it often represents the only possibility of healing. Endoscopic resection can be used only to treat cancers at a very early-stage, where the risk of spread to the lymph nodes is very low. For tumors localized in the upper and central part of the stomach the surgery usually consists in the removal of the whole organ (total gastrectomy), while for tumors localized in the lower part of the stomach only a part of the organ is removed (subtotal gastrectomy). In

association with gastrectomy, lymphoadenectomy (removal of regional lymph nodes) is currently performed.
Prophylactic gastrectomy is the removal of the entire stomach in the absence of cancer diagnosis and is strongly recommended for individuals at high risk of GC, such as *CDH1* gene mutation carriers.
Chemotherapy is performed when cancer is at an advanced stage, to selectively destroy cancer cells that have already given rise to metastases: neoadjuvant treatment (before radical surgery) is performed to reduce the tumor size, while adjuvant treatment (after radical surgery) is performed to avoid relapse and metastases. Unfortunately, stomach cancer has shown little sensitivity to available chemotherapeutic agents; consequently, chemotherapy is often used as a palliative, to reduce the tumor size and to prolong patient survival. Among the utilized drugs, only Trastuzumab and Ramucirumab are approved targeted therapies, targeting HER2 protooncogene and VEGFR2 angiogenic factor, respectively [Apicella M *et al.* 2017].
Radiotherapy is usually performed in combination with surgery and chemotherapy for palliative purposes.

1.7 Prognosis of gastric cancer

A complete healing depends on tumor staging and presence/localization of metastases, as well as on general conditions of the patient. As already mentioned, the main problem in GC treatment is a late diagnosis. Surgical treatment is effective in less than 50% of patients and, in case of metastases, it acts only as a palliative. The average survival rate 5 years after diagnosis is about 25%, but this is related to the cancer stage at the time of diagnosis. The percentage of survival after 5 years for non-surgically treated patients (about 18%) is minimal. The 5-year survival rates by stage for GCs treated with surgery are reported in the following table:

CANCER STAGE	5-YEAR SURVIVAL RATE
Stage 0	97%
Stage IA	94%
Stage IB	88%
Stage IIA	82%
Stage IIB	68%
Stage IIIA	54%
Stage IIIB	36%
Stage IIIC	18%
Stage IV	5%

1.8 Genetic classification of gastric cancer

From the genetic point of view, GC can be divided in three groups: sporadic, familial and hereditary (HDGC) (Fig. 11).

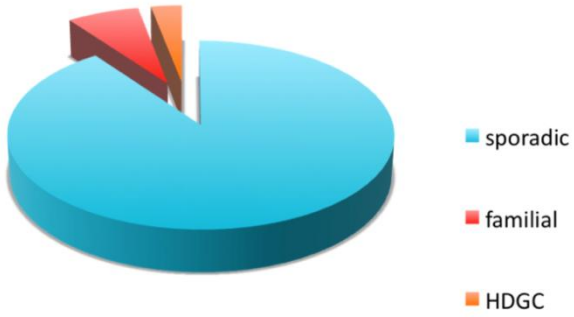


Fig. 11. Genetic classification of GC

- **Sporadic gastric cancer (SGC) - about 90% of all GC cases:** SGCs occur as isolated cases in families, without evidence of genetic predisposition factors. SGC usually occurs at an older age, is mainly of the intestinal-type and is related to environmental risk factors, including Helicobacter pylori infection, diet and smoking;

- **Familial gastric cancer (FGC) - about 10% of all GC cases:** more than one case of GC occurs in the same family, without a specific pattern of inheritance; familial clustering is likely due to the sharing of both genetic and environmental risk factors;

- **Hereditary gastric cancer (HGC) - about 1-3% of all GC cases:** several GC cases occur in the same family due to a germline mutation in a cancer predisposition gene; HGC usually occurs at a young age, is mainly of the diffuse-type and it can be associated with different predisposition genes.

To date, three syndromes associated with GC have been described:

- Hereditary Diffuse Gastric Cancer (HDGC);
- Familial Intestinal Gastric Cancer (FIGC);
- Gastric Adenocarcinoma and Proximal Polyposis of the Stomach (GAPPS).

1.8.1 Hereditary Diffuse Gastric Cancer (HDGC)

The major gene involved in GC predisposition is *CDH1* (OMIM *192090) [Berx G *et al.* 1995], the germline inactivating mutations of which are responsible for the Hereditary Diffuse Gastric Cancer syndrome (HDGC, OMIM #137215) [Kaurah P and Huntsman DG 2002].
CDH1 gene is located on chromosome 16q22.1, it covers around 100 kb and has a coding sequence of 2649 nucleotides distributed on 16 exons (Ensembl: ENSG00000039068). The 4.5 kb mRNA (RefSeq NM_004360) is translated in a 120 kDa protein, called E-cadherin (UniProt: P12830), which plays a key role in the formation of cell-cell junctions and in signal transduction pathways, regulating cell survival and differentiation (Fig. 12).

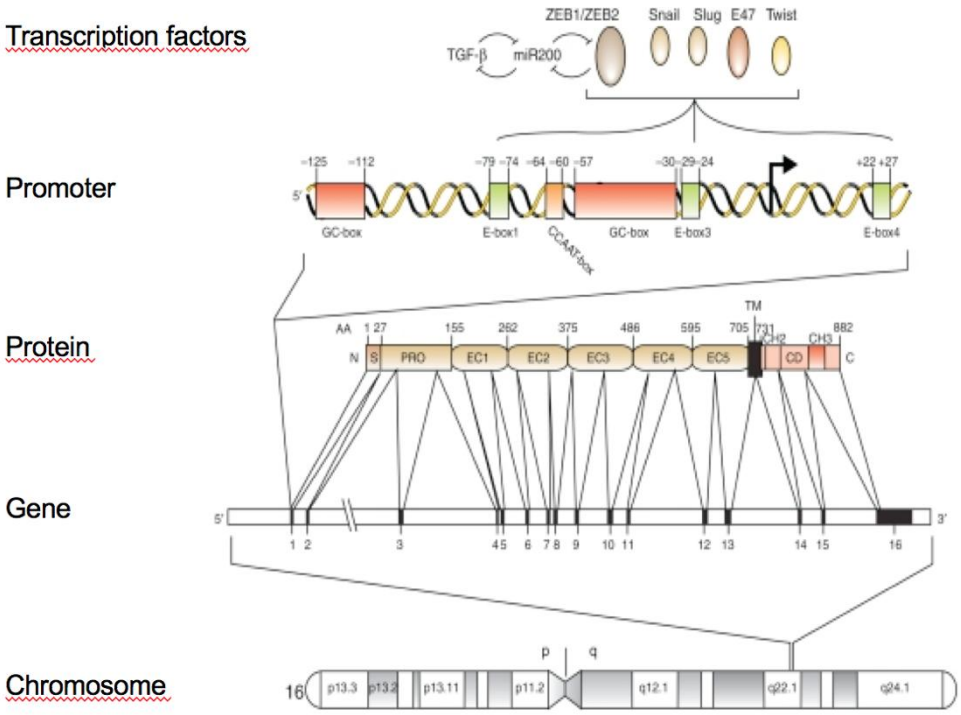


Fig. 12. Chromosomal localization of *CDH1* gene, E-cadherin protein and gene regulatory elements

E-cadherin is a homodimeric calcium-dependent transmembrane glycoprotein constituted by 882 amino acids forming different functional domains:

- a signal domain (amino acids 1-27);
- a precursor domain (amino acids 28-153);
- an extracellular portion with 5 extracellular (EC) domains binding 4 calcium ions (amino acids 154-708);
- a transmembrane domain (amino acids 709-731);
- a cytoplasmic domain with a highly phosphorylated region (amino acids 732-882).

The N-terminus of the extracellular domains of an E-cadherin dimer interacts with the N-terminus of another dimer on the membrane of the flanking cell (Fig. 13) [Canel M *et al.* 2013]. On the contrary, the C-terminus of the intracellular domains are associated with α-, β-, γ- and p120-catenins proteins to connect with the actin cytoskeleton (Fig. 14) [Kallakury BV *et al.* 2001].

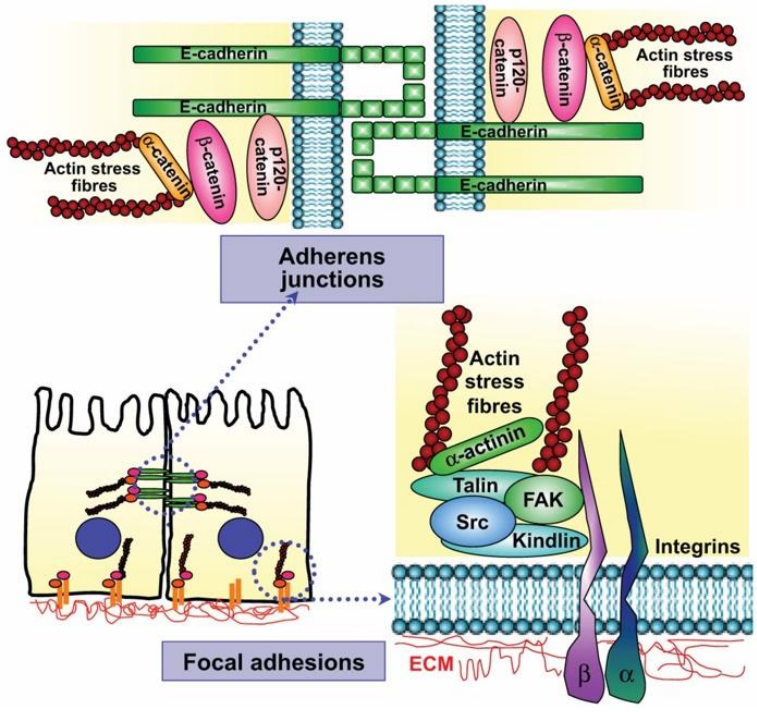


Fig.13. Schematic representation of E-cadherin-mediated cell–cell adherens junctions [Canel M *et al.* 2013]

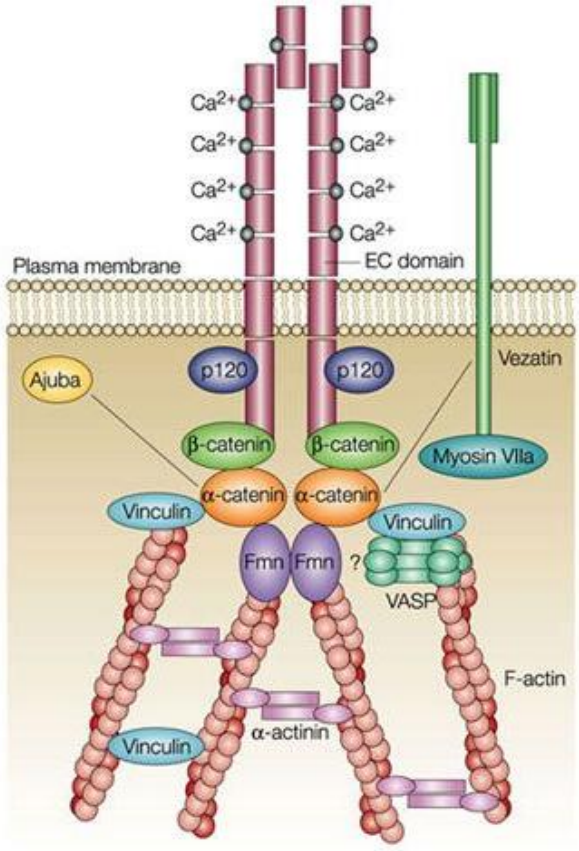


Fig. 14. Localization of E-cadherin and interactions with cellular proteins [Kobielak A and Fuchs E 2004]

The first *CDH1* germline mutations in GC were identified by linkage analysis in three Maori families from New Zeeland; Fig. 15 shows one of the families included in the study [Guilford P *et al.* 1998].

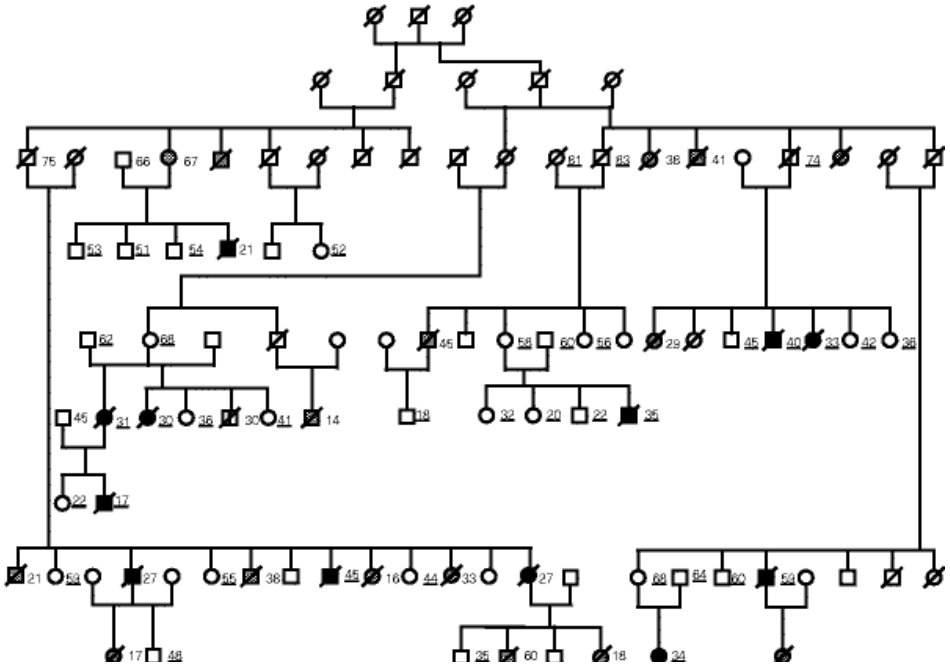


Fig. 15. Maori GC kindred. Dotted symbols indicate affected subjects; the individual's age is indicated to the right of the symbols. The pedigree pattern is consistent with the dominant inheritance of a susceptibility gene with incomplete penetrance [Guilford P *et al.* 1998]

To date, about 150 *CDH1* germline mutations have been reported, 80% of which are clearly pathogenic, while 20% remain of uncertain significance (VUS: Variants of Uncertain Significance) [Guilford P *et al.* 2010; Hansford S *et al.* 2015]. Although there are no major mutation hotspots, some mutations proved to be recurrent in unrelated families [Kaurah P *et al.* 2007]. The most common mutations are small insertions/deletions (35%) and missense mutations (28%); nonsense mutations (16%), splicing variants (16%) and large deletions (5%) [Oliveira C *et al.* 2009] have also been reported. Fig. 16 shows a schematic representation of *CDH1* mutations identified. Despite *CDH1* germline mutations are found in all ethnic groups, they are rare in countries with high GC incidence, such as Japan and Korea, suggesting that other genes and/or environmental factors can be responsible for GC familial clustering in those populations [Guilford P *et al.* 2010].

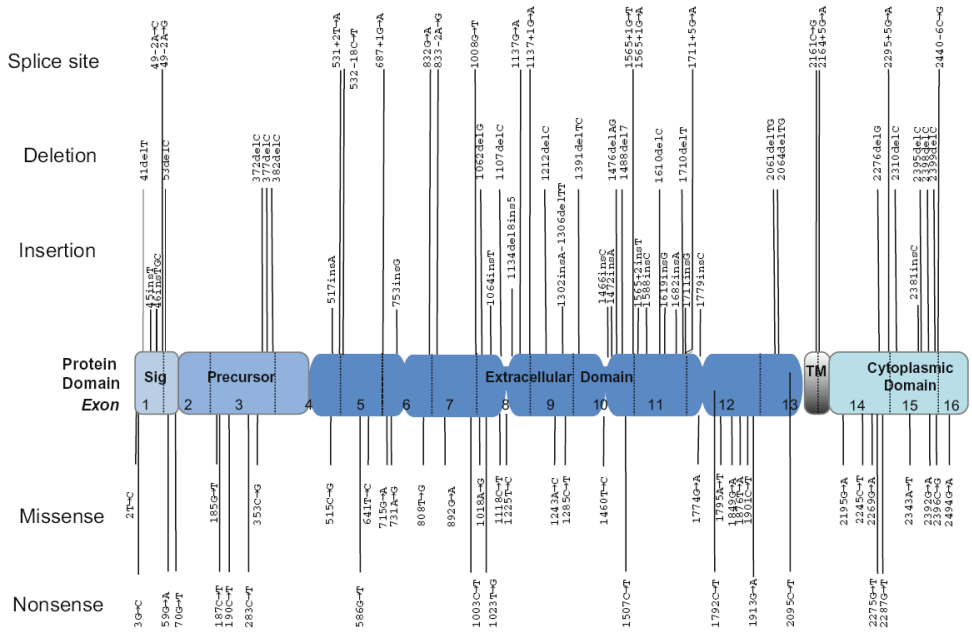


Fig. 16. Location and classification of *CDH1* mutations identified in HDGC families [Guilford P *et al.* 2010]

Selection criteria for genetic testing of patients are based on age of cancer onset and number of GC cases in first- and second-degree relatives. The International Gastric Cancer Linkage Consortium (IGCLC) established the following consensus criteria for selection of patients/families to be tested for germline defects [Caldas C *et al.* 1999; Brooks-Wilson AR *et al.* 2004; Fitzgerald RC *et al.* 2010; van der Post RS *et al.* 2015]:

- two or more GC cases regardless of age with at least one confirmed DGC;
- one case of DGC before 40 years;
- personal or family history of DGC and lobular breast cancer (LBC), one diagnosed before 50 years.

CDH1 testing should be also considered in subjects with [van der Post RS *et al.* 2015]:

- bilateral LBC or family history of 2 or more cases of LBC;
- a personal or family history of cleft lip/palate in addition to DGC;
- in situ signet ring cells and/or pagetoid spread of signet ring cells.

By using these criteria, *CDH1* mutation detection rate is 30-50% in countries with a low incidence of GC, but it decreases to 10-20% in countries with a high GC incidence, due to difficulties in discriminating hereditary cases from family clusters of sporadic cases.

CDH1 can be impaired by different types of lesions; accordingly, molecular methods utilized for genetic testing are relevant. Indeed, it has recently been demonstrated that complementary approaches, including DNA sequencing, Multiplex Ligation-dependent Probe Amplification (MLPA), single-nucleotide primer extension, bisulfite sequencing, reverse-transcription PCR, and bioinformatics tools, can significantly contribute to increase the mutation detection rate [Molinaro V *et al.* 2014].

CDH1-associated cancer predisposition is transmitted with an autosomal dominant pattern, with incomplete penetrance. In *CDH1* mutation carriers the cumulative risk of GC by the age of 80 is 70% for males and 56% for females. Furthermore, females have an increased risk of LBC, which is estimated to be 42% by the age of 80 years [Hansford S *et al.* 2015]. Due to the high penetrance of mutations and the early age of disease onset, prophylactic gastrectomy should be strongly advised in carriers of pathogenic mutations (Fig. 17).

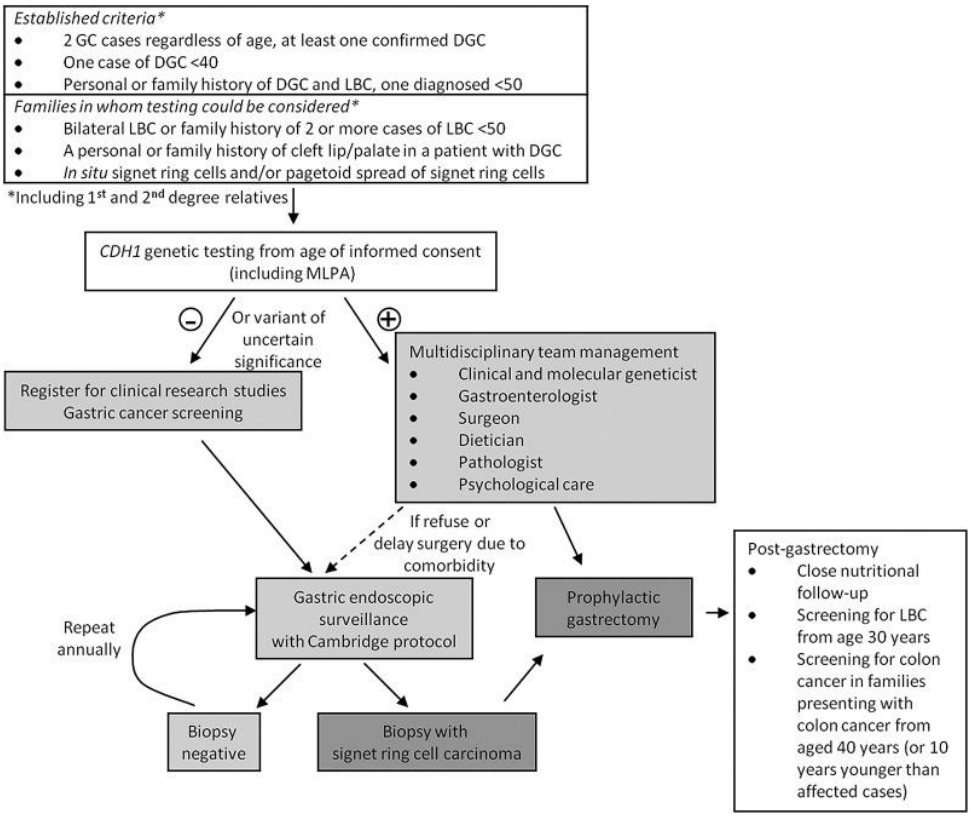


Fig. 17. Algorithm for HDGC management, including: clinical criteria for selection of patients, genetic testing, role of endoscopy and prophylactic gastrectomy [van der Post RS *et al.* 2015]

Of relevance, the analysis of prophylactic gastrectomy specimens always revealed the presence of multiple microscopic signet ring cell carcinomas (SRCC) (Fig. 18) [Huntsman DG *et al.* 2001; Charlton A *et al.* 2004; Guilford P *et al.* 2010; Molinaro V *et al.* 2014]. Mutation carriers in whom prophylactic gastrectomy is not feasible, should be offered an appropriate endoscopic surveillance, as well as mammography surveillance for women [van der Post RS *et al.* 2015].

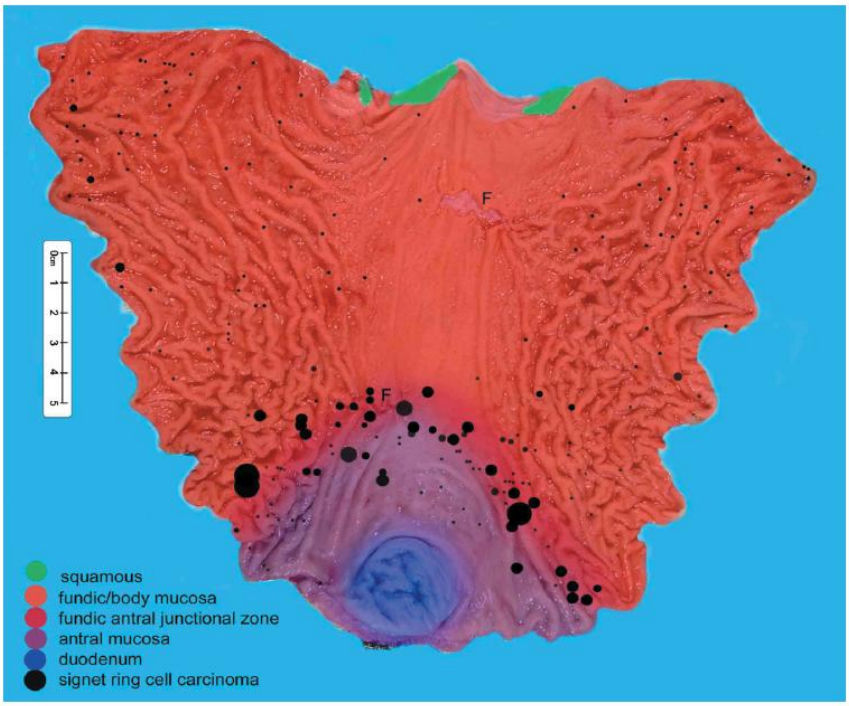


Fig. 18. Anatomical map of the stomach of a *CDH1* mutation carrier who underwent prophylactic gastrectomy: SRCC foci are represented by black spots [Guilford P *et al.* 2010]

CDH1 follows the so-called two-hit model (Knudson hypothesis) [Knudson AG Jr. 1971]: in HDGC patients, a first hit (inactivating one *CDH1* allele) occurs at the germline level, while the second one, occurring at the somatic level, is responsible for the inactivation of the wild-type allele in target cells. First and second hits cause the loss of E-cadherin protein function and its involvement in carcinogenic process [Guilford P *et al.* 1998] (Fig. 19). According to recent studies, the second allele is rarely inactivated by mutations or deletions (identifiable as loss of heterozygosity-LOH). On the contrary, epigenetic events appear to be frequent: indeed, *CDH1* gene silencing by promoter hypermethylation represents the main cause of inactivation of the wild type allele in HGDC cases. *CDH1* promoter hypermethylation has also been detected in most sporadic DGCs as cause of E-caherin impairment [Grady WM *et al.* 2000; Machado JC *et al.* 2001]. Globally, *CDH1* promoter hypermethylation is detectable in 50% of HGDC cases and in 40-80% of sporadic DGCs. Of relevance, this mechanism occurs less frequently in IGC cancers where cell-cell adhesion is commonly maintained or slightly impaired [To KF *et al.* 2002].

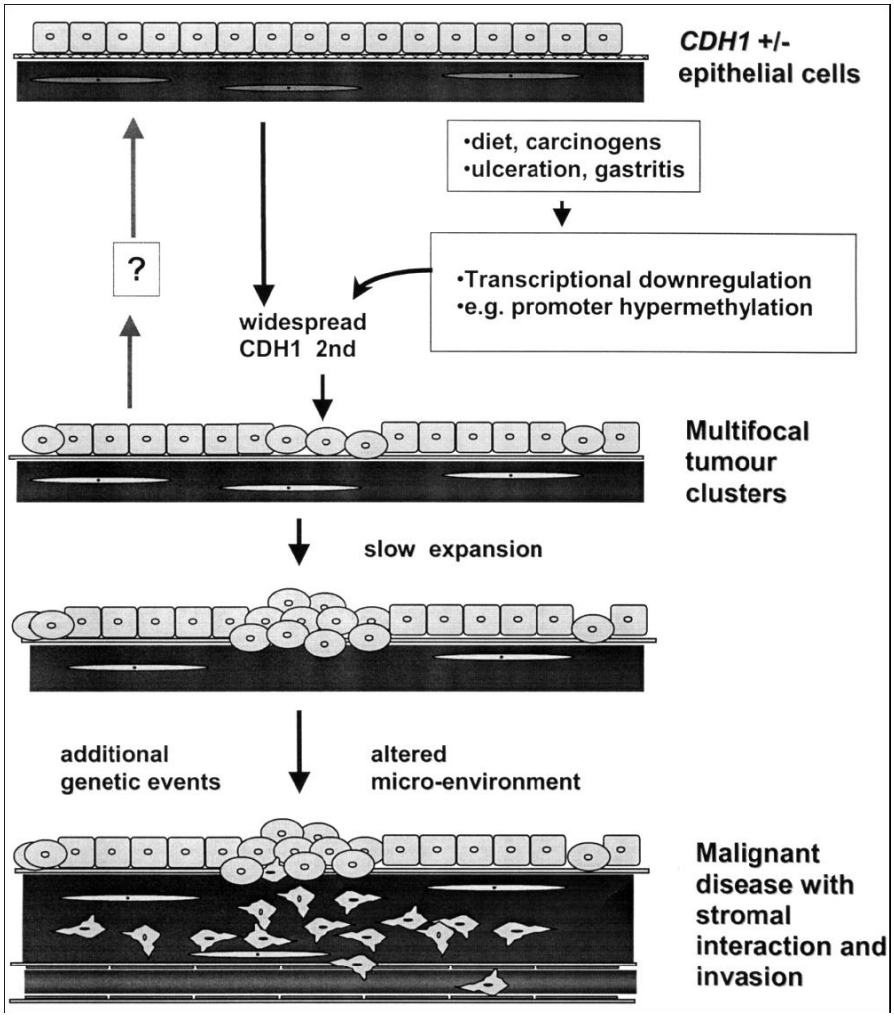


Fig. 19. Model for the development of HDG [Graziano F *et al.* 2003]

Given its role in downregulation of E-cadherin expression, *CDH1* promoter hypermethylation is considered as a possible target for new therapeutic strategies based on demethylating agents [Goffin J and Eisenhauer E 2002]. These strategies are not limited to GCs since epigenetic silencing of tumor suppressor genes due to hypermethylation of CpG sites along the promoter, represents a pivotal alteration in the development of different cancer types [Santini V *et al.* 2001].

1.8.2 Familial Intestinal Gastric Cancer (FIGC)

The counterpart of HDGC is the Familial Intestinal Gastric Cancer (FIGC), the selection criteria for which have been defined by the IGCLC depending on the GC incidence in the population [Caldas C *et al.* 1999; Oliveira C *et al.* 2004]. Countries with a high GC incidence, like Japan and Portugal, should use criteria analogous to those proposed for Lynch syndrome [Shinmura K *et al.* 1999]:

- at least three relatives should have IGC and one of them should be a first degree relative of the other two;
- at least two successive generations should be affected;
- in one of the relatives, GC should be diagnosed before the age of 50.

In countries with a low GC incidence, like USA and UK, FIGC selection criteria are:

- at least two first/second degree relatives affected by IGC, one diagnosed before the age of 50;
- three or more relatives with IGC at any age.

Of relevance, to date no germline defects have been found to be recurrently associated with FIGC predisposition.

1.8.3 Gastric Adenocarcinoma and Proximal Polyposis of the stomach

In 2012, a new autosomal dominantly transmitted syndrome, the gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS), was described [Worthley DL *et al.* 2012]. The key clinical features of GAPPS are fundic gland polyposis of the stomach with occasional hyperplastic and adenomatous polyps, sparing of the gastric antrum, development of intestinal-type gastric adenocarcinoma and autosomal dominant mode of inheritance.
For GAPPS, the following diagnostic criteria are recommended:

- gastric polyps restricted to the body and fundus with no evidence of colorectal or duodenal polyposis;
- >100 polyps carpeting the proximal stomach in the index case or >30 polyps in a first-degree relative of another case;

- predominantly fundic gland polyps, some having regions of dysplasia;
- an autosomal dominant pattern of inheritance.

In 2016, a targeted analysis of the main genes involved in GC and a subsequent whole genome sequencing failed to find causative mutations of GAPPS. However, linkage analysis on six selected families mapped the gene to the 5q22 chromosomal region. Through Sanger sequencing, point mutations in *APC* promoter 1B, that co-segregated with the disease in all six families, were then identified [Li J *et al.* 2016; Beer A *et al.* 2017]. Therefore, GAPPS is now considered as part of the broad phenotypic spectrum of inherited polyposis associated with *APC* germline defects.

1.8.4 Other genes associated with gastric cancer

Although *CDH1* remains the most relevant gene, other genes responsible for different cancer syndromes have been associated with GC predisposition [van der Post RS *et al.* 2015]. These genes include:

- *MLH1*, *MSH2*, *MSH6* and *PMS2* (Lynch syndrome, LS, or Hereditary nonpolyposis colorectal cancer, HNPCC);
- *TP53* (Li-Fraumeni syndrome, LFS);
- *APC* (familial adenomatous polyposis, FAP);
- *MUTYH* (*MUTYH*-associated polyposis, MAP);
- *BMPR1A* and *SMAD4* (juvenile polyposis syndrome, JPS);
- *STK11* (Peutz-Jeghers syndrome, PJS);
- *PTEN* (Cowden syndrome, CS).

In the last few years, thanks to the advent of Next-Generation Sequencing (NGS) approaches, new genes have been identified. In 2013, germline mutations of *CTNNA1* gene, encoding the α-catenin protein, have been described in HDGC families [Majewski IJ *et al.* 2013]; to date, *CTNNA1* is the only gene, besides *CDH1*, clearly associated with the HDGC syndrome [Weren RDA *et al.* 2018]. Germline mutations in *MAP3K6* and *MYD88* have also been reported in HDGC families [Gaston D *et al.* 2014; Vogelaar IP *et al.* 2016]; however, the specific role of these genes remains unclear and their involvement in GC predisposition is still questionable [Weren RDA *et al.* 2018]. In 2015, a targeted analysis with a panel of 55 cancer-related genes has been performed on 144 *CDH1*-negative cases: candidate mutations were identified in 16 probands (11%), including high and

moderate penetrance mutations of *CTNNA1*, *BRCA2*, *STK11*, *SDHB*, *PRSS1*, *ATM*, *MSR1*, and *PALB2* genes [Hansford S *et al.* 2015]. Very recently, a whole exome analysis on 54 *CDH1*-negative GC patients did not identify obvious candidates for GC predisposition [Vogelaar IP *et al.* 2017]. On the contrary, a gene panel-based analysis of 333 HDGC and non-HDGC cases identified 11 mutation carriers of *PALB2*, *BRCA1* and *RAD51C*, which are genes involved in DNA homologous recombination (HR) [Sahasrabudhe R *et al.* 2017].

2. Aims of the research

Patients selected for a given cancer syndrome can result as mutation-negative when analyzed for the corresponding gene, thus remaining without molecular diagnosis. Mutation-negative cases might be explained by rare high-penetrant mutations in new genes, or by mutations in known cancer predisposition genes that are unexpected based on patients' clinical features. On the other hand, in oncology, genetic transmission can be difficult to be identified due to the incomplete penetrance of gene mutations and the influence of both environmental factors and genetic background, making appropriate the molecular analysis of much more subjects compared to other genetic diseases.

In the last few years the advent of NGS has enabled the analysis of a great number of genes with lower costs and a wider access to molecular tests for patients with suspected genetic syndromes. Therefore, NGS represents a powerful tool to explain missing heritability and to rapidly increase our knowledge on the molecular basis of cancer predisposition.

First aim of this study was to analyze *CDH1* gene in patients selected for family history of GC in order to assess the possible presence of predisposing mutations.

Second aim of the study was to search for new genetic risk factors in *CDH1* mutation-negative cases. In particular, by using an NGS-based approach, we searched for alterations in a series of genes known to be involved in different carcinogenic processes and we performed an assessment of pathogenicity of the identified variants through bioinformatic analysis.

Third aim of the study was to perform a methylation analysis of *CDH1* promoter and enhancer sequences to deeply understand the mechanisms that regulate gene expression and to provide the rationale for investigating *CDH1* methylation in patients with suspected genetic predisposition to GC, proven to be mutation-negative by DNA sequencing. Indeed, not only a large body of data support the notion that DNA methylation is crucial in regulating gene expression, but recent findings also point to a link between this mechanism and cancer predisposition.

3. Materials and methods

3.1 Patients' selection

Patients referring to genetic counseling at the Cancer Prevention Unit of the Morgagni-Pierantoni Hospital (Forlì-Italy) from 2010 to 2018, and showing a personal and family history of GC and/or LBC were included in the study. Patient selection was performed according to the following criteria:

- I: 2 or more GC cases regardless of age, at least 1 confirmed DGC
- II: One case of DGC <40 years
- III: Personal or family history of DGC and LBC, one diagnosed <50 years
- IV: Bilateral LBC or family history of 2 or more cases of LBC <50 years
- V: GC ≤60 years with a family history of colorectal cancer
- VI: 2 or more GC cases ≤60 years in first-degree relatives
- VII: Several gastric polyps ≤60 years with a family history of at least 2 GC cases

The criteria I-IV are according to the updated guidelines for HDGC established by the IGCLC [van der Post RS *et al.* 2015]. The criterion V was adopted to select GC cases with suspected Lynch syndrome and the criterion VI to investigate families showing an aggregation of GC of different types. The criterion VII was adopted to investigate patients with gastric polyps (GPs) and family history of GC, given that GPs and GCs can occur in polyposis syndromes (GAPPS, FAP, MAP, JPS, PJS and CS).
Based on the above criteria, we selected 96 patients, including: 79 subjects with GC (57 with DGC, 14 with IGC, 8 with GC of mixed or unknown histotype), 14 with LBC and 3 with GPs.

3.2 Sample collection and DNA extraction

Peripheral blood was obtained from all selected patients after informed consent and as approved by the institutional review board.
Blood samples were transferred to cryovials and stored at -80°C until genomic DNA extraction.

Genomic DNA was purified by the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions.
DNA concentration was assessed with the Qubit 1.0 fluorometer and the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

3.3 Multigene panel (MGP) testing

Sequencing libraries were created starting from 50 ng of genomic DNA, following the enrichment protocol TruSight Cancer (Illumina) for simultaneous sequencing of 94 genes.
The utilized MGP covers all exons of the following genes associated with predisposition towards common and rare cancers:

Gene	Location	OMIM ID	Entrez ID	Ensembl ID	CCDS ID	Transcript ID	Protein ID	Uniprot ID
AIP	11q13.2	* 605555	9049	ENSG00000110711	CCDS8168	NM_003977	NP_003968	O00170
ALK	2p23.2-p23.1	* 105590	238	ENSG00000171094	CCDS33172	NM_004304	NP_004295	Q9UM73
APC	5q22.2	* 611731	324	ENSG00000134982	CCDS4107	NM_000038	NP_000029	P25054
ATM	11q22.3	* 607585	472	ENSG00000149311	CCDS31669	NM_000051	NP_000042	Q13315
BAP1	3p21.1	* 603089	8314	ENSG00000163930	CCDS2853	NM_004656	NP_004647	Q92560
BLM	15q26.1	* 604610	641	ENSG00000197299	CCDS10363	NM_000057	NP_000048	P54132
BMPR1A	10q23.2	* 601299	657	ENSG00000107779	CCDS7378	NM_004329	NP_004320	P36894
BRCA1	17q21.31	* 113705	672	ENSG00000012048	CCDS11453	NM_007294	NP_009225	P38398
BRCA2	13q13.1	* 600185	675	ENSG00000139618	CCDS9344	NM_000059	NP_000050	P51587
BRIP1	17q23.2	* 605882	83990	ENSG00000136492	CCDS11631	NM_032043	NP_114432	Q9BX63
BUB1B	15q15.1	* 602860	701	ENSG00000156970	CCDS10053	NM_001211	NP_001202	O60566
CDC73	1q31.2	* 607393	79577	ENSG00000134371	CCDS1382	NM_024529	NP_078805	Q6P1J9
CDH1	16q22.1	*192090	999	ENSG00000039068	CCDS10869	NM_004360	NP_004351	P12830
CDK4	12q14.1	* 123829	1019	ENSG00000135446	CCDS8953	NM_000075	NP_000066	P11802
CDKN1C	11p15.4	* 600856	1028	ENSG00000129757	CCDS7738	NM_000076	NP_000067	P49918
CDKN2A	9p21.3	* 600160	1029	ENSG00000147889	CCDS6510	NM_000077	NP_000068	P42771
					CCDS6511	NM_058195	NP_478102	Q8N726

CEBPA	19q13.11	* 116897	1050	ENSG00000245848	CCDS54243	NM_004364	NP_004355	P49715
CEP57	11q21	* 607951	9702	ENSG00000166037	CCDS8304	NM_014679	NP_055494	Q86XR8
CHEK2	22q12.1	+ 604373	11200	ENSG00000183765	CCDS13843	NM_007194	NP_009125	O96017
CYLD	16q12.1	* 605018	1540	ENSG00000083799	CCDS45482	NM_015247	NP_056062	Q9NQC7
DDB2	11p11.2	* 600811	1643	ENSG00000134574	CCDS7927	NM_000107	NP_000098	Q92466
DICER1	14q32.13	* 606241	23405	ENSG00000100697	CCDS9931	NM_030621	NP_085124	Q9UPY3
DIS3L2	2q37.1	* 614184	129563	ENSG00000144535	CCDS42834	NM_152383	NP_689596	Q8IYB7
EGFR	7p11.2	* 131550	1956	ENSG00000146648	CCDS5514	NM_005228	NP_005219	P00533
EPCAM	2p21	* 185535	4072	ENSG00000119888	CCDS1833	NM_002354	NP_002345	P16422
ERCC2	19q13.32	* 126340	2068	ENSG00000104884	CCDS33049	NM_000400	NP_000391	P18074
ERCC3	2q14.3	* 133510	2071	ENSG00000163161	CCDS2144	NM_000122	NP_000113	P19447
ERCC4	16p13.12	* 133520	2072	ENSG00000175595	CCDS32390	NM_005236	NP_005227	Q92889
ERCC5	13q33.1	* 133530	2073	ENSG00000134899	CCDS32004	NM_000123	NP_000114	P28715
EXT1	8q24.11	* 608177	2131	ENSG00000182197	CCDS6324	NM_000127	NP_000118	Q16394
EXT2	11p11.2	* 608210	2132	ENSG00000151348	CCDS7908	NM_207122	NP_997005	Q93063
EZH2	7q36.1	* 601573	2146	ENSG00000106462	CCDS56516	NM_001203247	NP_001190176	Q15910
FANCA	16q24.3	* 607139	2175	ENSG00000187741	CCDS32515	NM_000135	NP_000126	O15360
FANCB	Xp22.2	* 300515	2187	ENSG00000181544	CCDS14161	NM_001018113	NP_001018123	Q8NB91
FANCC	9q22.32	* 613899	2176	ENSG00000158169	CCDS35071	NM_000136	NP_000127	Q00597
FANCD2	3p25.3	* 613984	2177	ENSG00000144554	CCDS2595	NM_033084	NP_149075	Q9BXW9
FANCE	6p21.31	* 613976	2178	ENSG00000112039	CCDS4805	NM_021922	NP_068741	Q8NFG4
FANCF	11p14.3	* 613897	2188	ENSG00000183161	CCDS7857	NM_022725	NP_073562	Q9NPI8
FANCG	9p13.3	* 602956	2189	ENSG00000221829	CCDS6574	NM_004629	NP_004620	O15287
FANCI	15q26.1	* 611360	55215	ENSG00000140525	CCDS45346	NM_001113378	NP_001106849	Q9NVI1
FANCL	2p16.1	* 608111	55120	ENSG00000115392	CCDS1860	NM_018062	NP_060532	Q9NW38
FANCM	14q21.2	* 609644	57697	ENSG00000187790	CCDS32070	NM_020937	NP_065988	Q8IYD8
FH	1q43	* 136850	2271	ENSG00000091483	CCDS1617	NM_000143	NP_000134	P07954

FLCN	**17p11.2**	*** 607273**	**201163**	**ENSG00000154803**	**CCDS32579**	**NM_144997**	**NP_659434**	**Q8NFG4**
GATA2	**3q21.3**	*** 137295**	**2624**	**ENSG00000179348**	**CCDS3049**	**NM_001145661**	**NP_001139133**	**P23769**
GPC3	**Xq26.2**	*** 300037**	**2719**	**ENSG00000147257**	**CCDS14638**	**NM_004484**	**NP_004475**	**P51654**
HNF1A	**12q24.31**	*** 142410**	**6927**	**ENSG00000135100**	**CCDS9209**	**NM_000545**	**NP_000536**	**P20823**
HRAS	**11p15.5**	*** 190020**	**3265**	**ENSG00000174775**	**CCDS7698**	**NM_005343**	**NP_005334**	**P01112**
KIT	**4q12**	*** 164920**	**3815**	**ENSG00000157404**	**CCDS3496**	**NM_000222**	**NP_000213**	**P10721**
MAX	**14q23.3**	*** 154950**	**4149**	**ENSG00000125952**	**CCDS9771**	**NM_002382**	**NP_002373**	**P61244**
MEN1	**11q13.1**	*** 613733**	**4221**	**ENSG00000133895**	**CCDS8083**	**NM_000244**	**NP_000235**	**O00255**
MET	**7q31.2**	*** 164860**	**4233**	**ENSG00000105976**	**CCDS43636**	**NM_000245**	**NP_000236**	**P08581**
MLH1	**3p22.2**	*** 120436**	**4292**	**ENSG00000076242**	**CCDS2663**	**NM_000249**	**NP_000240**	**P40692**
MSH2	**2p21-p16.3**	*** 609309**	**4436**	**ENSG00000095002**	**CCDS1834**	**NM_000251**	**NP_000242**	**P43246**
MSH6	**2p16.3**	*** 600678**	**2956**	**ENSG00000116062**	**CCDS1836**	**NM_000179**	**NP_000170**	**P52701**
MUTYH	**1p34.1**	*** 604933**	**4595**	**ENSG00000132781**	**CCDS520**	**NM_012222**	**NP_036354**	**Q9UIF7**
NBN	**8q21.3**	*** 602667**	**4683**	**ENSG00000104320**	**CCDS6249**	**NM_002485**	**NP_002476**	**O60934**
NF1	**17q11.2**	*** 613113**	**4763**	**ENSG00000196712**	**CCDS42292**	**NM_001042492**	**NP_001035957**	**P21359**
NF2	**22q12.2**	*** 607379**	**4771**	**ENSG00000186575**	**CCDS13861**	**NM_000268**	**NP_000259**	**P35240**
NSD1	**5q35.3**	*** 606681**	**64324**	**ENSG00000165671**	**CCDS4412**	**NM_022455**	**NP_071900**	**Q96L73**
PALB2	**16p12.2**	*** 610355**	**79728**	**ENSG00000083093**	**CCDS32406**	**NM_024675**	**NP_078951**	**Q86YC2**
PHOX2B	**4p13**	*** 603851**	**8929**	**ENSG00000109132**	**CCDS3463**	**NM_003924**	**NP_003915**	**Q99453**
PMS1	**2q32.2**	*** 600258**	**5378**	**ENSG00000064933**	**CCDS2302**	**NM_000534**	**NP_000525**	**P54277**
PMS2	**7p22.1**	*** 600259**	**5395**	**ENSG00000122512**	**CCDS5343**	**NM_000535**	**NP_000526**	**P54278**
PRF1	**10q22.1**	*** 170280**	**5551**	**ENSG00000180644**	**CCDS7305**	**NM_005041**	**NP_005032**	**P14222**
PRKAR1A	**17q24.2**	*** 188830**	**5573**	**ENSG00000108946**	**CCDS11678**	**NM_002734**	**NP_002725**	**P10644**
PTCH1	**9q22.32**	*** 601309**	**5727**	**ENSG00000185920**	**CCDS6714**	**NM_000264**	**NP_000255**	**Q13635**
PTEN	**10q23.31**	*** 601728**	**5728**	**ENSG00000171862**	**CCDS31238**	**NM_000314**	**NP_000305**	**P60484**
RAD51C	**17q22**	*** 602774**	**5889**	**ENSG00000108384**	**CCDS11611**	**NM_058216**	**NP_478123**	**O43502**
RAD51D	**17q12**	*** 602954**	**5892**	**ENSG00000185379**	**CCDS11287**	**NM_002878**	**NP_002869**	**O75771**

RB1	**13q14.2**	*** 614041**	**5925**	**ENSG00000139687**	**CCDS31973**	**NM_000321**	**NP_000312**	**P06400**
RECQL4	**8q24.3**	*** 603780**	**9401**	**ENSG00000160957**	**CCDS75804**	**NM_004260**	**NP_004251**	**O94761**
RET	**10q11.21**	*** 164761**	**5979**	**ENSG00000165731**	**CCDS7200**	**NM_020975**	**NP_066124**	**P07949**
RHBDF2	**17q25.1**	*** 614404**	**79651**	**ENSG00000129667**	**CCDS32743**	**NM_024599**	**NP_078875**	**Q6PJF5**
RUNX1	**21q22.12**	*** 151385**	**861**	**ENSG00000159216**	**CCDS42922**	**NM_001001890**	**NP_001001890**	**Q01196**
SBDS	**7q11.21**	*** 607444**	**51119**	**ENSG00000126524**	**CCDS5537**	**NM_016038**	**NP_057122**	**Q9Y3A5**
SDHAF2	**11q12.2**	*** 613019**	**54949**	**ENSG00000167985**	**CCDS8007**	**NM_017841**	**NP_060311**	**Q9NX18**
SDHB	**1p36.13**	*** 185470**	**6390**	**ENSG00000117118**	**CCDS176**	**NM_003000**	**NP_002991**	**P21912**
SDHC	**1q23.3**	*** 602413**	**6391**	**ENSG00000143252**	**CCDS1230**	**NM_003001**	**NP_002992**	**Q99643**
SDHD	**11q23.1**	*** 602690**	**6392**	**ENSG00000204370**	**CCDS31678**	**NM_003002**	**NP_002993**	**O14521**
SLX4	**16p13.3**	*** 613278**	**84464**	**ENSG00000188827**	**CCDS10506**	**NM_032444**	**NP_115820**	**Q8IY92**
SMAD4	**18q21.2**	*** 600993**	**4089**	**ENSG00000141646**	**CCDS11950**	**NM_005359**	**NP_005350**	**Q13485**
SMARCB1	**22q11.23**	*** 601607**	**6598**	**ENSG00000099956**	**CCDS13817**	**NM_003073**	**NP_003064**	**Q12824**
STK11	**19p13.3**	*** 602216**	**6794**	**ENSG00000118046**	**CCDS45896**	**NM_000455**	**NP_000446**	**Q15831**
SUFU	**10q24.32**	*** 607035**	**51684**	**ENSG00000107882**	**CCDS7537**	**NM_016169**	**NP_057253**	**Q9UMX1**
TMEM127	**2q11.2**	*** 613403**	**55654**	**ENSG00000135956**	**CCDS2018**	**NM_017849**	**NP_060319**	**O75204**
TP53	**17p13.1**	*** 191170**	**7157**	**ENSG00000141510**	**CCDS11118**	**NM_000546**	**NP_000537**	**P04637**
TSC1	**9q34.13**	*** 605284**	**7248**	**ENSG00000165699**	**CCDS6956**	**NM_000368**	**NP_000359**	**Q92574**
TSC2	**16p13.3**	*** 191092**	**7249**	**ENSG00000103197**	**CCDS10458**	**NM_000548**	**NP_000539**	**P49815**
VHL	**3p25.3**	*** 608537**	**7428**	**ENSG00000134086**	**CCDS2597**	**NM_000551**	**NP_000542**	**P40337**
WRN	**8p12**	*** 604611**	**7486**	**ENSG00000165392**	**CCDS6082**	**NM_000553**	**NP_000544**	**Q14191**
WT1	**11p13**	*** 607102**	**7490**	**ENSG00000184937**	**CCDS7878**	**NM_024426**	**NP_077744**	**P19544**
XPA	**9q22.33**	*** 611153**	**7507**	**ENSG00000136936**	**CCDS6729**	**NM_000380**	**NP_000371**	**P23025**
XPC	**3p25.1**	*** 613208**	**7508**	**ENSG00000154767**	**CCDS46763**	**NM_004628**	**NP_004619**	**Q01831**

The MGP targets a total of 255 kb of the human genome, *i.e.* 1700 exons of the above genes, as well as their flanking regions (on average 50bp upstream and downstream each exon).

The genomic library was prepared with an enrichment protocol that includes DNA fragmentation, amplification of the fragments and selection of the region of interest through the use of biotinylated probes (Figure 20).

The sequencing was performed by means of the MiSeq platform (Illumina) with MiSeq Reagent Kit v2 configured 2x150 cycles, according to the manufacturer's instructions.

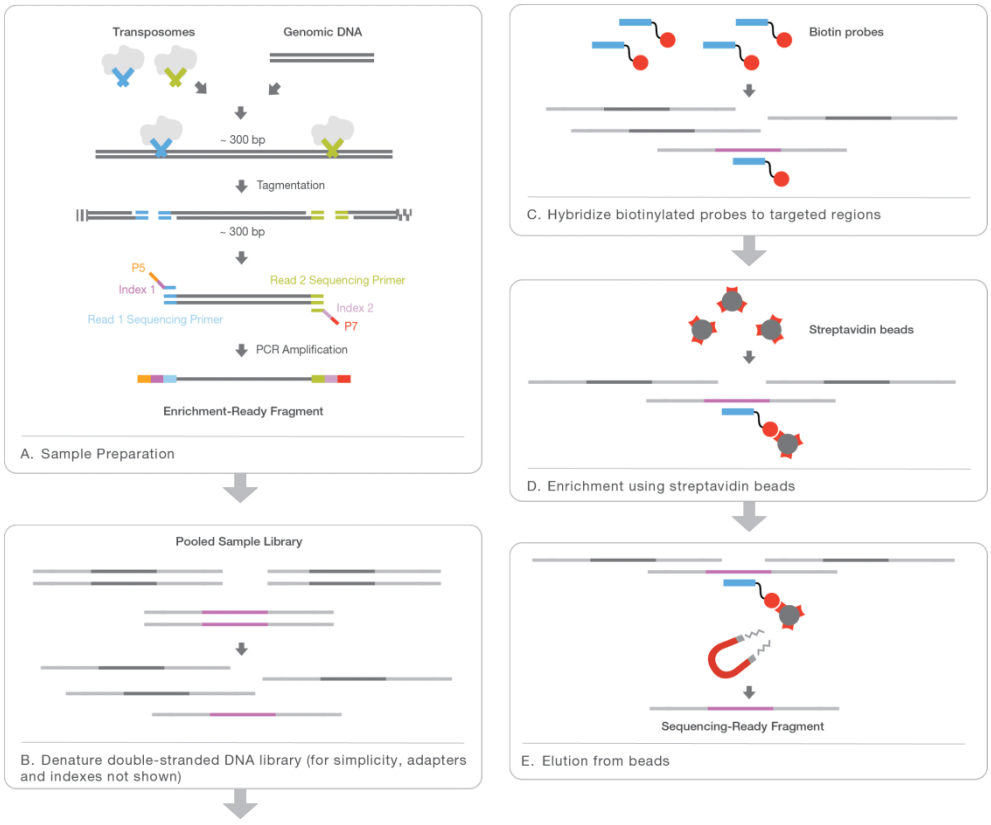


Fig. 20. Library preparation Workflow with Illumina TruSight Cancer [https://www.illumina.com]

3.4 Data analysis and variant calling

The bioinformatic analysis of the MGP results was performed with a customized pipeline (Figure 21).
Raw de-multiplexed reads from the MiSeq sequencer were aligned to the reference human genome (UCSC-Build37/hg19) using the Burrows-Wheeler algorithm [Li H and Durbin R 2009], running in paired-end mode. To ensure good call quality and to reduce the number of false positives, samples underwent Base Quality Score Recalibration (BQSR), using the Genome Analysis Toolkit GATK, version 3.2.2 [McKenna A *et al.* 2010]. After BQSR, sequences around regions with insertions and deletions (indels) were realigned locally with GATK. MarkDuplicates [https://broadinstitute.github.io/picard/] was used to remove duplicate read-

pairs arisen as artifacts during either polymerase chain reaction (PCR) amplification or sequencing. For variant analysis Unified Genotyper of GATK was used to search for SNVs and InDel. Genomic and functional annotations of detected variants were made by Annovar [Wang K *et al.* 2010]. Coverage statistics was performed by DepthOfCoverage utility of GATK. BASH and R custom scripts were used to obtain the list of low coverage (50X) regions per sample. The regions under this threshold were considered not evaluable.

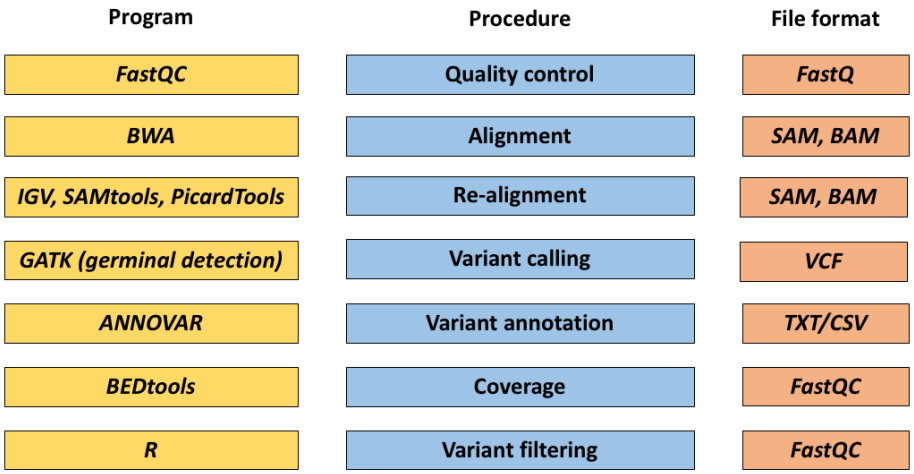


Fig. 21. Bioinformatic workflow with programs, procedures and file formats utilized.

3.5 Additional molecular analyses

CDH1 regions covered <50X were amplified by standard PCR with the Ex Taq DNA Polymerase (TaKaRa) and PCR products were sequenced by using the BigDye terminator v.3.1 cycle sequencing kit (Thermo Fisher Scientific) on an ABI-3130 Genetic analyzer (Applied Biosystems).
To identify the possible presence of *CDH1* extended deletions/duplications not detectable by sequencing, samples were analyzed by Multiplex Ligation-dependent Probe Amplification (MLPA) method (Figure 22), by using the P083-*CDH1* kit (MRC Holland).
Given the suspicion of HNPCC, the two patients fulfilling criterion V were also tested with the P003-*MLH1*/*MSH2* and P072-*MSH6* MLPA kits (MRC Holland), in order to identify deletions/duplications of *MLH1*, *MSH2* and *MSH6* genes.

All MLPA results were analyzed with the Coffalyser software (MRC Holland).

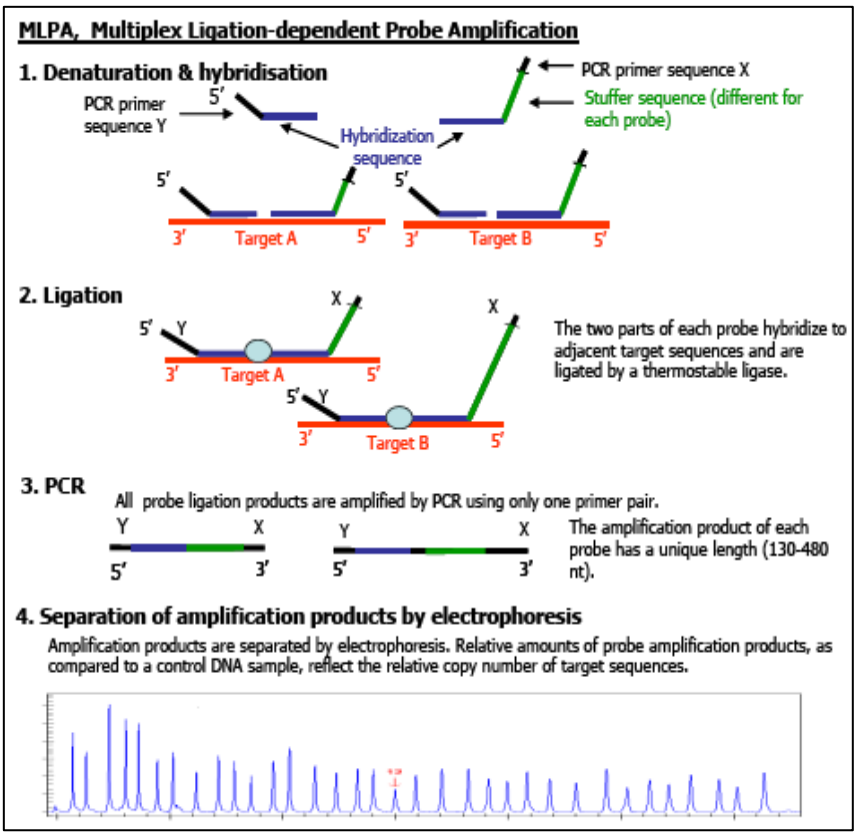


Fig. 22. Workflow of the MLPA analysis [Schouten JP *et al.* 2002]

3.6 Confirmation of variants

All *CDH1* variants of classes 3-5* identified by MGP testing were confirmed by Sanger sequencing with the same protocol used for the uncovered regions; (3: variants of uncertain significance; 4: likely pathogenic variants; 5: pathogenic variants)*
CDH1 and *MSH2* rearrangements identified by MLPA were confirmed through a second MLPA test.
All deleterious variants of classes 4-5 identified in genes other than *CDH1*, were confirmed through a second NGS-based analysis.

3.7 Variant classification

The identified genetic variants were divided into 5 classes according to the International Agency for Research on Cancer (IARC) recommendations [Plon SE *et al.* 2008].
For many genes a database for variant classification is not available and the only *CDH1* variant database is incomplete. Consequently, the classification of variants emerged from MGP testing was obtained by using the online databases dbSNP [https://www.ncbi.nlm.nih.gov/projects/SNP/] and ClinVar [https://www.ncbi.nlm.nih.gov/clinvar/].
Variants not included in any of these databases were classified on the basis of their characteristics: only variants producing premature stop codons and gross deletions were considered pathogenic (class 5) or likely-pathogenic (class 4) and classified in accordance with the guidelines of the American College of Medical Genetics (ACMG) [Richards S *et al.* 2015].
The potential impact of amino acid changes (MAPP p-value) was assessed with PolyPhen-2 HVAR [Adzhubei IA *et al.* 2010] and SIFT [Kumar P *et al.* 2009].

3.8 Sequencing of *APC* promoter 1B

The Illumina Trusight Cancer panel, used in the present work, contains probes for the exonic regions only of the 94 genes.
To verify the possible presence of alterations involved in GAPPS syndrome (paragraph 1.9.3), we performed Sanger sequencing of the promoter 1B of the *APC* gene (Figure 23).

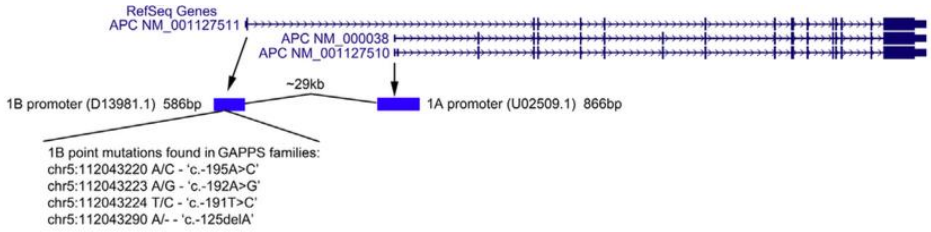


Fig. 23. Schematic representation of *APC* transcripts with promoters 1A and 1B represented by light blue bars (the mutations identified in GAPPS families are reported in the enlargement) [Li J *et al.* 2016]

The *APC* 1B promoter region was amplified by standard PCR with the Multiplex PCR kit (Qiagen) and the primers reported in literature (Figure 24) [Li J *et al.* 2016].

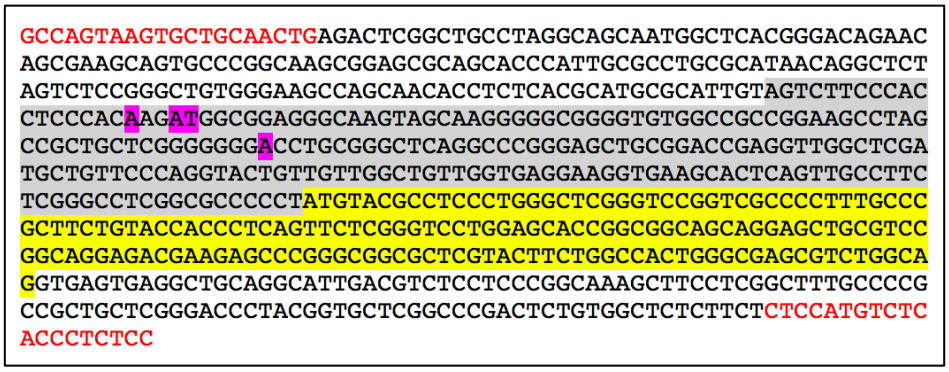


Fig. 24. Sequence of the *APC* promoter 1B. Red letters represent the primers used for the amplification and the sequencing; grey and yellow sequences represent 5'UTR and exon 1B of NM_001127511 transcript, respectively; the letters highlighted in purple represent the nucleotides found mutated in GAPPS families [Li J *et al.* 2016]

PCR products were sequenced by using the BigDye terminator v.3.1 cycle sequencing kit (Thermo Fisher Scientific) on an ABI-3130 Genetic analyzer (Applied Biosystems).
Due to the characteristics of GAPPS syndrome [Worthley DL *et al.* 2012], sequencing of *APC* promoter 1B was only performed on the 3 patients with gastric polyposis (GP) and the 14 patients with intestinal-type gastric cancer (IGC) from our case series (paragraph 3.1).
In addition, the analysis was performed on 47 cases of familial IGC (selected by Prof. Franco Roviello-University of Siena, Italy), previously tested negative for mutations of *CDH1* and of other cancer-predisposition genes (analysis performed by Prof. Carla Oliveira-University of Porto, Portugal).

3.9 Methylation analysis of *CDH1* promoter and enhancers

The promoter and the enhancers of *CDH1* gene were identified through the UCSC Genome Browser [https://genome.ucsc.edu/].
The *CDH1* promoter is located in a CpG island that overlaps exons 1 and 2 (Figure 25).

The FANTOM5 tool of the UCSC Genome Browser identified 7 enhancers in the *CDH1* region, one located 4724 bp upstream from the *CDH1* transcription start site (TSS), and six located within intron 2 (Figure 25).

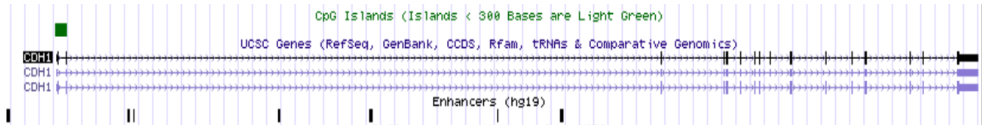


Fig. 25. UCSC representation of the *CDH1* gene with the CpG island symbolized by green bar and the enhancers represented by black bars

The coordinates of *CDH1* promoter and enhancers are reported in the following table (the enhancers were named from A to G starting from the most upstream one):

***CDH1* element**	**Genomic coordinates (hg19)**	**Size (bp)**
Promoter	chr16: 68771035 - 68772344	1310
Enhancer A	chr16: 68765919 - 68766309	391
Enhancer B	chr16: 68778759 - 68779020	262
Enhancer C	chr16: 68779435 - 68779559	125
Enhancer D	chr16: 68794776 - 68795028	253
Enhancer E	chr16: 68804488 - 68804868	381
Enhancer F	chr16: 68818215 - 68818347	133
Enhancer G	chr16: 68824914 - 68825217	304

The methylation analysis was performed on 6 DNA samples: 2 from peripheral blood of healthy individuals, 2 from frozen tissue samples obtained by gastric bariatric surgery, and 2 from MKN-74 and SNU-1 cell lines (with high and low *CDH1* gene expression, respectively).
DNA samples were treated with bisulfite using the Epitect Bisulfit kit (Qiagen).
CDH1 promoter and enhancers were amplified by standard PCR with the Multiplex PCR kit (Qiagen) and the PCR products were sequenced by the BigDye terminator v.3.1 cycle sequencing kit (Thermo Fisher Scientific) on an ABI-3130 Genetic analyzer (Applied Biosystems), with the exception of

enhancer C that was excluded from the analysis because of the absence of CpG sites.

The primers for amplification and sequencing were designed on the *in silico* bisulfite converted sequence and are reported in the table below.

***CDH1* element**	**Primer F (5'->3')**	**Primer R (5'->3')**	**Product size (bp)**
Promoter	GGTAGGTGAATTTTTAGTTAA	ACTCCAAAAACCCATAACTAACC	221
Enhancer A	GGGTGATGAATATTTGTGG	CACTTCCTAAATATTTTAACC	485
Enhancer B	GGAGGATTTGGTGTTGTTTG	CTAACAAATTCAAAATCATCCC	352
Enhancer D	GTTTTTTTAGAGTGGGGAG	CTACAATCCTACCAAACTC	330
Enhancer E	GGTAGTATATTTTAGAAGGATG	CTATCTCCTACACATCATTTC	504
Enhancer F	GGAGTTTTATATTTTTGTTGG	CCATACAAAATACAAAAAACC	272
Enhancer G	GTTGTTAAGAAGGGTATGG	CCAAAAACTTTATATAACAAAC	400

4. Results

4.1 *CDH1* pathogenic variants

We identified 9 *CDH1* pathogenic variants in 10 out of 96 patients (10.4%). Four were base deletions causing frameshift mutations, 3 were nonsense variants (one found in 2 unrelated subjects), 1 was a synonymous variant affecting RNA splicing and 1 was a gross deletion detected by MLPA method (Figure 26). Five out of 9 pathogenic variants had previously been reported [Berx G *et al.* 1995; Jonsson BA *et al.* 2002; Frebourg T *et al.* 2006; Oliveira C *et al.* 2009; Hansford S *et al.* 2015; www.ncbi.nlm.nih.gov/clinvar/] while 4 were novel. Among variant carriers, 9 patients had DGC only (mean age: 39.9 years) and one had LBC only (52 years of age). *CDH1* molecular data and clinical features of variant carriers are summarized in Table 1.

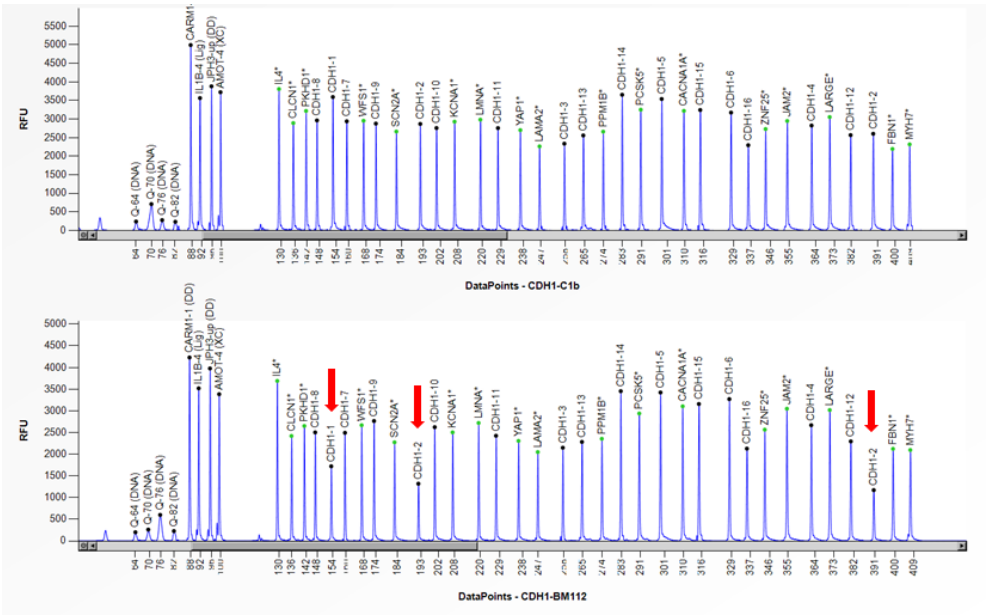


Fig. 26. MLPA electropherograms of *CDH1* gene: wild-type individual (upper panel) and patient with a deletion encompassing exons 1 and 2 (lower panel). Red arrows point to the peaks corresponding to *CDH1* probes hybridizing to exons 1 and 2

Table 1: Carriers of CDH1 deleterious variants.

Patient ID	Sex	Selection criteria	Cancer	Age at diagnosis	Gene	Exon	cDNA	Protein	IARC class	dbSNP	ClinVar	Literature
BM112	F	II	DGC	37	*CDH1*	1-2	c.1-?_163+?del	p.?	5	-	-	Oliveira C *et al.* 2009
BM73	F	III	LBC	52	*CDH1*	1	c.31delC	p.(Leu11Cys*fs**45)	4	-	-	-
BM37	F	II	DGC	37	*CDH1*	3	c.308G>A	p.Trp103*	5	-	pathogenic	-
BM100	M	I	DGC	58	*CDH1*	3	c.360delG	p.(His121Thr*fs**94)	4	-	-	-
BM81	F	II	DGC	18	*CDH1*	6	c.781G>T	p.Glu261*	5	rs121964873	pathogenic	Berx G *et al.* 1995
BM115	F	II	DGC	31								
BM60	M	II	DGC	39	*CDH1*	7	c.1003C>T	p.Arg335*	5	rs587780784	pathogenic	Jonsson BA *et al.* 2002
BM119	M	II	DGC	33	*CDH1*	8	c.1137G>A	p.Thr379=	4-5	rs587783050	pathogenic/likely pathogenic	Frebourg T *et al.* 2006
BM74	M	I	DGC	59	*CDH1*	13	c.1965delG	p.(Met656Trp*fs**3)	4	-	-	-
BM45	M	I	DGC	47	*CDH1*	13	c.2114delT	p.(Leu705Cys*fs**17)	4	-	-	-

DGC: diffuse-type gastric cancer; LBC: lobular breast cancer.

Figure 27 shows the localization of the 9 pathogenic variants we identified.

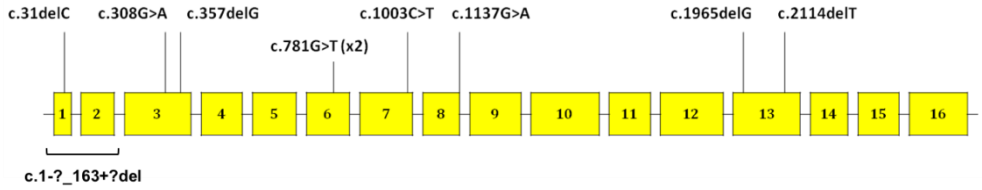


Fig. 27. Schematic representation of *CDH1* and localization of the 9 pathogenic variants identified in this work

4.2 Pathogenic variants in genes other than *CDH1*

In 11 out of 96 patients (11.5%), we found loss-of-function variants in genes other than *CDH1*, including: *ATM* (2 variants), *BRCA1*, *BRCA2*, *PALB2* (2 variants), *MSH2*, *PMS2*, *BMPR1A*, *PRF1* and *BLM*. Four out of 11 variants were frameshift deletions (*ATM*, *BLM*, *PMS2*, *BRCA1*), 1 was a frameshift insertion (*ATM*), 5 were nonsense variants (*PRF1*, *PALB2*, *BRCA2*, *BMPR1A*) and 1 was a gross deletion (*MSH2*) detected by MLPA. Seven out of 11 variants had previously been reported [Lakin ND *et al.* 1996; Wijnen J *et al.* 1998; Stepp SE *et al.* 1999; Meindl A *et al.* 2002; Saviozzi S *et al.* 2003; Casadei S *et al.* 2011; Kluska A *et al.* 2015; www.ncbi.nlm.nih.gov/clinvar/], while 4 were novel. Five variant carriers had developed a DGC (mean age: 54.2 years of age), one of whom after an LBC (50 years of age); three carriers had developed an IGC (mean age: 57.0 years), one had a bilateral LBC (at 62 and 66 years), one had an LBC (at 61 years of age), while one had a severe GP diagnosed at 52 years. Molecular data and clinical features of variant carriers are summarized in Table 2.

Table 2: Carriers of deleterious variants in cancer-related genes.

Patient ID	Sex	Selection criteria	Cancer(s)	Age at diagnosis	Gene	Exon	cDNA	Protein	IARC class	dbSNP	ClinVar	Literature
BM10	M	V	IGC	57	*MSH2*	3	c.367-?_645+?del	p.?	5	-	pathogenic	Wijnen J *et al.* 1998
BM90	M	I	DGC	73	*PMS2*	13	c.2182_2183delAC	p.(Thr728Ser*fs**7)	4	-	-	-
BM89	F	I	DGC	65	*PRF1*	3	c.1122G>A	p.Trp374*	5	rs104894176	pathogenic	Stepp SE *et al.* 1999
BM46	M	I	IGC	54	*ATM*	10	c.1564_1565delGA	p.Glu522Ile*fs**43	5	rs587779817	pathogenic	Lakin ND *et al.* 1996
BM76	F	II	DGC	32	*ATM*	14	c.2192dupA	p.(Tyr731*)	4	-	-	Saviozzi S *et al.* 2003
BM38	M	VI	IGC	60	*BRCA2*	11	c.6037A>T	p.Lys2013*	5	rs80358840	pathogenic	Meindl A *et al.* 2002
BM24	M	VII	GP	52	*BMPR1A*	3	c.34G>T	p.(Gly12*)	4	-	-	-
BM47	F	I	LBC, DGC	50, 54	*BLM*	11	c.2395delT	p.(Cys799Val*fs**16)	4	-	-	-
A530	F	IV	LBC, LBC	62, 66	*PALB2*	4	c.535C>T	p.(Gln179*)	4	-	-	-
BM126	F	IV	LBC	62	*PALB2*	7	c.2718G>A	p.Trp906*	4-5	rs180177122	pathogenic/likely pathogenic	Casadei S *et al.* 2011
BM110	F	I	DGC	47	*BRCA1*	7	c.406delA	p.Arg136Asp*fs**27	5	rs886040196	pathogenic	Kluska A *et al.* 2015

DGC: diffuse-type gastric cancer; IGC: intestinal-type gastric cancer; GP: gastric polyposis; LBC: lobular breast cancer.

The combination of MGP testing and MLPA analysis allowed us to detect pathogenic variants in 21/96 patients (21,9%). Figure 28 shows all pathogenic variants emerged from the analysis as well as the fraction of cases where no disease-associated variants were identified.

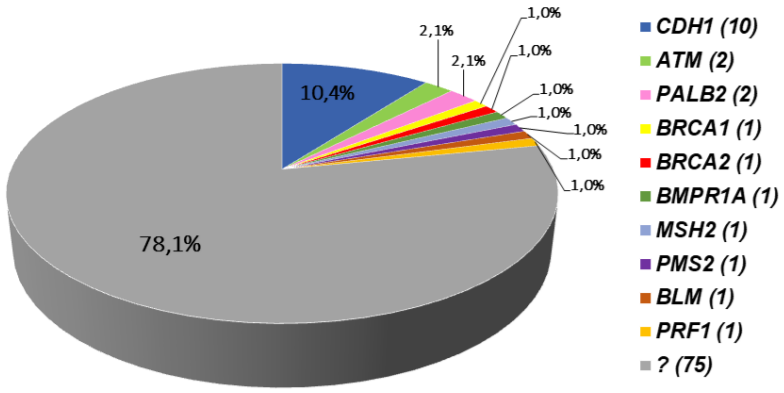


Fig. 28. Pie chart showing the fraction of cases with/without pathogenic variants; between brackets is reported the number of variant carriers

4.3 Exonic variants

In the remainder of the tested cohort, *i.e.* 75 out of 96 patients (78.1%), we did not find any variants with clear-cut impact on gene function and clinical relevance.
However, the 75 patients globally showed 7,489 exonic variants. To exclude polymorphisms, we considered the allelic frequencies reported in the 1000Genomes, Esp6500, and Exac03 databases. The 271 variants with frequencies <1% or n/a, included: 93 (34.3%) synonymous base changes, 173 (63.8%) missense variants, 3 (1.1%) small deletions in-frame and 2 (0.7%) small insertions in-frame.
We identified a total of 244 unique variants in 76 different genes. To assess their role in cancer development, we evaluated the 160 unique missense variants by PolyPhen-2 HVAR and SIFT bioinformatic tools that predict functional impact and pathogenicity of human variants. Sixty-six out of 160 variants (41.3%) were classified as benign by both PolyPhen-2 HVAR and SIFT, 63 (39.4%) were discordantly classified, and 31 (19.4%) were classified as probably damaging by both bioinformatics tools.

Four out of the 31 variants classified as probably damaging (*MET*, *WRN*, *NBN* and *TSC2* genes) were present in 2 patients (Table 3). Overall, these 31 variants were present in 28 patients, with 7/28 patients (BM58, BM61, BM67, BM75, BM93, BM118, BM122) carrying 2 different variants classified as probably damaging (Table 3).

Table 3: List of the 31 missense variants classified as probably damaging by both SIFT and PolyPhen2 identified in our case series and patients' characteristics.

Gene	Exon	cDNA	Protein	dbSNP	ClinVar	SIFT	Polyphen2 HVAR	Patient ID	Sex	Selection criteria	Cancer(s)	Age at diagnosis
FH	2	c.234C>A	p.Asn78Lys	-	-	D	D	BM32	F	I	DGC	49
EPCAM	6	c.605A>C	p.Lys202Thr	-	-	D	D	BM67	F	I	DGC	41
PMS1	3	c.224C>T	p.Thr75Ile	rs61756360	-	D	D	BM31	F	I	DGC	47
FANCD2	3	c.195G>C	p.Gln65His	rs36084488	VUS/Benign	D	D	BM71	M	II	DGC	35
GATA2	5	c.1010G>A	p.Arg337Gln	-	-	D	D	BM67	F	I	DGC	41
KIT	21	c.2867G>A	p.Arg956Gln	rs139694927	VUS	D	D	BM85	M	I	IGC	66
PMS2	2	c.52A>G	p.Ile18Val	rs63750123	VUS/Likely benign/Benign	D	D	BM56	F	I	DGC	55
MET	14	c.2975C>T	p.Thr992Ile	rs56391007	VUS/Likely benign/Benign	D	D	BM93	M	I	DGC	65
								BM50	F	IV	LBC, LBC	53, 58
EZH2	19	c.2151G>C	p.Gln717His	-	-	D	D	BM62	M	I	DGC	45
WRN	14	c.1717A>G	p.Thr573Ala	rs150148567	VUS/Likely benign	D	D	BM58	M	I	DGC	54
								BM75	F	I	IGC, OC, LBC	57, 60, 72
WRN	17	c.1909C>T	p.Arg637Trp	rs148286708	VUS	D	D	BM58	M	I	DGC	54
WRN	30	c.3523C>A	p.Pro1175Thr	-	-	D	D	BM84	F	I	LBC, DGC	61, 66
NBN	3	c.283G>A	p.Asp95Asn	rs61753720	VUS/Likely benign/Benign	D	D	BM41	F	III	LBC	45
								BM96	F	II	DGC	33
RET	11	c.1946C>T	p.Ser649Leu	rs148935214	VUS/Likely benign/Benign	D	D	BM69	F	I	GC	77
RET	11	c.1997A>T	p.Lys666Met	rs377767439	VUS	D	D	BM48	F	I	IGC	47

BMPR1A	11	c.1243G>A	p.Glu415Lys	rs140592056	VUS/Likely benign/Benign	D	D	BM79	F	III	LBC	52
EXT2	5	c.889C>T	p.Arg297Cys	rs146098187	-	D	D	BM93	M	I	DGC	65
CEP57	3	c.333G>C	p.Gln111His	rs117321017	Benign	D	D	BM75	F	I	IGC, OC, LBC	57, 60, 72
BRCA2	14	c.7225C>T	p.Pro2409Ser	-	-	D	D	BM72	M	II	DGC	25
ERCC5	14	c.2890C>T	p.Arg964Trp	rs574826021	VUS	D	D	BM61	F	I	DGC	49
FANCM	20	c.4931G>A	p.Arg1644Gln	rs138151018	Likely benign/Benign	D	D	BM61	F	I	DGC	49
FANCI	18	c.1813C>T	p.Leu605Phe	rs117125761	Likely benign/Benign	D	D	BM122	M	I	GC	59
BLM	12	c.2474C>T	p.Pro825Leu	rs749632465	VUS	D	D	BM118	F	I	DGC	54
TSC2	17	c.1747G>A	p.Ala583Thr	rs1800729	Benign/Likely benign	D	D	BM21	F	I	DGC	47
								BM63	F	I	DGC, DBC	41, 50
SLX4	6	c.1192C>T	p.Arg398Trp	rs138799572	VUS	D	D	BM114	F	IV	LBC, GC, LBC	36, 38, 52
ERCC4	7	c.1135C>T	p.Pro379Ser	rs1799802	VUS/Likely benign	D	D	BM121	F	I	DGC	40
ERCC4	11	c.2117T>C	p.Ile706Thr	rs1800069	VUS	D	D	BM117	F	II	DGC	36
FLCN	6	c.503G>A	p.Arg168His	-	-	D	D	BM118	F	I	DGC	54
NF1	34	c.4526G>A	p.Arg1509His	rs546073780	VUS	D	D	BM124	M	I	DGC	51
RAD51D	10	c.932T>A	p.Ile311Asn	rs145309168	VUS/Likely benign	D	D	BM122	M	I	GC	59
RHBDF2	5	c.478C>T	p.Arg160Cys	rs751482282	VUS	D	D	BM120	F	II	DGC	36

GC: gastric cancer; DGC: diffuse-type gastric cancer; IGC: intestinal-type gastric cancer; LBC: lobular breast cancer; DBC: ductal breast cancer; OC: ovarian cancer; VUS: variant of uncertain significance; D: damaging.

4.4 Variants in promoter 1B of the *APC* gene

The sequencing of *APC* promoter 1B we ferformed on 64 patients with IGC or GP (see paragraph 3.8) allowed us to detect polymorphic or benign variants only, with one exception. This was represented by BM23 patient, a male with severe GP, who proved to be carrier of a variant (c.-30357G>C; rs572582235) classified as of uncertain significance by ClinVar [https://www.ncbi.nlm.nih.gov/clinvar/]. The localization of the identified variant is shown in Figure 29.

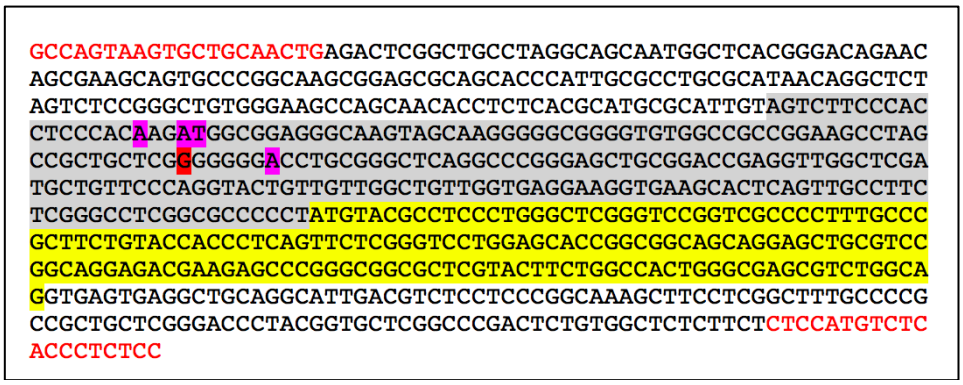


Fig. 29. Sequence of the *APC* promoter 1B: the position of the c.-30357G>C variant is highlighted in red.

4.5 Results of methylation analysis of the *CDH1* promoter/enhancers

The methylation analysis revealed different methylation patterns in the 2 cell lines (MKN-74 and SNU-1), in the 2 DNAs from blood of healthy controls (C11 and C16) and in the 2 frozen tissues obtained by gastric bariatric surgery (P4 and P7), with different CpG sites being methylated along promoter and enhancer sequences (Figure 30).

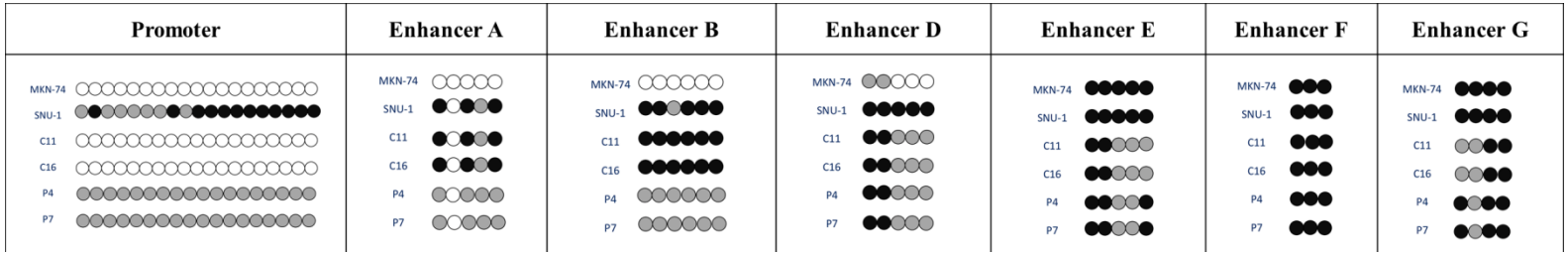


Fig. 30 Methylation analysis of *CDH1* promoter and enhancers on DNA samples from: GC cell lines (MKN-74 and SNU-1), peripheral blood of healthy individuals (C11 and C16), and gastric tissues from bariatric surgery (P4 and P7); open circles correspond to nonmethylated CpG sites; grey circles correspond to hemimethylated CpG sites; black circles correspond to methylated CpG sites.

5. Discussion

In the present work, we analyzed 96 Italian patients with suspected genetic predisposition to GC by sequencing 94 cancer-related genes.
CDH1 was confirmed as the major GC predisposition gene, with a mutation frequency of 10.4%. All carriers of *CDH1* pathogenic variants fell within the patient group selected according to criteria I-III for the HDGC [van der Post RS *et al.* 2015]. By only considering the 75 cases who fulfill criteria I-III, the percentage of *CDH1* patients with pathogenic variants was 13.3%.
For *CDH1* mutation carriers, the cumulative risk of developing GC by the age of 80 is 70% for men and 56% for women. In addition, women have an estimated risk of 42% of developing LBC by the age of 80 years [Hansford S *et al.* 2015]. Due to the high penetrance of *CDH1* pathogenic variants, the early age of onset and the poor prognosis of DGC, prophylactic total gastrectomy is strongly recommended in mutation carriers. Indeed, the analysis of gastrectomy specimens performed over the years almost invariably revealed the presence of multiple foci of signet ring cell carcinoma [Charlton A *et al.* 2004; Molinaro V *et al.* 2014]. Whenever prophylactic gastrectomy is not feasible, carriers should be offered an appropriate endoscopic surveillance, as well as mammography surveillance if women. However, endoscopic surveillance for *CDH1* mutation carriers is proven largely ineffective, essentially due to the highly focal nature of HDGC. In our survey, following genetic test on consenting relatives of *CDH1*-mutation carriers, 2 subjects with a pathogenic variant decided to undergo prophylactic gastrectomy. In both cases, pathological analysis of gastric specimens detected GC microscopic foci, making the management of the disease easier and the outcome more favorable.
As far as the 11 loss-of-function variants we found in genes other than *CDH1*, 6 were in genes associated with the hereditary breast and ovarian cancer (HBOC), including *BRCA1*, *BRCA2*, *ATM* and *PALB2*. *BRCA1* and *BRCA2* genes were found to be mutated in a single proband, while *ATM* and *PALB2* were both found to be mutated in 2 unrelated cases (6.2% of patients). The *BRCA1* variant was found in a subject with DGC and a family history of both GC and BC. The *BRCA2* pathogenic variant was found in two non-identical twins who both developed IGC at 60 years of age; their maternal cousin died of BC at less than 50 years of age. One *ATM* pathogenic variant was detected in an IGC patient with a strong family history of IGC and no BC cases in his family, while the second one was detected in an isolated patient with DGC at 32 years of age. One *PALB2* variant was carried by an LBC patient with GC and BC family history. The

carrier of the other *PALB2* variant developed two LBCs, at 62 and 66 years of age; her sister and mother died of ductal BC at 55 years of age and of DGC at 52 years of age, respectively. On the whole, only *ATM* variant carriers (2 out of 6 cases) had GC only and no family history of BC.

Apart from the two non-identical twins, due to compliance problems and difficulties in obtaining DNA samples, we could not perform co-segregation analysis for *BRCA1*, *BRCA2*, *ATM* and *PALB2* genes. However, these genes have already been implicated in rare GC cases (of both intestinal and diffuse histotypes) by at least three independent studies aimed at identifying genetic predisposition to GC [Hansford S *et al.* 2015; Sahasrabudhe R *et al.* 2017; Fewings E *et al.* 2018]. In addition, the *BRCA1* and *BRCA2* variants we identified have already proven to be disease-causative, at least in HBOC families [Meindl A *et al.* 2002; Kluska A *et al.* 2015; https://www.ncbi.nlm.nih.gov/clinvar/].

Three out of 11 loss-of-function variants in genes other than *CDH1*, were in *MSH2* and *PMS2* Lynch Syndrome (LS) genes and in *BMPR1A* Juvenile Polyposis Syndrome (JPS) gene, accounting for 3.1% of patients in our case series. Although colorectal cancer (CRC) is predominant in LS and JPS, both syndromes have also been associated with an increased risk of GC [Kohlmann W and Gruber SB 2004; Larsen Haidle J and Howe JR 2003]. In particular, LS mutation carriers have 40-80% risk of CRC and 11-19% lifetime risk of GC [Aarnio M *et al.* 1997; Bonadona V *et al.* 2011; Giardiello FM *et al.* 2014], while JPS mutation carriers have 17-22% risk of CRC and 10-21% lifetime risk of gastric and duodenal carcinoma [Brosens LA *et al.* 2007; Latchford AR *et al.* 2012; Aytac E *et al.* 2015]. In our case series, the *MSH2* variant co-segregated with different cancers of the LS spectrum, being colorectal cancer predominant in the mutated family. On the contrary, the family of the *PMS2* variant carrier was characterized by GC development. The *BMPR1A* variant carrier (52 years old) had only developed gastric polyps; interestingly, this same phenotype was shared by his two sisters, in whom, however, we could not perform genetic testing. On the other hand, by testing the proband for germline mutations in promoter 1B of the *APC* gene, we were able to exclude GAPPS [Worthley DL *et al.* 2012; Li J *et al.* 2016], thus reinforcing the causal link between *BMPR1A* and gastric polyposis.

Finally, we identified 2 loss-of-function variants (2.1% of the patients) in *BLM* and *PRF1* genes that have been implicated in susceptibility to multiple cancers, mainly leukemias and lymphomas [Ciambotti B *et al.* 2014; Cunniff C *et al.* 2017]. The *BLM* variant carrier had developed both a DGC and an LBC, she was *BRCA1/2* negative, and showed a family history of GC and BC. Similarly, the *PRF1* variant carrier showed a family history of GC.

Both genes make biological sense for GC development: somatic mutations of *BLM* gene have already been identified in GC [Calin G *et al.* 2001] and GC cases have been reported in families with *PRF1* germline mutations [El Abed R *et al.* 2011]. However, co-segregation data between cancers and germline variants are needed to definitely assess the role of *BLM* and *PRF1* genes in GC predisposition.

In 75 patients (78.1%), we did not find any variants with clear clinical relevance: in 28/75 (37.3%) cases we identified 31 rare missense variants (frequencies <1%) predicted to be damaging by two bioinformatic tools (Table 3). Besides refinements of criteria to improve selection of patients, further studies should be performed to assess the functional impact of all these variants, including *in vitro* tests, tumor analysis and segregation data.

On the whole, our results show that, in addition to *CDH1* genetic lesions, rare variants distributed across different genes can predispose to GC. Among these, there are also genes known to predispose to breast and ovarian cancer, thus reinforcing the emerging link between GC and BC predisposition. This last finding raises the question of clinical phenotypes associated with individual cancer susceptibility genes and add a new challenge for management and appropriate surveillance in some families.

Sequencing analysis of *APC* promoter 1B we performed on patients with IGC/GP to assess GAPPS syndrome, detected a rare unclassified variant in a patient with multiple hyperplastic polyps in the stomach. *In vitro* analyses (*e.g.* allelic imbalance and luciferase reporter assays) and segregation analysis will definitely clarify the impact of this variant on gene expression and on GC history in the family.

Bisulfite genomic sequencing we performed on different samples revealed variability of methylation pattern of *CDH1* promoter and enhancer sequences. In GC cell lines, *CDH1* promoter methylation (such as in SNU-1 cells) was associated with the absence of gene expression and, *vice versa*, the absence of methylation (such as in MKN-74 cells) was associated with a "normal" gene expression. However, this simple mechanism does not explain the very low gene expression level found in blood cells where promoter methylation is absent.

In blood cells, our analysis showed the presence of DNA methylation along the enhancer sequences, a pattern very similar to that we found in SNU-1 cells. This observation indicates that methylation of *CDH1* regulatory elements other than promoter plays an important role in regulating gene expression.

These preliminary results provide the rationale for investigating promoter/enhancers methylation in patients with suspected genetic predisposition to GC, tested to be *CDH1*-mutation negative by sequencing.

Indeed, a fraction of these patients might be attributable to *CDH1* allelic silencing due to perturbation of methylation patterns. Accordingly, studies aimed at linking genomic regions associated with cancer susceptibility and expression of genes involved in cancer development have already shown that promoters' and enhancers' sequence polymorphisms and methylation status can contribute to cancer risk [Aran D *et al.* 2013; Aran D and Hellman A 2013].

Of relevance, the methylation pattern we found in gastric tissue samples from bariatric surgery seems to be incompatible with a "normal" gene expression. This finding can be due to different causes: the methylation pattern of gastric mucosa in individuals with strong obesity might be different from that of healthy individuals; the frozen tissue samples used for DNA extraction might contain different types of cells (e.g. connective tissue or fat tissue cells) with heterogeneous methylation patterns compared to that of epithelial cells. Accordingly, we are currently setting up the conditions for methylation analysis in normal gastric mucosa by using DNAs extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples. Following selection of normal epithelial cells by experienced pathologists, we will be able to create a reference methylation pattern to then investigate methylation perturbance in GC patients.

References

Aarnio M, Salovaara R, Aaltonen LA, Mecklin JP, Järvinen HJ. Features of gastric cancer in hereditary non-polyposis colorectal cancer syndrome. Int J Cancer. 1997;74:551-555.

Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods. 2010;7:248-249.

AIOM-AIRTUM. I numeri del cancro in Italia 2017. Available on the website www.aiom.it

Apicella M, Corso S, Giordano S. Targeted therapies for gastric cancer: failures and hopes from clinical trials. Oncotarget. 2017;8:57654-57669.

Aran D, Sabato S, Hellman A. DNA methylation of distal regulatory sites characterizes dysregulation of cancer genes. Genome Biol. 2013;14:R21.

Aran D, Hellman A. DNA methylation of transcriptional enhancers and cancer predisposition. Cell. 2013;154:11-13.

Aytac E, Sulu B, Heald B, O'Malley M, LaGuardia L, Remzi FH, Kalady MF, Burke CA, Church JM. Genotype-defined cancer risk in juvenile polyposis syndrome. Br J Surg. 2015;102:114-118.

Beer A, Streubel B, Asari R, Dejaco C, Oberhuber G. Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) - a rare recently described gastric polyposis syndrome - report of a case. Z Gastroenterol. 2017;55:1131-1134.

Berx G, Cleton-Jansen AM, Nollet F, de Leeuw WJ, van de Vijver M, Cornelisse C, van Roy F. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. EMBO J 1995;14:6107-6115.

Berx G, Staes K, van Hengel J, Molemans F, Bussemakers MJ, van Bokhoven A, van Roy F. Cloning and characterization of the human invasion suppressor gene E-cadherin (*CDH1*). Genomics. 1995;26:281-289.

Bonadona V, Bonaïti B, Olschwang S, Grandjouan S, Huiart L, Longy M, Guimbaud R, Buecher B, Bignon YJ, Caron O, Colas C, Noguès C, Lejeune-Dumoulin S, Olivier-Faivre L, Polycarpe-Osaer F *et al.* Cancer risks associated with germline mutations in *MLH1*, *MSH2*, and *MSH6* genes in Lynch syndrome. JAMA. 2011;305:2304-2310.

Borrmann R. Geschwulste des Magens and Duodenums. In: Henke F, Lubarsch O, editors. Handbuch der Speziellen Pathologischen Anatomie and Histologie. Berlin Springer-Verlag. 1926;865.

Bosman FT, Carneiro F, Hruban RH, Theise ND. WHO Classification of Tumors of the Digestive System. IARC. 2010.

Brierley JD, Gospodarowicz MK, Christian Wittekind C (editors). TNM classification of malignant tumours. Union for International Cancer Control (UICC) 8th edition 2017.

Brooks-Wilson AR, Kaurah P, Suriano G, Leach S, Senz J, Grehan N, Butterfield YS, Jeyes J, Schinas J, Bacani J, Kelsey M, Ferreira P, MacGillivray B, MacLeod P, Micek M *et al.* Germline E-cadherin mutations in hereditary diffuse gastric cancer: assessment of 42 new families and review of genetic screening criteria. J Med Genet. 2004;41:508-517.

Brosens LA, van Hattem A, Hylind LM, Iacobuzio-Donahue C, Romans KE, Axilbund J, Cruz-Correa M, Tersmette AC, Offerhaus GJ, Giardiello FM. Risk of colorectal cancer in juvenile polyposis. Gut. 2007;56:965-967.

Caldas C, Carneiro F, Lynch HT, Yokota J, Wiesner GL, Powell SM, Lewis FR, Huntsman DG, Pharoah PD, Jankowski JA, MacLeod P, Vogelsang H, Keller G, Park KG, Richards FM *et al.* Familial gastric cancer: overview and guidelines for management. J Med Genet. 1999;36:873-880.

Calin G, Ranzani GN, Amadori D, Herlea V, Matei I, Barbanti-Brodano G, Negrini M. Somatic frameshift mutations in the Bloom syndrome *BLM* gene are frequent in sporadic gastric carcinomas with microsatellite mutator phenotype. BMC Genet. 2001;2:14.

Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513:202-209.

Canel M, Serrels A, Frame MC, Brunton VG. E-cadherin-integrin crosstalk in cancer invasion and metastasis. J Cell Sci. 2013;126:393-401.

Casadei S, Norquist BM, Walsh T, Stray S, Mandell JB, Lee MK, Stamatoyannopoulos JA, King MC. Contribution of inherited mutations in the BRCA2-interacting protein PALB2 to familial breast cancer. Cancer Res. 2011;71:2222-2229.

Charlton A, Blair V, Shaw D, Parry S, Guilford P, Martin IG. Hereditary diffuse gastric cancer: predominance of multiple foci of signet ring cell carcinoma in distal stomach and transitional zone. Gut. 2004;53:814-820.

Ciambotti B, Mussolin L, d'Amore ES, Pillon M, Sieni E, Coniglio ML, Ros MD, Cetica V, Aricò M, Rosolen A. Monoallelic mutations of the perforin gene may represent a predisposing factor to childhood anaplastic large cell lymphoma. J Pediatr Hematol Oncol. 2014;36:e359-e365.

Cunniff C, Bassetti JA, Ellis NA. Bloom's Syndrome: Clinical Spectrum, Molecular Pathogenesis, and Cancer Predisposition. Mol Syndromol. 2017;8:4-23.

El Abed R, Bourdon V, Voskoboinik I, Omri H, Youssef YB, Laatiri MA, Huiart L, Eisinger F, Rabayrol L, Frenay M, Gesta P, Demange L, Dreyfus H, Bonadona V, Dugast C *et al.* Molecular study of the perforin gene in familial hematological malignancies. Hered Cancer Clin Pract. 2011;9:9.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr

Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, Gavin A, Visser O, Bray F. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. Eur J Cancer. 2018.

Fewings E, Larionov A, Redman J, Goldgraben MA, Scarth J, Richardson S, Brewer C, Davidson R, Ellis I, Evans DG, Halliday D, Izatt L, Marks P, McConnell V, Verbist L *et al.* Germline pathogenic variants in PALB2 and other cancer-predisposing genes in families with hereditary diffuse gastric cancer without *CDH1* mutation: a whole-exome sequencing study. Lancet Gastroenterol Hepatol. 2018;3:489-498.

Fitzgerald RC, Hardwick R, Huntsman D, Carneiro F, Guilford P, Blair V, Chung DC, Norton J, Ragunath K, Van Krieken JH, Dwerryhouse S, Caldas C; International Gastric Cancer Linkage Consortium. Hereditary diffuse gastric cancer: updated consensus guidelines for clinical management and directions for future research. J Med Genet. 2010;47:436-444.

Frebourg T, Oliveira C, Hochain P, Karam R, Manouvrier S, Graziadio C, Vekemans M, Hartmann A, Baert-Desurmont S, Alexandre C, Lejeune Dumoulin S, Marroni C, Martin C, Castedo S, Lovett M *et al.* Cleft lip/palate and *CDH1*/E-cadherin mutations in families with hereditary diffuse gastric cancer. J Med Genet. 2006;43:138-142.

Gaston D, Hansford S, Oliveira C, Nightingale M, Pinheiro H, Macgillivray C, Kaurah P, Rideout AL, Steele P, Soares G, Huang WY, Whitehouse S, Blowers S, LeBlanc MA, Jiang H *et al.* Germline mutations in *MAP3K6* are associated with familial gastric cancer. PLoS Genet. 2014;10:e1004669.

Giardiello FM, Allen JI, Axilbund JE, Boland CR, Burke CA, Burt RW, Church JM, Dominitz JA, Johnson DA, Kaltenbach T, Levin TR, Lieberman DA, Robertson DJ, Syngal S, Rex DK. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-society Task Force on colorectal cancer. Am J Gastroenterol. 2014;109:1159-1179.

Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Akinyemiju TF, Al Lami FH, Alam T, Alizadeh-Navaei R, Allen C, Alsharif U, Alvis-Guzman N, Amini E, Anderson BO, Aremu O, Artaman A, Asgedom SW, Assadi R *et al.* Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 29 Cancer

Groups, 1990 to 2016: A Systematic Analysis for the Global Burden of Disease Study. JAMA Oncol. 2018.

Goffin J, Eisenhauer E. DNA methyltransferase inhibitors-state of the art. Ann Oncol. 2002;13:1699-1716.

Grady WM, Willis J, Guilford PJ, Dunbier AK, Toro TT, Lynch H, Wiesner G, Ferguson K, Eng C, Park JG, Kim SJ, Markowitz S. Methylation of the *CDH1* promoter as the second genetic hit in hereditary diffuse gastric cancer. Nat Genet. 2000;26:16-17.

Graziano F, Humar B, Guilford P. The role of the E-cadherin gene (*CDH1*) in diffuse gastric cancer susceptibility: from the laboratory to clinical practice. Ann Oncol. 2003;14:1705-1713.

Guilford P, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE. E-cadherin germline mutations in familial gastric cancer. Nature. 1998;392:402-405.

Guilford P, Humar B, Blair V. Hereditary diffuse gastric cancer: translation of *CDH1* germline mutations into clinical practice. Gastric Cancer. 2010;13:1-10.

Hansford S, Kaurah P, Li-Chang H, Woo M, Senz J, Pinheiro H, Schrader KA, Schaeffer DF, Shumansky K, Zogopoulos G, Santos TA, Claro I, Carvalho J, Nielsen C, Padilla S *et al.* Hereditary Diffuse Gastric Cancer Syndrome: *CDH1* Mutations and Beyond. JAMA Oncol. 2015;1:23-32.

Hu B, El Hajj N, Sittler S, Lammert N, Barnes R, Meloni-Ehrig A. Gastric cancer: Classification, histology and application of molecular pathology. J Gastrointest Oncol. 2012;3:251-261.

Huntsman DG, Carneiro F, Lewis FR, MacLeod PM, Hayashi A, Monaghan KG, Maung R, Seruca R, Jackson CE, Caldas C. Early gastric cancer in young, asymptomatic carriers of germ-line E-cadherin mutations. N Engl J Med. 2001;344:1904-1909.

Jonsson BA, Bergh A, Stattin P, Emmanuelsson M, Grönberg H. Germline mutations in E-cadherin do not explain association of hereditary prostate cancer, gastric cancer and breast cancer. Int J Cancer. 2002;98:838-843.

Kallakury BV, Sheehan CE, Winn-Deen E, Oliver J, Fisher HA, Kaufman RP Jr, Ross JS. Decreased expression of catenins (alpha and beta), p120 CTN, and E-cadherin cell adhesion proteins and E-cadherin gene promoter methylation in prostatic adenocarcinomas. Cancer. 2001;92:2786-2795.

Kaurah P, Huntsman DG. Hereditary Diffuse Gastric Cancer. GeneReviews. 2002 [updated 2018].

Kaurah P, MacMillan A, Boyd N, Senz J, De Luca A, Chun N, Suriano G, Zaor S, Van Manen L, Gilpin C, Nikkel S, Connolly-Wilson M, Weissman S,

Rubinstein WS, Sebold C *et al.* Founder and recurrent *CDH1* mutations in families with hereditary diffuse gastric cancer. JAMA. 2007;297:2360-2372.

Kluska A, Balabas A, Paziewska A, Kulecka M, Nowakowska D, Mikula M, Ostrowski J. New recurrent BRCA1/2 mutations in Polish patients with familial breast/ovarian cancer detected by next generation sequencing. BMC Med Genomics. 2015;8:19.

Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA. 1971;68:820-823.

Kobielak A, Fuchs E. Alpha-catenin: at the junction of intercellular adhesion and actin dynamics. Nat Rev Mol Cell Biol. 2004;5:614-625.

Kohlmann W, Gruber SB. Lynch Syndrome. GeneReviews. 2004 [updated 2018].

Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009;4:1073-1081.

Lakin ND, Weber P, Stankovic T, Rottinghaus ST, Taylor AM, Jackson SP. Analysis of the ATM protein in wild-type and ataxia telangiectasia cells. Oncogene. 1996;13:2707-2716.

Larsen Haidle J, Howe JR. Juvenile Polyposis Syndrome. GeneReviews. 2003 [updated 2017].

Latchford AR, Neale K, Phillips RK, Clark SK. Juvenile polyposis syndrome: a study of genotype, phenotype, and long-term outcome. Dis Colon Rectum. 2012;55:1038-1043.

Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal- type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand. 1965;64:31-49.

Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25:1754-1760.

Li J, Woods SL, Healey S, Beesley J, Chen X, Lee JS, Sivakumaran H, Wayte N, Nones K, Waterfall JJ, Pearson J, Patch AM, Senz J, Ferreira MA, Kaurah P *et al.* Point Mutations in Exon 1B of APC Reveal Gastric Adenocarcinoma and Proximal Polyposis of the Stomach as a Familial Adenomatous Polyposis Variant. Am J Hum Genet. 2016;98:830-842.

Ma K, Baloch Z, He TT, Xia X. Alcohol Consumption and Gastric Cancer Risk: A Meta-Analysis. Med Sci Monit. 2017;23:238-246.

Machado JC, Oliveira C, Carvalho R, Soares P, Berx G, Caldas C, Seruca R, Carneiro F, Sobrinho-Simões M. E-cadherin gene (*CDH1*) promoter methylation as the second hit in sporadic diffuse gastric carcinoma. Oncogene. 2001;20:1525-1528.

Majewski IJ, Kluijt I, Cats A, Scerri TS, de Jong D, Kluin RJ, Hansford S, Hogervorst FB, Bosma AJ, Hofland I, Winter M, Huntsman D, Jonkers J, Bahlo M, Bernards R. An α-E-catenin (*CTNNA1*) mutation in hereditary diffuse gastric cancer. J Pathol. 2013;229:621-629.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010;20:1297-1303.

Meindl A; German Consortium for Hereditary Breast and Ovarian Cancer. Comprehensive analysis of 989 patients with breast or ovarian cancer provides BRCA1 and BRCA2 mutation profiles and frequencies for the German population. Int J Cancer. 2002;97:472-480.

Molinaro V, Pensotti V, Marabelli M, Feroce I, Barile M, Pozzi S, Laghi L, Serrano D, Bernard L, Bonanni B, Ranzani GN. Complementary molecular approaches reveal heterogeneous *CDH1* germline defects in Italian patients with hereditary diffuse gastric cancer (HDGC) syndrome. Genes Chromosomes Cancer. 2014;53:432-445.

Oliveira C, Senz J, Kaurah P, Pinheiro H, Sanges R, Haegert A, Corso G, Schouten J, Fitzgerald R, Vogelsang H, Keller G, Dwerryhouse S, Grimmer D, Chin SF, Yang HK *et al.* Germline *CDH1* deletions in hereditary diffuse gastric cancer families. Hum Mol Genet. 2009;18:1545-1555.

Oliveira C, Suriano G, Ferreira P, Canedo P, Kaurah P, Mateus R, Ferreira A, Ferreira AC, Oliveira MJ, Figueiredo C, Carneiro F, Keller G, Huntsman D, Machado JC, Seruca R. Genetic screening for familial gastric cancer. Hered Cancer Clin Pract. 2004;2:51-64.

Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FB, Hoogerbrugge N, Spurdle AB, Tavtigian SV; IARC Unclassified Genetic Variants Working Group. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. Hum Mutat. 2008;29:1282-1291.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17:405-424.

Riihimäki M, Hemminki A, Sundquist K, Sundquist J, Hemminki K. Metastatic spread in patients with gastric cancer. Oncotarget. 2016;7:52307-52316.

Sahasrabudhe R, Lott P, Bohorquez M, Toal T, Estrada AP, Suarez JJ, Brea-Fernández A, Cameselle-Teijeiro J, Pinto C, Ramos I, Mantilla A, Prieto R, Corvalan A, Norero E, Alvarez C *et al.* Germline Mutations in *PALB2*, *BRCA1*,

and *RAD51C*, Which Regulate DNA Recombination Repair, in Patients With Gastric Cancer. Gastroenterology. 2017;152:983-986.

Santini V, Kantarjian HM, Issa JP. Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications, Annals of Internal Medicine, 2001;134:573-586.

Saviozzi S, Saluto A, Piane M, Prudente S, Migone N, DeMarchi M, Brusco A, Chessa L. Six novel ATM mutations in Italian patients with classical ataxia-telangiectasia. Hum Mutat. 2003;21:450.

Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002;30:e57.

Shinmura K, Kohno T, Takahashi M, Sasaki A, Ochiai A, Guilford P, Hunter A, Reeve AE, Sugimura H, Yamaguchi N, Yokota J. Familial gastric cancer: clinicopathological characteristics, RER phenotype and germline p53 and E-cadherin mutations. Carcinogenesis 1999;20:1127-1131.

Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, Henter JI, Bennett M, Fischer A, de Saint Basile G, Kumar V. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science. 1999;286:1957-1959.

Tan P, Yeoh KG. Genetics and Molecular Pathogenesis of Gastric Adenocarcinoma. Gastroenterology. 2015;149:1153-1162.e3.

To KF, Leung WK, Lee TL, Yu J, Tong JH, Chan MW, Ng EK, Chung SC, Sung JJ. Promoter hypermethylation of tumor-related genes in gastric intestinal metaplasia of patients with and without gastric cancer. Int J Cancer. 2002;102:623-628.

Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65:87-108.

van der Post RS, Vogelaar IP, Carneiro F, Guilford P, Huntsman D, Hoogerbrugge N, Caldas C, Schreiber KE, Hardwick RH, Ausems MG,Bardram L, Benusiglio PR, Bisseling TM, Blair V, Bleiker E *et al.* Hereditary diffuse gastric cancer: updated clinical guidelines with an emphasis on germline *CDH1* mutation carriers. J Med Genet. 2015;52:361-374.

Vogelaar IP, Ligtenberg MJ, van der Post RS, de Voer RM, Kets CM, Jansen TJ, Jacobs L, Schreibelt G; International Gastric Cancer Genetics Group, de Vries IJ, Netea MG, Hoogerbrugge N. Recurrent candidiasis and early-onset gastric cancer in a patient with a genetically defined partial MYD88 defect. Fam Cancer. 2016;15:289-296.

Vogelaar IP, van der Post RS, van Krieken JHJ, Spruijt L, van Zelst-Stams WA, Kets CM, Lubinski J, Jakubowska A, Teodorczyk U, Aalfs CM, van Hest LP, Pinheiro H, Oliveira C, Jhangiani SN, Muzny DM *et al.* Unraveling genetic

predisposition to familial or early onset gastric cancer using germline whole-exome sequencing. Eur J Hum Genet. 2017;25:1246-1252.

Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010;38:e164.

Wang K, Yuen ST, Xu J, Lee SP, Yan HH, Shi ST, Siu HC, Deng S, Chu KM, Law S, Chan KH, Chan AS, Tsui WY, Ho SL, Chan AK *et al.* Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. Nat Genet. 2014;46:573-582.

Weren RDA, van der Post RS, Vogelaar IP, van Krieken JH, Spruijt L, Lubinski J, Jakubowska A, Teodorczyk U, Aalfs CM, van Hest LP, Oliveira C, Kamping EJ, Schackert HK, Ranzani GN, Gómez García EB *et al.* Role of germline aberrations affecting *CTNNA1*, *MAP3K6* and *MYD88* in gastric cancer susceptibility. J Med Genet. 2018.

Wijnen J, van der Klift H, Vasen H, Khan PM, Menko F, Tops C, Meijers Heijboer H, Lindhout D, Møller P, Fodde R. MSH2 genomic deletions are a frequent cause of HNPCC. Nat Genet. 1998;20:326-328.

Worthley DL, Phillips KD, Wayte N, Schrader KA, Healey S, Kaurah P, Shulkes A, Grimpen F, Clouston A, Moore D, Cullen D, Ormonde D, Mounkley D, Wen X, Lindor N *et al.* Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS): a new autosomal dominant syndrome. Gut. 2012;61:774-779.

Zali H, Rezaei-Tavirani M, Azodi M. Gastric cancer: prevention, risk factors and treatment. Gastroenterol Hepatol Bed Bench. 2011;4:175-185.

List of original manuscripts

Gianluca Tedaldi, Valentina Zampiga, Michela Tebaldi, Rita Danesi, Valentina Arcangeli, Mila Ravegnani, Ilaria Cangini, Francesca Pirini, Raefa Abou Khouzam, Chiara Molinari, Dino Amadori, Fabio Falcini, Guglielmina Nadia Ranzani, Daniele Calistri. Multigene panel testing increases the number of loci associated with gastric cancer predisposition.
Manuscript in preparation

Simone Giacopuzzi, Maria Bencivenga, Lorenzo Scorsone, Gianluca Tedaldi, Daniele Marrelli, Anna Tomezzoli, Giuseppe Verlato, Giovanni De Manzoni, Franco Roviello. Results of *CDH1* germline mutation screening in a large Clinical Centre.
Manuscript in preparation

Part two

Germline alterations in hereditary breast cancer

Abstract

With the advent of NGS-based approaches, new genes have emerged as breast and ovarian cancer (BC/OC) susceptibility genes and their number is progressively increasing due to clinical applications of multigene panel testing for BC/OC risk assessment. The identification of a large number of new variants in high/medium penetrant genes allows for deeper knowledge of cancer predisposition, although raising questions about genetic counseling and patient management.

To search for germline variants predisposing to cancer, we perfomed a study on 255 BC/OC patients referring to the genetic counseling at the Cancer Prevention Unit of the Morgagni-Pierantoni Hospital (Forlì-Italy) in the years from 2012 through 2015. We collected clinical features and cancer family history of patients, as well as peripheral blood samples to extract DNA. DNA samples were utilized to screen for germline variants in 94 genes associated with predisposition towards common and rare cancers.

Among the 255 analyzed patients, 57 proved to carry pathogenic variants in *BRCA1/2* genes, while 17 proved to carry pathogenic variants in other genes, including *PALB2*, *ATM*, *BRIP1*, *RAD51D*, *MSH6*, *PPM1D*, *RECQL4*, *ERCC3*, *TSC2*, *SLX4* and other Fanconi anemia genes.

Patients with pathogenic variants in genes other than *BRCA1/2* did not show significant differences from *BRCA1/2*-mutation carriers with respect to age at cancer diagnosis and clinical features, suggesting that these variants are likely to be associated with a high risk of BC/OC development. Of relevance, patients with variants in genes other than *BRCA1/2* showed a much higher frequency of bilateral breast cancer (BBC) and a lower frequency of OC compared to *BRCA1/2*-mutation carriers and to patients without pathogenic mutations. On this basis, surveillance protocols should be customized accordingly to patient-specific genetic backgrounds.

Abbreviations

ACMG: American College of Medical Genetics
BASC: BRCA1-associated genome surveillance complex
BBC: bilateral breast bancer
BC: breast cancer
BIC: Breast Cancer Information Core
BQSR: Base Quality Score Recalibration
DBC: ductal breast cancer
DCIS: ductal carcinoma in situ
CA15-3: carcinoma antigen 15-3
CA125: carcinoma antigen 125
CEA: carcinoembryonic antigen
CT: computed tomography
ER: estrogen receptor
FA: Fanconi anemia
FDA: Food and Drug Administration
FONCaM: Forza Operativa Nazionale sul Carcinoma Mammario
HBOC: Hereditary Breast and Ovarian Cancer
HER2: human epidermal growth factor receptor 2
HR: homologous recombination
IARC: International Agency for Research on Cancer
IDC: invasive ductal carcinoma
ILC: invasive lobular carcinoma
LBC: lobular breast cancer
LCIS: lobular carcinoma in situ
LOVD: Leiden Open Variation Database
MBC: male breast cancer
MLPA: Multiplex Ligation-dependent Probe Amplification
MMR: mismatch repair
MRI: magnetic resonance imaging
MRN: MRE11-RAD50-NBS1 complex
NGS: next-generation sequencing
OC: ovarian cancer
OMIM: Online Mendelian Inheritance in Men
PARP: poly ADP ribose polymerase
PCR: polymerase chain reaction
PET: positron-emission tomography
PR: progesterone receptor
SLNB: sentinel lymph node biopsy TDLU: terminal ductal-lobular unit
TDLU: terminal ductal-lobular unit

TNBC: triple negative breast cancer
TNM: tumor, node, metastasis
VUS: variant of uncertain significance
WES: whole-exome sequencing
WGS: whole-genome sequencing
WHO: World Health Organization

1. Introduction

1.1 Epidemiology of breast cancer

In 2016 breast cancer (BC) globally ranked third for cancer incidence and fifth for cancer deaths, with 1.702.000 incident cases and 546.000 deaths having occurred. Moreover, it was the first most common cancer and the first cause of cancer-related death among women [Global Burden of Disease Cancer Collaboration *et al.* 2018]. Incidence rates are high in Northern America, Australia/New Zealand, and Northern and Western Europe, intermediate in Central and Eastern Europe, Latin America and the Caribbean, and low in most countries of Africa and Asia (Figure 1) [Torre LA *et al.* 2015].

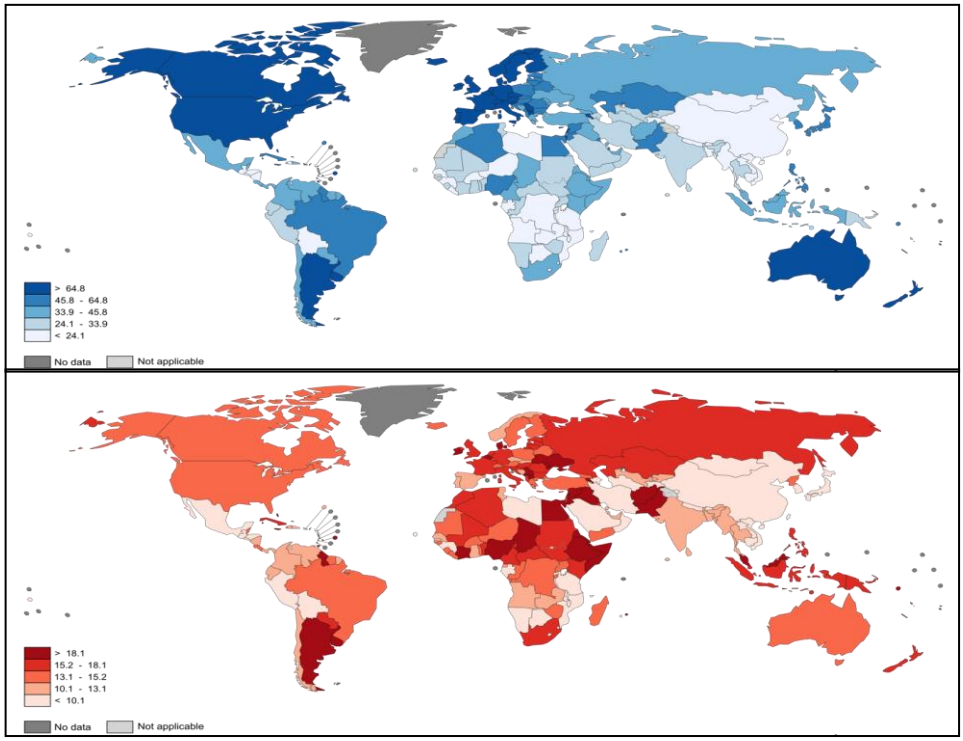


Fig 1 Global estimated age-standardised incidence (upper panel) and mortality (lower panel) per 100.000 women in 2012 [Ferlay J *et al.* 2013].

Male breast cancer (MBC) accounts for less than 1% of all BCs [Giordano SH *et al.* 2002; Ruddy KJ *et al.* 2013]. MBC is frequently diagnosed at more advanced stages compared to women BC, but prognostic factors and survival rates are similar for cancers at the same age and stage of progression.
In Europe, it is estimated that 523.000 women will develop BC and 138.000 will die for the disease in 2018, placing BC at the first position for incidence and mortality in women [Ferlay J *et al.* 2018].
In Italy, it is estimated that about 50.000 new cases of BC are diagnosed every year, confirming BC as the most common cancer also in Italian women [AIOM-AIRTUM 2017]. Of relevance, in Italy the overall incidence of BC is slightly increasing (+ 0,9% per year), with an estimated number of women living with BC of 767.000. BC also represents the first cause of cancer death among Italian women, with 12.201 deaths occurred in 2014 [AIOM-AIRTUM 2017]; however, the mortality is now significantly decreasing (-2.2% per year) tanks to the screening programs allowing early cancer detection, and to the improvement of therapeutic programs.

1.2 Breast cancer risk factors

As for other cancers, BC is the result of a combination of environmental and genetic risk factors.
The main risk factors for BC are:

- age: BC risk increases exponentially with age until the menopause, when it reaches a plateau, and increases again after 60 years of age; this is likely due to stimulation by hormones until the menopause of breast epithelium proliferation and then to the accumulation of DNA damages with aging;
- diet: high consumption of animal fats and low consumption of vegetables are associated with a higher risk;
- tobacco smoking: cigarette smoke contains potent carcinogens and both active and passive exposure to it are linked to a higher risk;
- obesity and lack of physical exercise: a sedentary lifestyle, often associated with overweight, is associated with a higher risk;
- alcohol consumption: alcohol increases the level of estrogen and other hormones associated with BC and can also induce DNA damages;
- hormonal and reproductive factors: a long fertile period (with an early menarche and a late menopause), nulliparity or late pregnancies, lack of breastfeeding, use of oral contraceptives or

hormone replacement therapy are all associated with an increased disease risk;

- exposure to specific carcinogens: the exposure to ionizing radiations or chemical compounds, such as xenoestrogens and aromatic amines, is associated with an increased risk;
- genetic predisposition: about 10-30% of BCs show familial clustering and 5-10% of cases are estimated to be hereditary [Gage M *et al.* 2012].

1.3 Classification of breast cancers

The breasts are grandular organs secreting milk to feed infants. Breasts are present both in males and females, but in females, at puberty, estrogens cause breast development both in the adipous and glandular components and, after childbirth, prolactin hormone promotes milk secretion from mammary glands for lactation and breastfeeding.
The breast is made up of subcutaneous fat that envelops a network of lobules and ducts that converge on the nipple, a raised region of tissue on the surface of the breast, surrounded by a pigmented area of skin called areola (Figure 2). The lobules are clusters of alveoli, small cavities where the milk is produced, stored and drained through the lactiferous ducts out of the nipples.

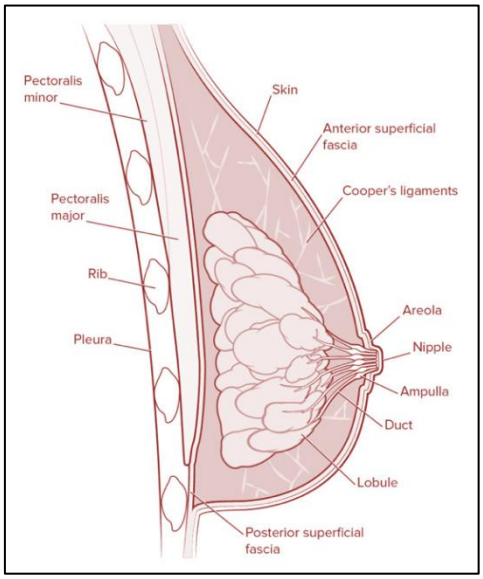


Fig. 2. Schematic representation of human breast anatomy

1.3.1 Histopathological classification of breast cancers

BC originates from the epithelial cells of the mammary glands, giving rise to different types of tumors, the most common of which are ductal breast cancer (DBC) and lobular breast cancer (LBC). These two terms have been coined some decades ago, acknowledging that the site of origin of DBC was in the ducts, while and the site of origin LBC was in the lobules. However, the majority of BCs actually originate in the terminal ductal-lobular units (TDLU) and then evolve to different types by mechanisms not yet fully clarified. For both DBC and LBC there is a non-invasive (*in situ*) and an invasive (infiltrating) variant. Accordingly, there are four main types of breast neoplasms (Figure 3):

- **ductal carcinoma *in situ* (DCIS:** 85% of *in situ* carcinomas**)**: area of abnormal cells growing in the breast ducts without traversing the basement membrane;
- **lobular carcinoma in situ (LCIS:** 9% of in situ carcinomas**)**: area of abnormal cells growing in the breast lobules without traversing the basement membrane;
- **invasive ductal carcinoma (IDC:** 80% of invasive carcinomas**)**: area of cancer cells growing in the breast ducts invading the surrounding tissues;
- **invasive lobular carcinoma (ILC:**10% of invasive carcinomas**)**: area of cancer cells growing in the breast lobules invading the surrounding tissues.

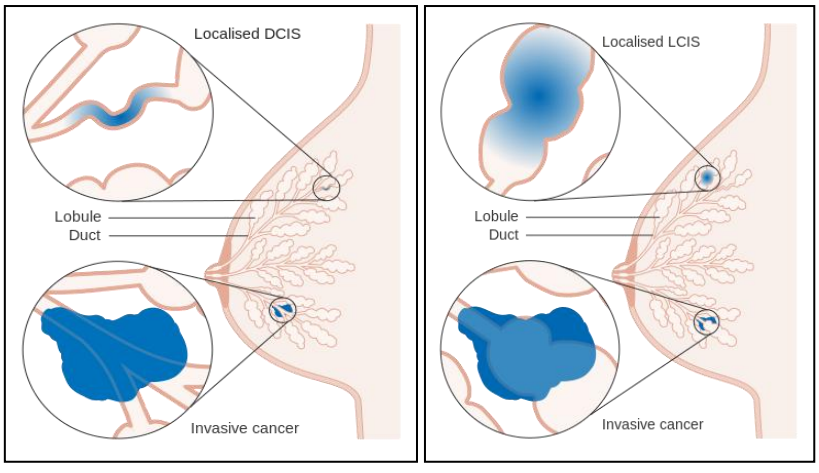


Fig. 3. Schematic representation of: ductal carcinoma in situ, and invasive ductal carcinoma (left panel); lobular carcinoma in situ, and invasive lobular carcinoma (right panel).

The World Health Organization (WHO) has recently updated the detailed classification of all the different histotypes of breast tumors, as reported in the following table [Lakhani SR *et al.* 2012]:

EPITHELIAL TUMORS

Invasive breast carcinomas
Invasive carcinoma of no special type
Invasive lobular carcinoma (ILC)
Tubular carcinoma
Cribriform carcinoma
Mucinous carcinoma
Carcinoma with medullary features
Carcinoma with apocrine differentiation
Carcinoma with signet-ring-cell differentiation
Invasive micropapillary carcinoma
Metaplastic carcinoma of no special type

Rare types
Carcinoma with neuroendocrine features
Secretory carcinoma
Invasive papillary carcinoma
Acinic cell carcinoma
Mucoepidermoid carcinoma
Polymorphous carcinoma
Oncocytic carcinoma
Lipid-rich carcinoma
Glycogen-rich clear cell carcinoma
Sebaceous carcinoma
Salivary gland/skin adnexal tumors
Epithelial-myoepithelial tumors

Precursor lesions
Ductal carcinoma *in situ* (DCIS)
Lobular neoplasia:
Lobular carcinoma *in situ* (LCIS):
- Classic lobular carcinoma *in situ*
- Pleomorphic lobular carcinoma *in situ*

Atypical lobular hyperplasia

Intraductal proliferative lesions Usual ductal hyperplasia Columnar cell lesions including flat epithelial atypia Atypical ductal hyperplasia **Papillary lesions** Intraductal papilloma Intraductal papillary carcinoma Encapsulated papillary carcinoma Solid papillary carcinoma **Benign epithelial proliferations** Sclerosing adenosis Apocrine adenosis Microglandular adenosis Radial scar/Complex Sclerosing Lesion Adenomas
MESENCHYMAL TUMORS
FIBROEPITHELIAL TUMORS Fibroadenoma Phyllodes tumor Amartoma
TUMORS OF THE NIPPLE Adenoma of the nipple Syringomatous adenoma of the nipple Paget's disease of the nipple
MALIGNANT LYMPHOMAS
METASTATIC TUMORS
MALE BREAST TUMORS Gynecomastia Invasive carcinoma Carcinoma *in situ*
SPECIAL TYPES Microinvasive carcinoma Inflammatory carcinoma Bilateral breast carcinoma (BBC)

1.3.2 Molecular classification of breast cancers

BC is a heterogeneous disease and tumors with similar clinicopathological features can be quite different from the molecular point of view.
In clinical practice, BCs are classified in different subtypes by immunohistochemical analysis of the following markers: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and antigen Ki-67, a nuclear protein associated to cellular proliferation. This analysis identifies four main subtypes of invasive carcinoma [van de Vijver MJ *et al.* 2002], subsequently revised by the St Gallen International Expert Consensus [Goldhirsch A *et al.* 2013]:

- **luminal A:** cancers with expression of hormone receptors (ER/PR-positive), low levels of Ki-67, and HER2-negative;
- **luminal B:** carcers with expression of hormone receptors (ER/PR-positive), high levels of Ki-67, HER2-overexpression (luminal B1) or HER2-negative (luminal B2);
- **HER2-positive:** cancers without expression of hormone receptors (ER/PR-negative), with overexpressed or amplified HER2;
- **basal-like:** cancers without expression of hormone receptors (ER/PR-negative), and HER2-negative.

Basal-like BCs are usually referred as Triple Negative Breast Cancers (TNBC); of note, some cases with low-positive ER staining can cluster with basal-like BCs and TNBCs also include some special histological types such as adenoid cystic carcinoma [Prat A *et al.* 2013].
In 2012, The Cancer Genome Atlas (TCGA), a project to catalogue genetic mutations responsible for cancer, published the results of a comprehensive molecular characterization (whole exome sequencing, array-based somatic copy number analysis, array-based DNA methylation profiling, messenger RNA sequencing, microRNA sequencing and reverse-phase protein array) of 466 primary BCs [Cancer Genome Atlas Research Network 2012]. This extensive analysis introduced new highlights on genomic, clinical and proteomic features of BC subtypes (Figure 4).

Subtype	Luminal A	Luminal B	Basal-like	HER2E
ER$^+$/HER2$^-$ (%)	87	82	10	20
HER2$^+$ (%)	7	15	2	68
TNBCs (%)	2	1	80	9
TP53 pathway	*TP53* mut (12%); gain of *MDM2* (14%)	*TP53* mut (32%); gain of *MDM2* (31%)	*TP53* mut (84%); gain of *MDM2* (14%)	*TP53* mut (75%); gain of *MDM2* (30%)
PIK3CA/PTEN pathway	*PIK3CA* mut (49%); *PTEN* mut/loss (13%); *INPP4B* loss (9%)	PIK3CA mut (32%) PTEN mut/loss (24%) INPP4B loss (16%)	PIK3CA mut (7%); PTEN mut/loss (35%); INPP4B loss (30%)	PIK3CA mut (42%); PTEN mut/loss (19%); INPP4B loss (30%)
RB1 pathway	Cyclin D1 amp (29%); *CDK4* gain (14%); low expression of *CDKN2C*; high expression of *RB1*	Cyclin D1 amp (58%); *CDK4* gain (25%)	*RB1* mut/loss (20%); cyclin E1 amp (9%); high expression of *CDKN2A*; low expression of *RB1*	Cyclin D1 amp (38%); *CDK4* gain (24%)
mRNA expression	High ER cluster; low proliferation	Lower ER cluster; high proliferation	Basal signature; high proliferation	HER2 amplicon signature; high proliferation
Copy number	Most diploid; many with quiet genomes; 1q, 8q, 8p11 gain; 8p, 16q loss; 11q13.3 amp (24%)	Most aneuploid; many with focal amp; 1q, 8q, 8p11 gain; 8p, 16q loss; 11q13.3 amp (51%); 8p11.23 amp (28%)	Most aneuploid; high genomic instability; 1q, 10p gain; 8p, 5q loss; *MYC* focal gain (40%)	Most aneuploid; high genomic instability; 1q, 8q gain; 8p loss; 17q12 focal *ERRB2* amp (71%)
DNA mutations	*PIK3CA* (49%); *TP53* (12%); *GATA3* (14%); *MAP3K1* (14%)	*TP53* (32%); *PIK3CA* (32%); *MAP3K1* (5%)	*TP53* (84%); *PIK3CA* (7%)	*TP53* (75%); *PIK3CA* (42%); *PIK3R1* (8%)
DNA methylation	–	Hypermethylated phenotype for subset	Hypomethylated	–
Protein expression	High oestrogen signalling; high MYB; RPPA reactive subtypes	Less oestrogen signalling; high FOXM1 and MYC; RPPA reactive subtypes	High expression of DNA repair proteins, PTEN and INPP4B loss signature (pAKT)	High protein and phospho-protein expression of EGFR and HER2

Percentages are based on 466 tumour overlap list. Amp, amplification; mut, mutation.

Fig. 4. Summary of TCGA general findings on BC subtypes

1.4 Clinical manifestations and diagnosis of breast cancer

The first symptom usually detected is the presence of a lump in the breast. However, in more than 80% of cases the lump is the sign of a benign disease such as mastitis and fibroadenoma of the breast. A hard lump with irregular margins can be a sign of malignancy. Other symptoms can be present, including skin dimpling, edema, axillary lymphadenopathy, changes in skin color or texture, alterations in the nipple position or shape, and clear or bloody fluid that leaks out of the nipple. Breast pain is not a common symptom of cancer, especially in young women, and is usually associated with benign breast diseases.

Early diagnosis is one of the most important aspects for successful treatment of the disease. The breast self-examination is not a sensitive method for the identification of lesions in a preclinical phase; however, if correctly performed, it has an important role in early detection of the disease. Accordingly, it is advisable that women know how to correctly perform self-examination, at the end of each menstrual cycle or monthly for the women in menopause. Clinical examination can occasionally detect some signs of malignancy (hard lump with irregular margins, dimpling of the skin, edema, secretion of blood or serum, alteration of the nipple, axillary lymphadenopathy).

Mammography is a technique that uses low-energy X-rays to examine the whole breast. This is the exam with the highest sensitivity for BC detection, especially for cancers in the early stages when a lump is not yet perceptible. However, mammography has proven to be inadeguate for BC diagnosis in young women and in women with dense breasts.

Breast ultrasonography is complementary to mammography: it is performed in the presence of a palpable abnormality, or when a previous mammogram has revealed a focal lesion, to verify if the lump is a solid mass or a fluid-filled cyst. This exam is particularly useful in cases of a dense breast.
Breast magnetic resonance imaging (MRI) is complementary to mammography and ultrasonography and is based on the use of magnetic fields to produce a detailed image of the breast after the injection of a contrast medium. MRI can be used after a woman has been diagnosed with cancer to check the other breast for cancer or to find out how much the disease has grown throughout the breast. It may also be used before surgery to find out if chemotherapy is able to shrink the tumor. MRI is also a screening option for women with a very high risk of developing breast cancer.
The definitive BC diagnosis is usually obtained by a biopsy, a procedure during which a needle is guided to the location with the help of an imaging technique, such as mammography, ultrasound, or MRI, and a tissue sample is picked up for a subsequent pathological analysis.
Furthermore, the computed tomography (CT) scan, the positron-emission tomography (PET) and the radiological investigation of the chest are useful tools to evaluate the cancer spread in the nearby tissues and the presence of metastases.
It is also possible to perform blood tests to search for tumor markers, including carcinoembryonic antigen (CEA), carcinoma antigen 15-3 (CA15-3), and carcinoma antigen 125 (CA125). Although these markers are not useful in the initial diagnosis of BC, they are important during the follow-up, since their level increase is highly predictive of disease relapse.

1.5 Evolution of breast cancer

BC, during its development, tends to grow invading the chest wall, to spread to the regional lymph nodes and finally to generate metastases in distant organs.
As for other cancers, the TNM (tumor, node, metastasis) classification of malignant tumors is a globally recognised standard classification for measuring the extent of cancer spread [Brierley JD *et al.* 2017].
It is based on the combination of three factors:

- **T (tumor)** describes the size of the primary tumor and the invasion of nearby tissues:
  - TX: size and invasion of the primary tumor cannot be assessed;

- T0: no evidence of primary tumor;
- Tis: cancer cells are growing in the epithelium without infiltrating the basement membrane (carcinoma *in situ*);
- T1: the cancer is 2 cm or less; in particular, the cancer is 0.1 cm or less (T1mi), or is more than 0.1 cm but no more than 0.5 cm (T1a), or is more than 0.5 cm but no more than 1 cm (T1b), or is more than 1 cm but no more than 2 cm (T1c);
- T2: the cancer is more than 2 cm but no more than 5 cm across;
- T3: the cancer is bigger than 5 cm across;
- T4: the cancer has spread into the skin (T4a), underlying chest wall (T4b) or both (T4c), or is an inflammatory carcinoma in which the overlying skin is red, swollen and painful (T4d).

- **N (node)** describes the involvement of the regional lymph nodes:
  - NX: the presence of metastases in the regional lymph nodes cannot be assessed;
  - N0: absence of metastases in the regional lymph nodes;
  - N1: the cancer has spread into the lymph nodes of the armpit generating micrometastases that are 0.2-2 mm (N1mi), or the cancer has spread into 1-3 lymph nodes and at least one is larger than 2 mm (N1a), or into the lymph nodes behind the breastbone (N1b), or into 1-3 lymph nodes in the armpit and in the lymph nodes behind the breastbone (N1c);
  - N2: the cancer has reached the lymph nodes of the armpit (N2a), or the lymph nodes behind the breast bone (N2b);
  - N3: the cancer has reached the lymph nodes below the collarbone (N3a), or the lymph nodes in the armpit and behind the breastbone (N3b), or the lymph nodes above the collarbone (N3c).

- **M (metastasis)** describes the presence of distant metastases:
  - MX: the presence of metastases cannot be assessed;
  - M0: absence of distant metastases;
  - M1: presence of distant metastases.

Of note, there are different types of TNM classification depending on the time of assessment and on the technique used to assess the stage.

Cancer staging is a classification of BC based on cancer size and spread that identifies 5 stages, some of which are further divided into sub-stages (Figure 5):

- **stage 0**: there are abnormal cells in the epithelium, but they are not invading neighboring normal tissue (Tis, N0, M0);

- **stage IA**: the cancer is 2 cm or smaller and has not spread outside the breast (T1, N0, M0);

- **stage IB** means one of the following:
  - there is no evidence of primary tumor but there are micrometastases (0.2-2 mm) in the lymph nodes of the armpit (T0, N1mi, M0);
  - the cancer is less than 2 cm and there are micrometastases (0.2-2 mm) in the lymph nodes of the armpit (T1, N1mi, M0);

- **stage IIA** means one of the following:
  - there is no evidence of primary tumor but there are metastases in 1-3 lymph nodes of the armpit (T0, N1, M0);
  - the cancer is less than 2 cm and there are metastases in 1-3 lymph nodes of the armpit (T1, N1, M0);
  - the cancer is 2-5 cm but there are no metastases in the lymph nodes of the armpit (T2, N0, M0);

- **stage IIB** means one of the following:
  - the cancer is 2-5 cm and there are metastases in 1-3 lymph nodes of the armpit (T2, N1, M0);
  - the cancer is bigger than 5 cm but there are no metastases in the lymph nodes of the armpit (T3, N0, M0);

- **stage IIIA** means one of the following:
  - there is no evidence of primary tumor but there are metastases in 4-9 lymph nodes of the armpit (T0, N2, M0);
  - the cancer is less than 2 cm and there are metastases in 4-9 lymph nodes of the armpit (T1, N2, M0);
  - the cancer is 2-5 cm and there are metastases in 4-9 lymph nodes of the armpit (T2, N2, M0);
  - the cancer is bigger than 5 cm and there are metastases in 1-3 lymph nodes of the armpit (T3, N1, M0);
  - the cancer is bigger than 5 cm and there are metastases in 4-9

lymph nodes of the armpit (T3, N2, M0);

- **stage IIIB** means one of the following:
  - the cancer has spread to the chest wall and/or skin of the breast but there are no metastases in the lymph nodes of the armpit (T4, N0, M0);
  - the cancer has spread to the chest wall and/or skin of the breast and there are metastases in 1-3 lymph nodes of the armpit (T4, N1, M0);
  - the cancer has spread to the chest wall and/or skin of the breast and there are metastases in 4-9 lymph nodes of the armpit (T4, N2, M0);

- **stage IIIC**: the cancer has generated metastases in 10 or more lymph nodes of the armpit (any T, N3, M0);

- **stage IV**: the cancer has generated metastases in distant organs as bones, lungs, liver or brain (any T, any N, M1).

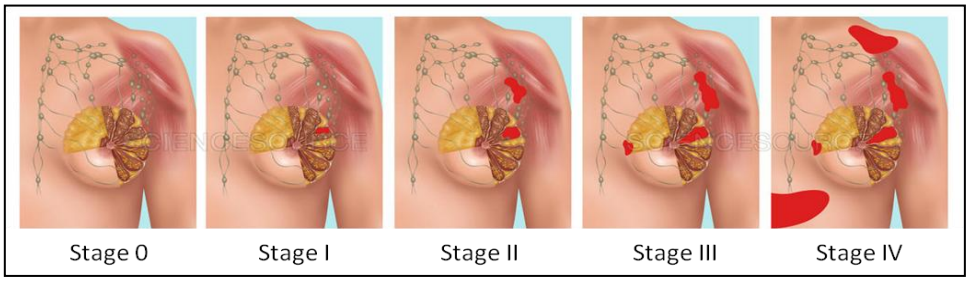


Fig. 5. Schematic representation of the 5 stages of BC (cancer cells in red).

According to this classification the 5 stages can be summarized as reported in the following table:

STAGE	TNM
Stage 0	Tis N0 M0
Stage IA	T1 N0 M0
Stage IB	T0 N1mi M0 T1 N1mi M0
Stage IIA	T0 N1 M0 T1 N1 M0 T2 N0 M0

Stage IIB	T2 N1 M0 T3 N0 M0
Stage IIIA	T0 N2 M0 T1 N2 M0 T2 N2 M0 T3 N1 M0 T3 N2 M0
Stage IIIB	T4 N0 M0 T4 N1 M0 T4 N2 M0
Stage IIIC	any T N3 M0
Stage IV	any T any N M1

In pathology, the tumor grading, also called the Broder's classification, represents a measure of the degree of tumor cell differentiation. Accordingly, it is a measure of the tumor aggressiveness and is different from staging, that is a measure of tumor spread.
The most used BC grading system includes 3 different grades:

- **G1:** well differentiated (low grade), with cells that appear very similar to normal cells and grow slowly;
- **G2:** moderately differentiated (intermediate grade), with intermediate growing cells that are slightly different from normal cells;
- **G3:** poorly differentiated (high grade), with fast growing cancer cells that look quite different from normal cells.

Tumor grading and cancer staging are used together to evaluate the best treatment strategy and to predict prognosis for each cancer patient.

1.6 Breast cancer treatment

The BC therapeutic choice depends on various factors, including cancer stage and age of patient. BC is usually treated with surgery, that can be followed by chemotherapy and/or radiotherapy.
The surgery implies the removal of cancer and surrounding tissues. In particular, during surgery, a sentinel lymph node biopsy (SLNB) is usually performed. The surgeon injects a radioactive substance or a blue dye near the tumor to locate the sentinel lymph node, *i.e.* the first lymph node to which cancer cells are most likely to spread from the primary tumor. The

sentinel lymph node is then removed and examined by a pathologist to verify the presence of cancer cells. If the sentinel lymph node is cancer-free, only the cancer is removed; on the contray, if the sentinel lymph node contains cancer cells, additional lymph nodes are removed.
There are different types of surgery that are performed on BC patients, depending on the size and spread of the tumor:

- lumpectomy: only tumor and surrounding tissue are removed from the breast; it is usually performed in early stages, such as *in situ* carcinomas;
- partial mastectomy: only a part of the breast is removed, usually a quadrant (quadrantectomy); it is usually performed when the cancer is located in a limited area of the breast;
- simple mastectomy: the entire breast is removed (total mastectomy) leaving the axillary lymph nodes undisturbed; it is performed when the cancer involves an extensive area of the breast without lymph node involvement (negative SLNB);
- radical mastectomy: the entire breast is removed along with the underlying chest muscle and the axillary lymph nodes; it is usually performed in advanced BCs with lymph node involvement (positive SLNB);
- prophylactic mastectomy: in this type of surgery both breasts are removed in the absence of a cancer diagnosis; it is usually performed in patients with a very high risk of developing BC, such as *BRCA1/2* mutation carriers.

Chemotherapy in BC is used as an adjuvant treatment (after surgery) or as a neoadjuvant treatment (before surgery). The most common drugs, used in different combinations, are: cyclophosphamide, methotrexate, 5-fluorouracil, doxorubicin, cyclophosphamide, docetaxel, paclitaxel, cisplatin, carboplatin, capecitabine, gemcitabine and epirubicin.
Hormone therapy can be used in patients with ER-positive and/or PR-positive BCs. The most common drugs are tamoxifen and aromatase inhibitors (letrozole, anastrozole, exemestane).
Chemotherapy also includes target therapies for specific BC types. HER2-positive BCs can be treated with monoclonal antibodies (trastuzumab, pertuzumab) or kinase inhibitors (lapatinib, neratinib). ER-positive and/or PR-positive BCs can be treated with CDK4/6 inhibitors (palbociclib, ribociclib, abemaciclib) or mTOR inhibitors (everolimus).
Some inhibitors of poly ADP ribose polymerase (PARP), a protein family involved in DNA repair, have proven to be effective in the treatment of

cancers with *BRCA1/2* mutations [Fong PC *et al.* 2009]. Until recently, PARP inhibitors (olaparib, rucaparib, niraparib) were only approved for the treatment of ovarian cancers with *BRCA1/2* germline or somatic mutations. In January 2018, olaparib became the first PARP inhibitor to be approved by the Food and Drug Administration (FDA) for metastatic BC in patients with *BRCA1/2* germline mutations.
Radiotherapy is used in BC as an adjuvant treatment, to eliminate cancer cells that may remain in the tumor area following surgery, or as a neoadjuvant treatment, to reduce the tumor size before surgery.

1.7 Breast cancer prognosis

There are several prognostic factors for BC that have to be considered for the choice of treatment [Fitzgibbons PL *et al.* 2000], including:

- tumor size: it is one of the most powerful predictors of tumor behavior since directly related with the frequency of nodal metastases;
- axillary lymph nodes status: it is the single most important predictor of disease-free survival and overall survival;
- histological grade: a high histological grade is associated with a worse prognosis;
- proliferative activity (Ki67): a high proliferative activity is associated with a worse prognosis;
- histological type: tubular, medullary, apocrine and adenoid cystic carcinomas have a better prognosis;
- vascular invasion: it is associated with worse disease-free survival and overall-survival;
- HER2 status: HER2 overexpression is associated with high histological grade, reduced survival, lower responsiveness to methotrexate-based treatment regimens and hormone receptor modulators such as tamoxifen, and higher responsiveness to doxorubicin-based regimens;
- hormone receptor status: cancers with high levels of hormone receptors can benefit from hormone therapy;
- patient's age: patients who are <35 years old have a worse prognosis;
- gene expression profiles: luminal A cancers have a good prognosis, while basal-like and HER2 positive tumors have a poor prognosis.

The 5-year survival rates by stage, calculated for people diagnosed with BC between 2007 and 2013 in the United States of America [National Cancer Institute's SEER database] are reported in the following table:

CANCER STAGE	5-YEAR SURVIVAL RATE
Stage 0	100%
Stage I	100%
Stage II	93%
Stage III	72%
Stage IV	22%

1.8 Genetic predisposition to breast cancer

BC predisposition is often associated with ovarian cancer (OC) risk. OC is the fourth cause of female cancer death in the developed world, also called "the silent killer" given the high mortality rate often due to late diagnosis [Jayson GC *et al.* 2014]. About 10-30% of BCs and OCs shows a familial aggregation, but it is estimated that 5-10% only are hereditary, namely due to pathogenic variants in high penetrant genes transmitted to the progeny [Gage M *et al.* 2012; Prat J *et al.* 2005].
The main genes involved in Hereditary Breast and Ovarian Cancer syndrome (HBOC) are *BRCA1* [Miki Y *et al.* 1994] and *BRCA2* [Wooster R *et al.* 1995]. Cancer predisposition associated with these genes is transmitted as an autosomal dominant condition with incomplete penetrance, *i.e.* mutation carriers have a high risk of developing BC and/or OC during their lifetime, but not all the carriers will develop the disease.
In addition to *BRCA1/2*, other genes have been associated with BC risk with different penetrance estimates [Apostolou P and Fostira F 2013]. The best-known genes, for which a more precise lifetime risk has been estimated, are *PALB2*, *TP53*, *ATM*, *CHEK2*, *CDH1*, *PTEN*, *STK11* [Easton *et al.* 2015; Kwong A *et al.* 2016] (Figure 6).

Syndrome	Gene or locus (chromosomal location)	Neoplasm	Lifetime risk
Genes with high-penetrance mutations			
Hereditary breast/ovarian cancer syndrome	*BRCA1* (17q12–21)	Female breast, ovarian cancer	40–80%
	BRCA2 (13q12-13)	Male and female breast, ovarian, prostate, and pancreatic cancer	20–85%
Li-Fraumeni syndrome	*TP53* (17p13.1)	Breast cancer, sarcomas, leukemia, brain tumours, adrenocortical carcinoma, lung cancers	56–90%
Cowden syndrome	*PTEN* (10q23.3)	Breast, thyroid, endometrial cancer Other: benign hamartomas, macrocephaly	25–50%
Peutz-Jeghers syndrome	*STK11* (19p13.3)	Breast, ovarian, cervical, uterine, testicular, small bowel, and colon carcinoma Other: Hamartomatous polyps of the small intestine, mucocutaneous pigmentation	32–54%
Hereditary gastric cancer	*CDH1* (16q22.1)	Hereditary diffuse gastric, lobular breast, colorectal cancer	60%
Moderate-penetrance mutations			
ATM- related	*ATM* (11q22.3)	Breast and ovarian cancers	15–20%
CHEK2- related	*CHEK2* (22q12.1)	Breast, colorectal, ovarian, bladder cancers	25–37%
PALB2-related	*PALB2* (16p12.1)	Breast, pancreatic, ovarian cancer, male breast cancers	20–40%
Moderate risk breast/ovarian cancer	*BARD1* (2q34-q35), *BRIP1* (17q22–q24), *MRE11A* (11q21), *NBN* (8q21), *RAD50* (5q31), *RAD51C* (17q25.1), *XRCC2* (7q36.1), *RAD51D* (17q11), *ABRAXAS* (4q21.23)	Breast and ovarian cancers	variable

Fig. 6. Breast cancer susceptibility genes with the corresponding estimated lifetime risk [Apostolou P and Fostira F 2013]

1.8.1 *BRCA1*

The *BRCA1* gene (OMIM *113705) is located on chromosome 17q21.31 and encodes a nuclear protein involved in DNA repair, cell cycle checkpoint control, and maintenance of genomic stability [Roy R *et al.* 2011; Foulkes WD and Shuen AY 2013].

The BRCA1 protein is a tumor suppressor acting with other tumor suppressors, DNA damage sensors, and signal transducers to form a large multi-subunit protein complex known as BRCA1-associated genome surveillance complex (BASC) [Wang Y *et al.* 2000; Hedenfalk IA *et al.* 2002] (Figures 7 and 8).

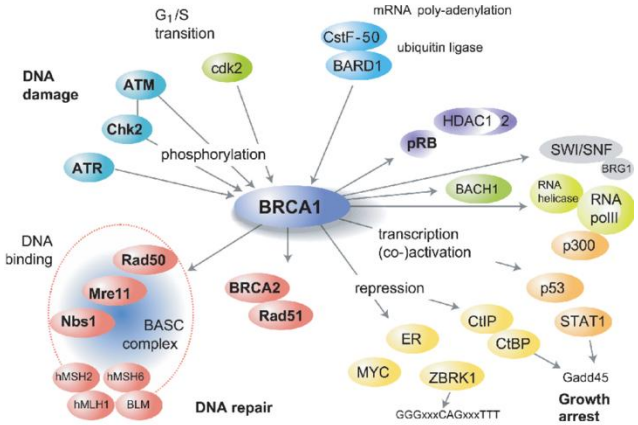


Fig. 7. Schematic view of BRCA1 pathways and BASC (BRCA1 associated genome surveillance complex): BRCA1 is involved in different cellular pathways, including DNA repair, cell cycle control, and growth arrest [Hedenfalk IA *et al.* 2002].

Germline pathogenic variants in *BRCA1* gene are associated with a 57-65% and 39-44% risk of developing BC and OC by the age of 70, respectively [Antoniou *et al.* 2003; Chen S and Parmigiani G 2007; Mavaddat N *et al.* 2013]. *BRCA1* mutations have also been associated with an increased risk of breast cancer in males (MBC), that is estimated to be 1.2% by the age of 70 [Tai YC *et al.* 2007]. In addition, *BRCA1* mutations have been associated with an increased risk of colon cancer [Ford D *et al.* 1994], prostate cancer [Leongamornlert D *et al.* 2012] and pancreatic cancer [Thompson D *et al.* 2002; Ghiorzo P *et al.* 2012].

1.8.2 *BRCA2*

The *BRCA2* gene (OMIM *600185) is located on chromosome 13q13.1 and encodes a nuclear protein involved in repairing damaged DNA through homologous recombination (HR) [Roy R *et al.* 2011; Foulkes WD and Shuen AY 2013].
BRCA2 protein mediates the recruitment of the recombinase RAD51 to the DNA double-strand breaks through the formation of a BRCA1-PALB2-BRCA2 complex (Figure 7). The BRCA2 DNA-binding domain contains a helical domain (H), three oligonucleotide binding (OB) folds and a tower domain (T), which may facilitate BRCA2 binding to both single-stranded DNA and double-stranded DNA (Figure 8) [Xia B *et al.* 2006; Buisson R *et al.* 2010; Roy R *et al.* 2011].

Germline mutations in *BRCA2* gene are associated with a 45-55% and 11-18% risk of developing BC and OC by the age of 70 years, respectively [Antoniou *et al.* 2003; Chen S and Parmigiani G 2007; Mavaddat N *et al.* 2013].
BRCA2 mutations have also been associated with an increased risk of breast cancer in males (MBC), that is estimated 6.8% by the age of 70 years [Tai YC *et al.* 2007].
In addition, *BRCA2* mutations have been associated to an increased risk of prostate cancer [Kote-Jarai Z *et al.* 2011], pancreatic cancer [Iqbal J *et al.* 2012; Ghiorzo P *et al.* 2012], and uveal melanoma [Moran A *et al.* 2012; Breast Cancer Linkage Consortium 1999].

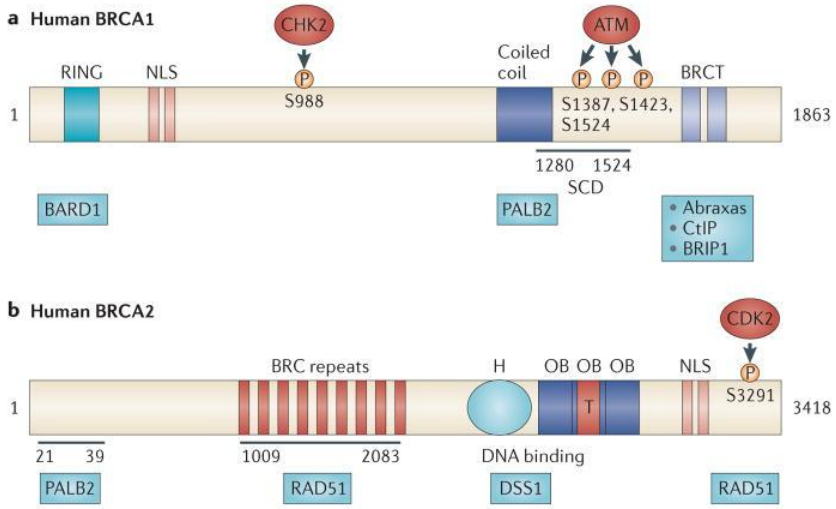


Fig. 8. *BRCA1* and *BRCA2* functional domains and protein interaction sites [Roy R *et al.* 2011]

1.8.3 *PALB2*

The *PALB2* gene (OMIM *610355) is located on chromosome 16p12.2 and encodes a protein that colocalizes with BRCA2 in nuclear foci, promotes its localization and stability in nuclear structures, and enables its recombinational repair and checkpoint functions [Xia B *et al.* 2006].
Biallelic mutations in *PALB2* gene are associated with Fanconi anemia (OMIM #610832), an autosomal recessive disorder characterized by developmental abnormalities in major organ systems, early-onset bone marrow failure, and high predisposition to cancer [Mehta PA and Tolar J 2002].

On the contrary, single mutations in *PALB2* gene lead to a higher risk of BC, that is estimated 35% by the age 70 years [Antoniou AC *et al.* 2014]. Of note, the risk of OC is estimated to be low or none [Ramus SJ *et al.* 2015].

1.8.4 *ATM*

The *ATM* gene (OMIM *607585) is located on chromosome 11q22.3 and encodes a phosphatidylinositol 3-kinase protein that respond to DNA damage by phosphorylating key substrates involved in DNA repair and cell cycle control [Zaki-Dizaji M *et al.* 2017].
Biallelic mutations in *ATM* gene are associated with ataxia-telangiectasia (OMIM #208900), an autosomal recessive disorder, characterized by cerebellar ataxia, telangiectases, immune defects, and a predisposition to malignancy [Savitsky K *et al.* 1995; Gatti R and Perlman S 1999].
On the opposite, monoallelic mutations are associated with an increased risk of BC that is estimated to be 15-60% [Thompson D *et al.* 2005; Renwick A *et al.* 2006; Goldgar DE *et al.* 2011; Marabelli M *et al.* 2016; van Os NJ *et al.* 2016].

1.8.5 *TP53*

The *TP53* gene (OMIM *191170) is located on chromosome 17p13.1 and encodes p53 protein, a tumor suppressor that responds to different cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or metabolism changes [Harris CC 1996].
Mutation in *TP53* gene are associated with Li-Fraumeni syndrome (OMIM #151623), a rare autosomal dominant disorder characterized by a high predisposition to several types of cancer, in particular brain tumors, sarcomas, acute leukaemia and adrenocortical tumors [Li FP and Fraumeni JF Jr 1969; Schneider K *et al.* 1999].
The risk of BC is estimated to be 25-60% [Birch JM *et al.* 2001; Masciari S *et al.* 2012].

1.8.6 *CHEK2*

The *CHEK2* gene (OMIM +604373) is located on chromosome 22q12.1 and encodes a nuclear Ser/Thr kinase involved in different cellular processes. In response to DNA double-strand breaks, CHEK2 protein is phosphorylated by ATM and catalyses the phosphorylation of CDC25C, down-regulating it and preventing entry into mitosis [Matsuoka S *et al.* 1998]. Furthermore, after DNA damage, CHEK2 phosphorylates the p53 tumor suppressor protein and prevents its degradation, leading to cell cycle arrest in G1 [Chehab NH *et al.* 2000]. Under gamma irradiation, CHEK2 also phosphorylates BRCA1 on Ser-988, activating the DNA repair process [Lee JS *et al.* 2000]. Finally, CHEK2 has been shown to induce apoptosis independently from p53, *via* phosphorylation of the PML tumor suppressor protein [Yang S *et al.* 2002].
The first *CHEK2* germline mutations identified have been associated with the Li-Fraumeni syndrome (see paragraph 1.9.5) [Bell DW *et al.* 1999; Vahteristo P *et al.* 2001]; subsequently, this association has been questioned because of phenotype differences among Li-Fraumeni patients and CHEK2 mutation carriers [Evans DG *et al.* 2008].
Germline mutations in this gene are associated with an increased risk of breast cancer [Meijers-Heijboer H *et al.* 2002; Adank MA *et al.* 2011; Walsh T *et al.* 2006; Desrichard A *et al.* 2011; Tedaldi *et al.* 2014]. The cumulative BC risk in mutation carriers is estimated to be 20-44% [Meijers-Heijboer H *et al.* 2002; Weischer M *et al.* 2008; Cybulski C *et al.* 2011].
In addition to BC, *CHEK2* mutations have also been associated with other cancers [Cybulski C *et al.* 2004], including prostate [Dong X *et al.* 2003; Cybulski C *et al.* 2004; Cybulski C *et al.* 2006], colorectal [Meijers-Heijboer H *et al.* 2003], and gastric cancers [Teodorczyk U *et al.* 2013].

1.8.7 *PTEN*

The *PTEN* gene (OMIM *601728) is located on chromosome 10q23.31 and encodes a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, that antagonizes the PI3K signaling pathway through its lipid phosphatase activity and negatively regulates the MAPK pathway through its protein phosphatase activity [Pezzolesi MG *et al.* 2007].
Germline mutations in *PTEN* gene are associated with the Cowden syndrome (OMIM #158350), an autosomal dominant disorder characterized by benign hamartomas as well as by an increased lifetime risk of breast, thyroid, uterine, and other cancers [Eng C 2001; Mester J and Eng C 2015].

The risk of BC in *PTEN* mutation carriers is estimated to be 85% by 70 years of age, while the risk of OC is low or none [Cho MY *et al.* 2008; Tan MH *et al.* 2012; Ngeow J *et al.* 2017].

1.8.8 *CDH1*

The *CDH1* gene (OMIM *192090) is located on chromosome 16q22.1 and encodes E-cadherin, a transmembrane calcium-dependent protein involved in cell-cell adhesion [van Roy F and Berx G 2008].
Germline mutations in *CDH1* gene are associated with the Hereditary Diffuse Gastric Cancer syndrome (OMIM #137215), an autosomal dominant condition predisposing to diffuse-type gastric cancer (DGC) and lobular breast cancer (LBC) [Kaurah P and Huntsman DG 2002]. The cumulative risk of LBC for women with a *CDH1* germline mutation is estimated 42% by 80 years of age [van der Post RS *et al.* 2015].
CDH1 alterations have been extensively treated in the first part of this thesis (see Part one, Paragraph 1.8.1).

1.8.9 *STK11*

The *STK11* gene (OMIM *602216) is located on chromosome 19p13.3 and encodes a serine/threonine kinase that regulates energy metabolism and cell polarity [Xu X *et al.* 2013].
Germline mutations in *STK11* gene are associated with the Peutz-Jeghers syndrome (OMIM #175200), an autosomal dominant disorder characterized by melanocytic macules of the lips, buccal mucosa, and digits, multiple gastrointestinal hamartomatous polyps, and an increased risk of various neoplasms [McGarrity TJ *et al.* 2001].
In mutation carriers, the BC risk is estimated to be 32% by the age of 60, and the risk of gynaecological cancers (cervical, ovarian, uterine) 13% by the age of 60 [Lim W *et al.* 2004].

1.8.10 Other genes associated with breast cancer risk

In the last few years the advent of Next-Generation Sequencing (NGS) has enabled the analysis of a great number of genes with the advantage of lower costs and a wider access to molecular tests for patients with suspected genetic syndromes [Walsh T *et al.* 2010; Weaver JM and Edwards PA 2011;

Gracia-Aznarez FJ *et al.* 2013; Kurian AW *et al.* 2014; Desmond A *et al.* 2015; Easton DF *et al.* 2015; Kurian AW *et al.* 2016; Nielsen FC *et al.* 2016; Kraus C *et al.* 2017].

The following genes, in addition to the ones reported above, have been associated with BC and/or OC.

- ***BARD1*** [Ghimenti C *et al.* 2002; Ratajska M *et al.* 2012; Apostolou P and Fostira F 2013; Ramus SJ *et al.* 2015];
- ***BLM*** [de Voer RM *et al.* 2015; Bogdanova N *et al.* 2015; Kurian AW *et al.* 2016]
- ***BRIP1*** [Seal S *et al.* 2006; Rafnar T *et al.* 2011; Catucci I *et al.* 2012; Apostolou P and Fostira F 2013; Ramus SJ *et al.* 2015; Easton DF *et al.* 2016; Weber-Lassalle N *et al.* 2018];
- ***FAM175A*** [Wang B *et al.* 2007; Solyom S *et al.* 2012; Apostolou P and Fostira F 2013; Kurian AW *et al.* 2016];
- ***FANCC*** [Berwick M *et al.* 2007; Barroso E *et al.* 2009; Kurian AW *et al.* 2016]
- ***FANCM*** [Kiiski JI *et al.* 2014; Economopoulou P *et al.* 2015; Peterlongo P *et al.* 2015; Silvestri V *et al.* 2018];
- Mismatch repair (MMR) genes (***MLH1***, ***MSH2***, ***MSH6***, ***PMS2***, ***EPCAM***) [Walsh MD *et al.* 2010; Bonadona V *et al.* 2011; Engel C *et al.* 2012; Win AK *et al.* 2013; Economopoulou P *et al.* 2015; Harkness EF *et al.* 2015; ten Broeke SW *et al.* 2015];
- MRN complex (MRE11-RAD50-NBS1) genes (***MRE11***, ***RAD50***, ***NBN***) [Bartkova J *et al.* 2008; Bogdanova N *et al.* 2008; Hollestelle A *et al.* 2010; Apostolou P and Fostira F 2013; Damiola F *et al.* 2014; Pennington KP *et al.* 2014; Ramus SJ *et al.* 2015; Kurian AW *et al.* 2016];
- ***NF1*** [Madanikia SA *et al.* 2012; Seminog OO and Goldacre MJ 2015; Kurian AW *et al.* 2016];
- ***PPM1D*** [Ruark E *et al.* 2013];
- ***RAD51B***, ***RAD51C***, ***RAD51D*** [Loveday C *et al.* 2011; Loveday C *et al.* 2012; Apostolou P and Fostira F 2013; Song H *et al.* 2015];
- ***WRN*** [Lauper JM *et al.* 2013];
- ***XRCC2*** [Lin WY *et al.* 2011; Park DJ *et al.* 2012; Hilbers FS *et al.* 2012; Apostolou P and Fostira F 2013].

Figure 9 summarizes the most relevant fingings on BC predisposition obtained by NGS-based approaches on selected patients, showing the proportion of gene mutations, incuding (panel A) and excluding (panel B)

results on *BRCA1/2* testing: apart from *BRCA1/2*, BC predisposition appears clearly distributed over many genes, with only few genes being recurrently mutated in unrelated patients. On the other hand, NGS-based approaches have also highlighted unexpected overlappings among genetic syndromes predisposing to breast/ovarian cancer or to colorectal and gastric cancers, raising the question of phenotypes associated with individual cancer susceptibility genes (Figure 10).

On the whole, it is undoubted that NGS has deeply increased our knowledge on BC predisposition by increasing the number of susceptibility genes. However, in consequence of the growing demand for higher throughput and lower costs, quality data and standardized procedures need to be carefully assessed. Moreover, genetic counseling and risk evaluation, as well as clinical management of patients and families at risk are becoming more and more challenging. In particular, all health-care professionals who offer genetic testing must engage in constant education as the field is continuously evolving with new data becoming available.

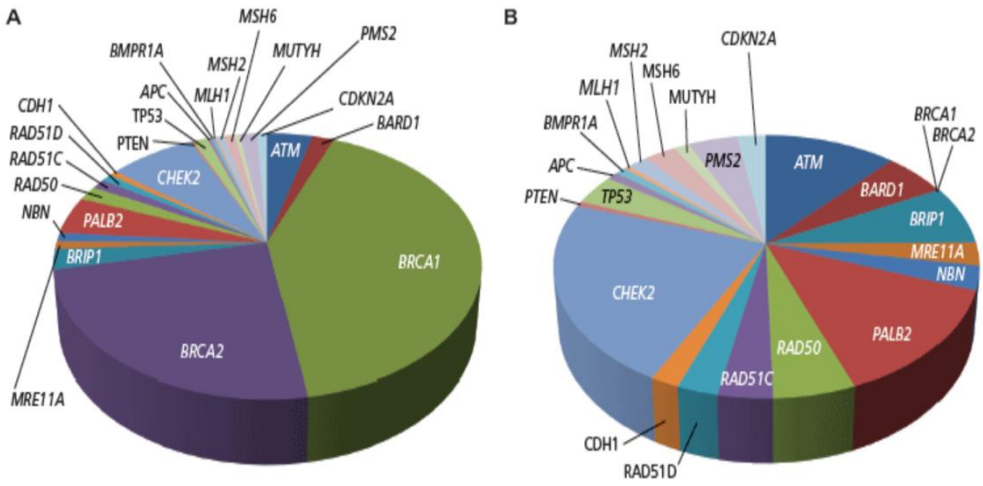


Fig. 9. Proportion of gene mutations predisposing to BC, incuding (pie chart A) and excluding (pie chart B) results on *BRCA1/2* testing [Plichta JK *et al.* 2016]

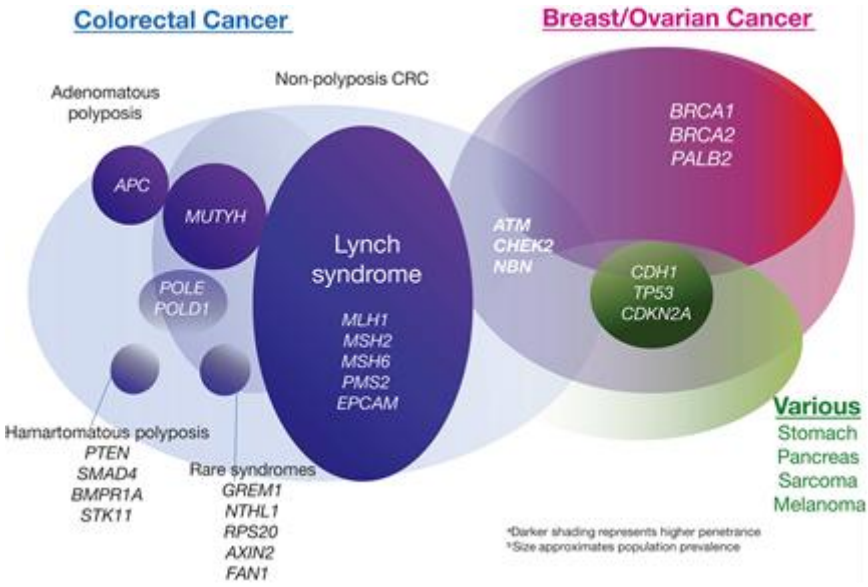


Fig.10 Genes implicated in high and moderate penetrance hereditary predisposition to cancer [Stoffel EM *et al.* 2018]

2. Aims of the research

In the last few years the advent of Next-Generation Sequencing (NGS) has enabled the simultaneous analysis of a great number of genes with the advantage of lower costs and a wider access to molecular tests for patients with suspected genetic syndromes predisposing to cancer.
BRCA1 and *BRCA2* have been known for decades as predisposition genes to BC and OC. Consequently, for mutation carriers in these genes accurate cancer risk estimates are available, as well as surveillance protocols for cancer prevention and early detection of the disease.
New genes are constantly emerging from NGS studies as predisposition factors to BC and OC. However, genetic testing other than of *BRCA1* and *BRCA2* are not routinely performed, due to lack of information about the actual risks for mutation carriers and the unavailability of surveillance programs.
In this study, we utilized a panel of 94 cancer-predisposition genes to screen 255 females affected with BC and/or OC and selected according to the *BRCA1/2* testing criteria. Aim of this NGS-based approach was to search for germline mutations explaining the personal and family history of cancer of these patients.
Moreover, to evaluate genotype-phenotype correlations, we compared age at diagnosis, BC/OC clinicopathological features, and disease family history among: *BRCA1/2* mutation carriers; patients with pathogenic variants in genes other than *BRCA1/2*; patients without clear disease-causing mutations.

3. Materials and methods

3.1 Selection of patients

Patients referring to genetic counseling at the Cancer Prevention Unit of the Morgagni-Pierantoni Hospital (Forlì-Italy) in the years from 2012 through 2015, with a history of BC and/or OC were included in the study.
We selected 255 patients according to the FONCaM (Forza Operativa Nazionale sul Carcinoma Mammario) guidelines [Collegio Italiano dei Senologi 2016], based on the age of BC/OC onset and on the number of cancer cases in I- and II-degree relatives.
The FONCaM guidelines for *BRCA1/2* testing also include affected individuals without family history:

- individuals with BC <36 years
- individuals with BC and OC at any age
- individuals with BBC <50 years
- individuals with TNBC <50 years
- individuals with MBC at any age
- individuals with OC (or Fallopian tube cancer) <50 years or high-grade serous OC at any age

The guidelines also include affected individuals with family history:

- individuals with BC <50 years and:
  - 1 relative* with BC <50 years;
  - 1 relative* with BBC at any age;
  - 1 relative* with OC (or Fallopian tube cancer) at any age;
- individuals with BC >50 years and:
  - 2 relatives* with BC/OC at any age;
- individuals with OC (or Fallopian tube cancer) and:
  - 1 relative* with OC (or Fallopian tube cancer) at any age.

∗ The relatives must be part of either the paternal or the maternal branch and the affected relatives of the 2 branches must not be added up.
Patients must be I-degree relatives; *e.g.* in the paternal branch, paternal aunts, paternal grandmother and paternal cousins (daughters of father's brothers) are considered I-degree relatives.
When it is difficult to gather information about family members (deceased or unknown parents), in families with few members (only

children, only child parents) or in families with numerous male members, the genetic test can be proposed even if selection criteria are not completely fulfilled.

FONCaM guidelines for *BRCA1/2* testing also include healthy subjects with high family history of BC and/or OC; however, only females diagnosed with BC and/or OC and fulfilling the above criteria were included in this study.

For each patient, the following data were collected:

- age at diagnosis;
- cancer histotype (see paragraph 1.3.1);
- cancer grading (see paragraph 1.6);
- cancer stage (see paragraph 1.6);
- tumor invasiveness (see paragraph 1.3.1);
- occurrence of a second BC, a second OC or other malignancies;
- family history of BC/OC and other cancers.

BCs were clustered into 4 histological subtypes:

- in situ carcinomas;
- invasive ductal carcinomas;
- invasive lobular carcinomas;
- other invasive histotypes.

OCs were clustered into 3 histological subtypes:

- serous carcinomas;
- other malignant histotypes;
- borderline tumors (semi-malignant tumors).

For BCs, information about the status of Ki-67 antigen and ER, PR, HER2 receptors was collected.

According to the St Gallen guidelines [Goldhirsch A *et al.* 2013], available information was used to divide BCs in 5 different subtypes (see paragraph 1.3.2):

- luminal A;
- luminal B1;
- luminal B2;
- HER2-positive;
- Triple negative (TNBC).

The information about cancer family history (I- and II-degree relatives) was organized as follows:

- positive or negative family history of BC/OC in I-degree relatives;
- positive or negative family history of BC/OC in I- and II-degree relatives;
- positive or negative family history of other cancers in I-degree relatives;
- positive or negative family history of other cancers in I- and II-degree relatives.

3.2 Samples and DNA extraction

After obtaining informed consent from patients, we collected peripheral blood samples in vacutainer tubes.
Blood samples were transferred to cryovials and stored at -80°C until DNA extraction. Genomic DNA was extracted by using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was assessed with the Qubit 1.0 fluorometer and the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

3.3 NGS analysis

Sequencing libraries were created by using 50 ng of genomic DNA and following the enrichment protocol Trusight Cancer (Illumina) for the simultaneous sequencing of 94 genes (Part one, Paragraph 3.3).
The gene panel covers a total of 355 kb and includes exons and flanking intronic regions (50bp upstream and downstream each exon).
DNA sequencing was performed by using the MiSeq platform (Illumina) with MiSeq Reagent Kit v2 configured 2×150 cycles, according to the manufacturer's instructions.

3.4 Bioinformatic analysis

NGS results were analyzed by a customized bioinformatics pipeline.
Raw de-multiplexed reads from the MiSeq sequencer were aligned to the reference human genome (UCSC-Build37/hg19) using the Burrows–Wheeler algorithm [Li H and Durbin R 2009], running in paired-end mode.

To ensure good call quality and to reduce the number of false positives, samples underwent Base Quality Score Recalibration (BQSR), using the Genome Analysis Toolkit GATK, version 3.2.2 [McKenna A *et al.* 2010].
After BQSR, sequences around regions with insertions and deletions (indels) were realigned locally with GATK.
MarkDuplicates [http://broadinstitute.github.io/picard/] was used to remove duplicate read-pairs arisen as artifacts during either polymerase chain reaction amplification or sequencing.
For variant analysis Unified Genotyper of GATK was used to search for SNVs and indels.
Genomic and functional annotations of detected variants were made by Annovar [Wang K *et al.* 2010].
Coverage statistics was performed by DepthOfCoverage utility of GATK.
BASH and R custom scripts were used to obtain the list of low coverage (<50X) regions per sample.
The regions under this threshold were considered not evaluable.
The potential impact of amino acid changes (MAPP P value) was assessed with PolyPhen-2 HVAR [Adzhubei IA *et al.* 2010] and SIFT [Kumar P *et al.* 2009].

3.5 *BRCA1/2* analysis

BRCA1/2 regions covered <50X were amplified by standard polymerase chain reaction (PCR). PCR products were sequenced using the BigDye terminator v.3.1 cycle sequencing kit (Thermo Fisher Scientific) on an ABI-3130 Genetic analyzer (Applied Biosystems).
To complete *BRCA1/2* analysis and to identify gross deletions/insertions undetectable by sequencing, we performed the Multiplex Ligation-dependent Probe Amplification (MLPA) assay with *BRCA1*-P002 and *BRCA2*-P045 kits (MRC Holland). MLPA results were analyzed with the Coffalyser software (MRC Holland).

3.6 Confirmation of mutations

All mutations of classes 3-5 identified in *BRCA1* and *BRCA2* genes were confirmed by Sanger sequencing with the same protocol used for the uncovered regions.
All deleterious mutations (classes 4-5) identified in other genes were confirmed by a second NGS analysis.

3.7 Variant classification

Genetic variants identified in this work were divided into 5 classes according to the IARC recommendations [Plon SE *et al.* 2008].
The classification of *BRCA1/2* variants was performed using the main *BRCA* mutation databases: Breast Cancer Information Core (BIC) [Szabo C *et al.* 2000], *BRCA* Share (formerly Universal Mutation Database) [Caputo S *et al.* 2012] and Leiden Open Variation Database (LOVD) [Fokkema IF *et al.* 2011].
Sequence variants in the remaining 92 genes were classified using dbSNP [https://www.ncbi.nlm.nih.gov/projects/SNP/] and ClinVar [https://www.ncbi.nlm.nih.gov/clinvar/] databases.
Variants not present in any of these databases were classified on the basis of their characteristics. Only mutations causing a premature stop codon (frameshift and nonsense) and gross deletions were considered pathogenic/likely-pathogenic and classified in accordance with the guidelines of the American College of Medical Genetics (ACMG) [Richards S *et al.* 2015].

3.8 Statistical analysis

Patient characteristics and sequencing results were tabulated, with descriptive statistics including median and range for continuous data, and natural frequencies and percentages for categorical data.
Proportions were compared using either the Pearson Chi-square test or the Fisher Exact test, as appropriate.
The Wilcoxon-Mann Whitney or the Kruskal-Wallis test, as appropriate, were used for the continuous variables.
All P values were two-tailed. Analyses were performed using STATA Release 14.0.

4. Results

By using a panel of 94 cancer-syndrome genes (Part one, Paragraph 3.3), we performed an NGS-based analysis on a case series of 255 women. The cohort included 227 (89.0%) patients with BC (median age at diagnosis 41 years) and 28 (11.0%) patients with OC (median age at diagnosis 49.5 years). BC and OC features are summarized in Tables 1 and 2, respectively.
Among the 227 BC patients, 52 (22.9%) had bilateral breast cancer (BBC), 8 (3.5%) had a subsequent OC and 22 (9.7%) had subsequent other malignancies (reported in Table 1 as "Second BC", "Second OC" and "Other tumors", respectively). Of the 28 OC patients, 7 (25.0%) had a subsequent BC (reported in Table 2 as "Second BC"), and none had other malignancies (reported in Table 2 as "Other tumors").
DNA analysis performed on the case series showed a mean target coverage of 399,7X and a 95.5% mean percentage of target covered >50X.
We focused at first on *BRCA* genes. According to databases and guidelines (see Materials and Methods), 57 (22.4%) patients proved to be carriers of a pathogenic/likely-pathogenic variants in *BRCA* genes. In particular, 31 (12.2%) were carriers of a *BRCA1* mutation, 25 (9.8%) of a *BRCA2* mutation and 1 (0.4%) of pathogenic variants in both *BRCA1* and *BRCA2* genes (Table 3).
As far as the remaining 92 genes is concerned, we found 23 pathogenic/likely-pathogenic variants in 14 genes in 21/255 (8.2%) patients (Table 6). Among these 21 patients, 4 were *BRCA*-positive and 17 *BRCA*-negative.
As far as the 181 (71.0%) patients without pathogenic/likely-pathogenic variants is concerned, we found 23,882 exonic and splicing (± 5bp) variants of uncertain significance. We used the allelic frequencies present in 1000Genomes, Esp6500 and Exac03 databases to exclude polymorphic variants (frequency >1%) from further analysis. Among the 1,026 variants with frequency <1% or n/a, we focused the attention on missense variants and we evaluated their role in cancer predisposition by predicting their pathogenicity with PolyPhen-2 HVar and SIFT bioinformatic tools.

Table 1: BC patients: clinical features and personal/family history of cancer

BREAST CANCER (BC)	**All patients**	**Patients with *BRCA1/2* pathogenic variants**	**Patients with extra-*BRCA* pathogenic variants**	**Patients without pathogenic variants**	***P***
	N (%)	***N (%)***	***N (%)***	***N (%)***	
N. of patients	227	48	17	162	
Age at diagnosis, years					
Median Age [Min-Max]	41 [25-79]	39 [25-70]	43 [26-74]	42 [25-79]	0.140
Missing	0	0	0	0	
Histotype					
In situ carcinoma	22 (10.14)	3 (6.52)	3 (17.65)	16 (10.39)	0.810
Invasive ductal carcinoma	148 (68.20)	33 (71.74)	10 (58.82)	105 (68.18)	
Invasive lobular carcinoma	26 (11.98)	6 (13.04)	3 (17.65)	17 (11.04)	
Other invasive histotypes	21 (9.68)	4 (8.70)	1 (5.88)	16 (10.39)	
Missing	10	2	0	8	
Grading					
Well-differentiated	18 (9.68)	0 (0.00)	1 (6.67)	17 (12.98)	0.005
Moderately differentiated	85 (45.70)	13 (32.50)	7 (46.67)	65 (49.62)	
Poorly differentiated	83 (44.62)	27 (67.50)	7 (46.67)	49 (37.40)	
Missing	41	8	2	31	
Stage					
0	22 (12.50)	3 (8.82)	3 (23.08)	16 (12.40)	0.375
I	92 (52.27)	15 (44.12)	5 (38.46)	72 (55.81)	
II	45 (25.57)	13 (38.24)	3 (23.08)	29 (22.48)	
III-IV	17 (9.66)	3 (8.82)	2 (15.38)	12 (9.30)	
Missing	51	14	4	33	
Tumor invasiveness					
In situ	22 (10.09)	3 (6.38)	3 (17.65)	16 (10.39)	0.420
Invasive	196 (89.91)	44 (93.62)	14 (82.35)	138 (89.61)	
Missing	9	1	0	8	
Ki-67					
High (≥14)	115 (70.55)	37 (90.24)	6 (75.00)	72 (63.16)	0.003
Low (<14)	48 (29.45)	4 (9.76)	2 (25.00)	42 (36.84)	
Missing	64	7	9	48	
St Gallen subtype					
Luminal A	29 (20.14)	1 (2.86)	2 (28.57)	26 (25.49)	0.005

Luminal B1	56 (38.89)	13 (37.14)	2 (28.57)	41 (40.20)	
Luminal B2	26 (18.06)	8 (22.86)	1 (14.29)	17 (16.67)	
Her2 positive	9 (6.25)	1 (2.86)	1 (14.29)	7 (6.86)	
Triple negative	24 (16.67)	12 (34.29)	1 (14.29)	11 (10.78)	
Missing	83	13	10	60	
Second BC					
No	175 (77.09)	36 (75.00)	9 (52.94)	130 (80.25)	0.036
Yes	52 (22.91)	12 (25.00)	8 (47.06)	32 (19.75)	
Median Age [Min-Max] [a]	55 [32-82]	46 [37-70]	57 [36-77]	58.5 [32-82]	0.041
Second OC					
No	219 (96.48)	46 (95.83)	15 (88.24)	158 (97.53)	0.103
Yes	8 (3.52)	2 (4.17)	2 (11.76)	4 (2.47)	
Median Age [Min-Max] [a]	66.5 [51-77]	69 [68-70]	52.5 [51-54]	68.5 [55-77]	0.135
Other tumors					
No	205 (90.31)	45 (93.75)	17 (100.00)	143 (88.27)	0.254
Yes	22 (9.69)	3 (6.25)	0 (0.00)	19 (11.73)	
BC/OC in I degree relatives					
No	81 (35.68)	17 (35.42)	10 (58.82)	54 (33.33)	0.113
Yes	146 (64.32)	31 (64.58)	7 (41.18)	108 (66.67)	
BC/OC in I/II degree relatives					
No	43 (18.94)	10 (20.83)	7 (41.18)	26 (16.05)	0.039
Yes	184 (81.06)	38 (79.17)	10 (58.82)	136 (83.95)	
Other cancers in I degree relatives					
No	144 (63.44)	33 (68.75)	11 (64.71)	100 (61.73)	0.670
Yes	83 (36.56)	15 (31.25)	6 (35.29)	62 (38.27)	
Other cancers in I/II degree relatives					
No	81 (35.68)	22 (45.83)	7 (41.18)	52 (32.10)	0.193
Yes	146 (64.32)	26 (54.17)	10 (58.82)	110 (67.90)	

[a] Median age, in years, refers to age at second cancer diagnosis

Table 2: OC patients: clinical features and personal/family history of cancer

OVARIAN CANCER (OC)	**All patients**	**Patients with *BRCA1/2* pathogenic variants**	**Patients with extra-*BRCA* pathogenic variants**	**Patients without pathogenic variants**	***P***
	N (%)	***N (%)***	***N (%)***	***N (%)***	
N. of patients	28	9	0	19	
Age at diagnosis, years					
Median Age [Min-Max]	49.5 [28-81]	50 [38-68]	-	47 [28-81]	0.640
Missing	0	0	-	0	
Histotype					
Serous carcinoma	18 (64.29)	7 (77.78)	-	11 (57.89)	0.700
Other malignant histotypes	7 (25.00)	2 (22.22)	-	5 (26.32)	
Borderline tumors	3 (10.71)	0 (0.00)	-	3 (15.79)	
Missing	0	0	-	0	
Grading					
Well-differentiated	2 (8.00)	0 (0.00)	-	2 (12.50)	0.772
Moderately differentiated	2 (8.00)	1 (11.11)	-	1 (6.25)	
Poorly differentiated	21 (84.00)	8 (88.89)	-	13 (81.25)	
Missing	3	0	-	3	
Stage					
0	0 (0.00)	0 (0.00)	-	0 (0.00)	0.343
I	7 (31.82)	1 (12.50)	-	6 (42.86)	
II	2 (9.09)	1 (12.50)	-	1 (7.14)	
III-IV	13 (59.09)	6 (75.00)	-	7 (50.00)	
Missing	6	1	-	5	
Tumor invasiveness					
Borderline	3 (10.71)	0 (0.00)	-	3 (15.79)	0.530
Invasive	25 (89.29)	9 (100.00)	-	16 (84.21)	
Missing	0	0	-	0	
Second BC					
No	21 (75.0)	7 (77.78)	-	14 (73.68)	1.000
Yes	7 (25.0)	2 (22.22)	-	5 (26.32)	
Median Age [Min-Max] [a]	55 [45-81]	58.5 [53-64]	-	55 [45-81]	1.000
Other tumors					
No	28 (100.00)	9 (100.00)	-	19 (100.00)	-
Yes	0 (0.00)	0 (0.00)	-	0 (0.00)	

BC/OC in I degree relatives					
No	12 (42.86)	1 (11.11)	-	11 (57.89)	0.039
Yes	16 (57.14)	8 (88.89)	-	8 (42.11)	
BC/OC in I/II degree relatives					
No	9 (32.14)	1 (11.11)	-	8 (42.11)	0.195
Yes	19 (67.86)	8 (88.89)	-	11 (57.89)	
Other cancers in I degree relatives					
No	16 (57.14)	6 (66.67)	-	10 (52.63)	0.687
Yes	12 (42.86)	3 (33.33)	-	9 (47.37)	
Other cancers in I/II degree relatives					
No	12 (42.86)	5 (55.56)	-	7 (36.84)	0.432
Yes	16 (57.14)	4 (44.44)	-	12 (63.16)	

[a] Median age, in years, refers to age at second cancer diagnosis

4.1 Pathogenic variants in *BRCA1/2* genes

We identified 32 pathogenic/likely-pathogenic variants along the *BRCA1* gene and 26 along the *BRCA2* gene. In one patient, a pathogenic variant was present in both *BRCA1* and *BRCA2* genes (Table 3).

All carriers of *BRCA1* pathogenic/likely-pathogenic variants (31 patients) developed a BC. In particular, 23 (74.2%) patients had BC first; among these, 6 (26.1%) had a BBC, and 1 (4.3%) a subsequent OC. The remaining 8 (25.8%) patients had OC first; among these, 2 (25.0%) had a subsequent BC. Similarly, all carriers of *BRCA2* pathogenic/likely-pathogenic variants (25 patients) developed a BC. In particular, 24 (96.0%) patients had BC first; among these, 6 (25.0%) had a BBC, and 1 (4.2%) a subsequent OC. One patient only (4.0%) had OC first. The only patient with pathogenic variants in both *BRCA1* and *BRCA2* genes developed a BC.

We compared the clinical features of the 31 *BRCA1*-mutation carriers with those of the 25 *BRCA2*-mutation carriers. We did not find any significant difference between the two groups, except for BC grading with a higher number of poorly differentiated tumors among *BRCA1*-mutation carriers (Tables 4 and 5). On this basis we pooled the two groups (also including the patient with both *BRCA1* and *BRCA2* mutations) to evaluate differences between *BRCA1/2* mutated and non-mutated subjects.

For *BRCA1/2* mutation-positive patients, the median age at the onset of BC was 39 years for the first BC and 46 for the subsequent BC, while the median age at the onset of OC was 50 years. The number of triple-negative

BCs was significantly higher in *BRCA1/2* mutation-positive patients (34.3%) than in mutation-negative patients (11.0%).

The BC/OC family history in I- and II-degree relatives was significantly higher in *BRCA1/2* mutation-positive patients and in *BRCA1/2* wild type subjects with BC than in patients with mutations in genes other than *BRCA1/2* (P=0.039, Table 1). Similarly, *BRCA1/2* mutation-positive patients with OC had a higher BC/OC family history in I-degree relatives compared to *BRCA1/2* wild type patients (P=0.039, Table 2).

Table 3: *BRCA1* and *BRCA2* pathogenic/likely-pathogenic variants detected in 57 unrelated patients

Sample ID	Cancer (age)[a]	Gene	chr	start	end	ref	alt	Mutation type	HGVS[b]	depth	VAF[c]	IARC class	BRCA Share-BIC-LOVD[d]	dbSNP/ClinVar[e]
A004	IDC (33y), IDC (37y)	*BRCA2*	13	32954142	32954142	A	G	splicing	NM_000059: exon 24, c.9118-2A>G	321	0.49	class 5	Pathogenic	rs81002862/Pathogenic
A034	IDC (43y)	*BRCA2*	13	32953453	32953453	G	A	splicing	NM_000059: exon 22, c.8755-1G>A	368	0.42	class 4	VUS/Pathogenic	rs81002812/Likely pathogenic
A154	IDC (32y)	*BRCA1*	17	41197784	41197784	G	A	nonsense	NM_007294: exon 24, c.5503C>T p.Arg1835Ter	336	0.38	class 5	Pathogenic	rs41293465/Pathogenic
A194	IDC (51y), IDC (54y)	*BRCA1*	17	41245647	41245647	-	GTGGGCTTAGATTT	frameshift insertion	NM_007294: exon 11, c.1887_1900dupAAATCTAAGCCCAC p.Pro634fs	583	0.18	class 5	-	rs886039977/Pathogenic
A200	OPC (66y)	*BRCA2*	13	32930609	32930609	C	T	nonsense	NM_000059: exon 15, c.7480C>T p.Arg2494Ter	545	0.43	class 5	Pathogenic	rs80358972/Pathogenic
A207	IDC (48y)	*BRCA1*	17	41228505	41228505	C	A	missense	NM_007294: exon 14, c.4484G>T p.Arg1495Met	1125	0.52	class 5	Pathogenic	rs80357389/Pathogenic
A236	BC (37y)	*BRCA2*	13	32911684	32911687	AATT	-	frameshift deletion	NM_000059: exon 11, c.3192_3195delAATT p.Ser1064fs	436	0.37	class 5	Pathogenic	rs80359375/Pathogenic
A284	IDC (39y)	*BRCA1*	17	41209079	41209079	-	G	frameshift insertion	NM_007294: exon 20, c.5266dupC p.Gln1756fs	1340	0.33	class 5	Pathogenic	rs80357906/Pathogenic
A305	ILC (44y)	*BRCA2*	13	32890665	32890665	G	A	splicing	NM_000059: exon 2, c.67+1G>A	740	0.45	class 5	Pathogenic	rs81002796/Pathogenic
A311	IDC (27y)	*BRCA1*	13	-	-	exons 1-2	-	gross deletion	NM_007294: exons 1-2, c.1-?_80+?del p.?	-	-	class 5	Pathogenic	-
A391	IDC (41y)	*BRCA1*	17	41226450	41226450	G	A	nonsense	NM_007294: exon 15, c.4573C>T p.Gln1525Ter	725	0.52	class 5	-	rs886040237/Pathogenic
A392	IDC (34y)	*BRCA1*	17	-	-	exon 24	-	gross deletion	NM_007294: exon 24, c.5468-?_5592+?del p.?	-	-	class 5	Pathogenic	-
A396	IDC (39y)	*BRCA2*	13	32912354	32912357	ATAA	-	frameshift deletion	NM_000059: exon 11, c.3860_3863delATAA p.Asn1287fs	488	0.49	class 5	Pathogenic	rs80359410/Pathogenic
A407	ILC (39y)	*BRCA1*	17	41246566	41246567	AT	-	frameshift deletion	NM_007294: exon 11, c.981_982del p.Thr327fs	925	0.47	class 5	Pathogenic	rs80357772/Pathogenic
A414	IDC (30y)	*BRCA2*	13	32921023	32921023	-	T	frameshift insertion	NM_000059: exon 13,c.6998dupT p.Pro2334fs	272	0.30	class 5	Pathogenic	rs754611265/Pathogenic
A420	OSC (41y)	*BRCA1*	17	41209079	41209079	-	G	frameshift insertion	NM_007294: exon 20, c.5266dupC p.Gln1756fs	1218	0.18	class 5	Pathogenic	rs80357906/Pathogenic
A427	IDC (26y)	*BRCA1*	17	41215920	41215920	G	T	missense	NM_007294: exon 18, c.5123C>A p.Ala1708Glu	319	0.42	class 5	Pathogenic	rs28897696/Pathogenic
A433	IDC (34y)	*BRCA2*	13	32914894	32914898	TAACT	-	frameshift deletion	NM_000059: exon 11, c.6402_6406delTAACT p.Asn2134fs	633	0.46	class 5	Pathogenic	rs80359584/Pathogenic
A467	IDC (45y)	*BRCA1*	17	41245251	41245252	CT	-	frameshift deletion	NM_007294: exon 11, c.2296_2297del p.Ser766fs	433	0.42	class 5	Pathogenic	rs80357780/Pathogenic

A469	IDC (37y)	*BRCA1*	17	41245861	41245861	G	A	nonsense	NM_007294: exon 11, c.1687C>T p.Gln563Ter	434	0.49	class 5	Pathogenic	rs80356898/Pathogenic
A482	IDC (37y), IDC (37y)	*BRCA2*	13	32903605	32903606	TG	-	frameshift deletion	NM_000059: exon 8, c.658_659delGT p.Val220fs	554	0.41	class 5	Pathogenic	rs80359604/Pathogenic
A497	OSC (50y)	*BRCA1*	17	41246764	41246764	G	-	frameshift deletion	NM_007294: exon 11, c.784delC p.Gln262fs	362	0.52	class 4	VUS	rs886040318/Pathogenic
A500	OAC (38y)	*BRCA1*	17	41251790	41251790	A	T	splicing	NM_007294: exon 8, c.547+2T>A	343	0.43	class 5	Pathogenic	rs80358047/Pathogenic
A503	IDC (25y)	*BRCA2*	13	32911143	32911146	CAGA	-	frameshift deletion	NM_000059: exon 11, c.2653_2656delGACA p.Asp885fs	484	0.48	class 5	Pathogenic	rs80359340/Pathogenic
A525	IDC (48y)	*BRCA2*	13	32914529	32914529	A	-	frameshift deletion	NM_000059: exon 11, c.6039delA p.Val2014fs	473	0.43	class 5	-	rs876660637/Pathogenic
A575	OSC (40y), IDC (64y)	*BRCA1*	17	-	-	exon 20	-	gross deletion	NM_007294: exon 20, c.5194-?_5277+?del p.?	-	-	class 5	Pathogenic	Pathogenic
A576	IDC (37y), IDC (54y)	*BRCA1*	17	41228505	41228505	C	A	missense	NM_007294: exon 14, c.4484G>T p.Arg1495Met	714	0.54	class 5	Pathogenic	rs80357389/Pathogenic
A598	ILC (28y)	*BRCA1*	17	41219660	41219664	ATTAG	-	frameshift deletion	NM_007294: exon 17, c.5035_5039del p.Leu1679fs	481	0.43	class 5	Pathogenic	rs80357623/Pathogenic
A601	OSC (68y)	*BRCA1*	17	41243789	41243792	AGAC	-	frameshift deletion	NM_007294: exon 11, c.3756_3759del p.Leu1252fs	314	0.34	class 5	Pathogenic	rs80357868/Pathogenic
A606	ILC (34y)	*BRCA2*	13	32915083	32915084	TG	-	frameshift deletion	NM_000059: exon 11, c.6591_6592delTG p.Thr2197fs	226	0.42	class 5	Pathogenic	rs80359605/Pathogenic
A616	IDC (41y), IDC (41y)	*BRCA1*	17	41243789	41243792	AGAC	-	frameshift deletion	NM_007294: exon 11, c.3756_3759del p.Leu1252fs	305	0.48	class 5	Pathogenic	rs80357868/Pathogenic
A619	IDC (32y)	*BRCA2*	13	32912338	32912339	TG	-	frameshift deletion	NM_000059: exon 11, c.3847_3848delGT p.Val1283fs	470	0.48	class 5	Pathogenic	rs746229647/Pathogenic
A620	DCIS (41y)	*BRCA2*	13	32907421	32907421	A	-	frameshift deletion	NM_000059: exon 10, c.1806delA p.Gly602fs	465	0.50	class 5	Pathogenic	rs80359307/Pathogenic
A623	IDC (53y)	*BRCA1*	17	32910537	32910538	TC	-	frameshift deletion	NM_000059: exon 11, c.2049_2050del p.Ile684fs	683	0.33	class 5	Pathogenic	rs80359319/Pathogenic
		BRCA2	13	41209079	41209079	-	G	frameshift insertion	NM_007294: exon 20, c.5266dupC p.Gln1756fs	811	0.41	class 5	Pathogenic	rs80357906/Pathogenic
A629	IDC (32y)	*BRCA2*	13	-	-	exons 1-2	-	gross deletion	NM_000059: exons 1-2, c.1-?_67+?del p.?	-	-	class 5	Pathogenic	-
A630	OSC (55y)	*BRCA1*	17	41258504	41258504	A	C	missense	NM_007294: exon 5, c.181T>G p.Cys61Gly	314	0.50	class 5	Pathogenic	rs28897672/Pathogenic
A633	DCIS (38y), IDC(45y)	*BRCA2*	13	32944695	32944695	G	A	splicing	NM_000059: exon 19, c.8487+1G>A	116	0.58	class 5	Pathogenic	rs81002798/Pathogenic
A634	IDC (40y)	*BRCA1*	17	41209079	41209079	-	G	frameshift insertion	NM_007294: exon 20, c.5266dupC p.Gln1756fs	509	0.34	class 5	Pathogenic	rs80357906/Pathogenic
A643	IDC (38y), OSC (70y)	*BRCA2*	13	32912386	32912390	TGAAA	-	frameshift deletion	NM_000059: exon 11, c.3894_3898delTGAAA p.Ile1298fs	368	0.41	class 4	-	-
A651	BC (35y)	*BRCA1*	17	41219660	41219664	ATTAG	-	frameshift deletion	NM_007294: exon 17, c.5035_5039del p.Leu1679fs	337	0.39	class 5	Pathogenic	rs80357623/Pathogenic
A655	OSC (51y), IDC (53y)	*BRCA1*	17	41228505	41228505	C	A	missense	NM_007294: exon 14, c.4484G>T p.Arg1495Met	513	0.50	class 5	Pathogenic	rs80357389/Pathogenic

A677	IDC (39y), IDC (45y)	*BRCA1*	17	41244309	41244309	A	T	nonsense	NM_007294: exon 11, c.3239T>A p.Leu1080Ter	198	0.53	class 5	Pathogenic	rs80357145/Pathogenic
A678	BMC (39y), IDC (47y)	*BRCA1*	17	-	-	exon 20	-	gross deletion	NM_007294: exon 20, c.5194-?_5277+?del p.?	-	-	class 5	Pathogenic	Pathogenic
A691	IDC (53y)	*BRCA2*	13	32907062	32907062	-	CAGT	frameshift insertion	NM_000059: exon 10, c.1448_1451dupCAGT p.Lys485fs	528	0.30	class 5	-	rs886040366/Pathogenic
A696	OSC (51y)	*BRCA1*	17	41228505	41228505	C	A	missense	NM_007294: exon 14, c.4484G>T p.Arg1495Met	383	0.49	class 5	Pathogenic	rs80357389/Pathogenic
A708	DCIS (70y)	*BRCA2*	13	32910437	32910437	C	T	nonsense	NM_000059: exon 11, c.1945C>T p.Gln649Ter	558	0.53	class 5	-	rs398122735/Pathogenic
A726	IDC (51y), ILC (61y)	*BRCA2*	13	32930689	32930689	A	-	frameshift deletion	NM_000059: exon 15, c.7561delA p.Ile2521fs	572	0.40	class 5	Pathogenic	rs886040717/Pathogenic
A728	BC (45y), IDC (56y)	*BRCA2*	13	32954022	32954022	-	A	frameshift insertion	NM_000059: exon 23, c.9097dupA p.Thr3033fs	318	0.32	class 5	Pathogenic	rs397507419/Pathogenic
A741	IDC (34y), BC (39y)	*BRCA1*	17	41209079	41209079	-	G	frameshift insertion	NM_007294: exon 20, c.5266dupC p.Gln1756fs	177	0.36	class 5	Pathogenic	rs80357906/Pathogenic
A758	ILC (34y)	*BRCA2*	13	32914529	32914529	A	T	nonsense	NM_000059: exon 11, c.6037A>T p.Lys2013Ter	755	0.52	class 5	Pathogenic	rs80358840/Pathogenic
A762	BMC (59y), IDC (70y)	*BRCA2*	13	-	-	exon 3	-	gross deletion	NM_000059: exon 3, c.68-?_316+?del p.?	-	-	class 5	Pathogenic	-
A763	IDC (46y)	*BRCA2*	13	32914174	32914174	C	G	nonsense	NM_000059: exon 11, c.5682C>G p.Tyr1894Ter	594	0.50	class 5	Pathogenic	rs41293497/Pathogenic
A768	IDC (57y), OCCA (68y)	*BRCA1*	17	41246698	41246698	G	A	nonsense	NM_007294: exon 11, c.850C>T p.Gln284Ter	621	0.53	class 5	Pathogenic	rs397509330/Pathogenic
A775	IDC (41y)	*BRCA1*	17	41222949	41222967	TCTTCTGGGGTCAGGCCAG	-	frameshift deletion	NM_007294: exon 16, c.4964_4982del p.Ser1655fs	265	0.45	class 5	Pathogenic	rs80359876/Pathogenic
T088	ILC (47y)	*BRCA1*	17	41197751	41197751	G	A	nonsense	NM_007294: exon 24, c.5536C>T p.Gln1846Ter	348	0.50	class 5	Pathogenic	rs80356873/Pathogenic
TR69	IDLC (43y)	*BRCA2*	13	32930609	32930609	C	T	nonsense	NM_000059: exon 15, c.7480C>T p.Arg2494Ter	721	0.45	class 5	Pathogenic	rs80358972/Pathogenic
TR86	IDC (41y)	*BRCA1*	17	41244057	41244067	CTAGTATCTTC	-	frameshift deletion	NM_007294: exon 11, c.3481_3491del p.Glu1161fs	340	0.46	class 5	Pathogenic	rs80357877/Pathogenic

Gross deletions identified by MLPA miss information about start/end and VAF due to the technique utilized.

[a] Tumor histotype: BC (breast cancer), BMC (breast medullary carcinoma), DCIS (ductal carcinoma in situ), IDC (invasive ductal carcinoma), IDLC (infiltrating ductal and lobular carcinoma), ILC (invasive lobular carcinoma), OAC (ovarian adenosquamous carcinoma), OCCA (ovarian clear cell adenocarcinoma), OPC (ovarian papillary cystadenocarcinoma), OSC (ovarian serous carcinoma)

[b] Mutation nomenclature according to the *Human Genome Variation Society* (HGVS)

[c] Variant Frequency

[d] Mutation classification according to *BRCA Share*, *Breast Cancer Information Core* (BIC) and *Leiden Open Variation Database* (LOVD)

[e] Mutation classification according to *Single Nucleotide Polymorphism Database* (dbSNP) and *Clinical Variant* (ClinVar)

Table 4: BC patients with *BRCA1/2* pathogenic variants: clinical features and personal/family history of cancer

BREAST CANCER (BC)	***BRCA1*-mutated** ***N (%)***	***BRCA2*-mutated** ***N (%)***	***P***
N. of patients	23	24	
Age at diagnosis, years			
Median Age [Min-Max]	39 [26-57]	38.5 [25-70]	0.766
Missing	0	0	
Histotype			
In situ carcinoma	0 (0.00)	3 (13.64)	0.410
Invasive ductal carcinoma	18 (78.26)	14 (63.64)	
Invasive lobular carcinoma	3 (13.04)	3 (13.64)	
Other invasive histotypes	2 (8.70)	2 (9.09)	
Missing	0	2	
Grading			
Well-differentiated	0 (0.00)	0 (0.00)	0.019
Moderately differentiated	3 (15.00)	10 (52.63)	
Poorly differentiated	17 (85.00)	9 (47.37)	
Missing	3	5	
Stage			
0	0 (0.00)	3 (20.00)	0.169
I	10 (55.56)	5 (33.33)	
II	7 (38.89)	5 (33.33)	
III-IV	1 (5.56)	2 (13.33)	
Missing	5	9	
Tumor invasiveness			
In situ	0 (0.00)	3 (13.04)	0.233
Invasive	23 (100.00)	20 (86.96)	
Missing	0	1	
Ki-67			
High (≥14)	19 (90.48)	17 (89.47)	1.000
Low (<14)	2 (9.52)	2 (10.53)	
Missing	2	5	
St Gallen subtype			
Luminal A	0 (0.00)	1 (6.67)	0.286
Luminal B1	6 (31.58)	7 (46.67)	

Luminal B2	4 (21.05)	3 (20.00)	
Her2 positive	0 (0.00)	1 (6.67)	
Triple negative	9 (47.37)	3 (20.00)	
Missing	4	9	
Second BC			
No	17 (73.91)	18 (75.00)	1.000
Yes	6 (26.09)	6 (25.00)	
Median Age [Min-Max] [a]	46 [39-54]	50 [37-70]	0.830
Second OC			
No	22 (95.65)	23 (95.83)	1.000
Yes	1 (4.35)	1 (4.17)	
Median Age [Min-Max] [a]	68	70	-
Other tumors			
No	22 (95.65)	22 (91.67)	1.000
Yes	1 (4.35)	2 (8.33)	
BC/OC in I degree relatives			
No	9 (39.13)	8 (33.33)	0.766
Yes	14 (60.87)	16 (66.67)	
BC/OC in I/II degree relatives			
No	7 (30.43)	3 (12.50)	0.168
Yes	16 (69.57)	21 (87.50)	
Other cancers in I degree relatives			
No	19 (82.61)	14 (58.33)	0.111
Yes	4 (17.39)	10 (41.67)	
Other cancers in I/II degree relatives			
No	12 (52.17)	10 (41.67)	0.564
Yes	11 (47.83)	14 (58.33)	

[a] Median age, in years refers to age at diagnosis of the second cancer

Table 5: OC patients with *BRCA1/2* pathogenic variants: clinical features and personal/family history of cancer

OVARIAN CANCER (OC)	***BRCA1*-mutated** *N (%)*	***BRCA2*-mutated** *N (%)*	***P***
N. of patients	8	1	
Age at diagnosis, years			
Median Age [Min-Max]	50 [38-68]	66	-
Missing	0	0	
Histotype			
Serous carcinoma	7 (87.50)	0 (0.00)	-
Other malignant histotypes	1 (12.50)	1 (100.00)	
Borderline tumors	0 (0.00)	0 (0.00)	
Missing	0	0	
Grading			
Well-differentiated	0 (0.00)	0 (0.00)	-
Moderately differentiated	1 (21.50)	0 (0.00)	
Poorly differentiated	7 (87.50)	1 (100.00)	
Missing	0	0	
Stage			
0	0 (0.00)	0 (0.00)	-
I	1 (14.29)	0 (0.00)	
II	1 (14.29)	0 (0.00)	
III-IV	5 (71.43)	1 (100.00)	
Missing	1	0	
Tumor invasiveness			
Borderline	0 (0.00)	0 (0.00)	-
Invasive	8 (100.00)	1 (100.00)	
Missing	0	0	
Second BC			
No	6 (75.00)	1 (100.00)	-
Yes	2 (25.00)	0 (0.00)	
Median Age [Min-Max] [a]	58.5 [53-64]	-	-
Other tumors			
No	8 (100.00)	1 (100.00)	-
Yes	0 (0.00)	0 (0.00)	

BC/OC in I degree relatives			
No	1 (12.50)	0 (0.00)	-
Yes	7 (87.50)	1 (100.00)	
BC/OC in I/II degree relatives			
No	1 (12.50)	0 (0.00)	-
Yes	7 (87.50)	1 (100.00)	
Other cancers in I degree relatives			
No	5 (62.50)	1 (100.00)	-
Yes	3 (37.50)	0 (0.00)	
Other cancers in I/II degree relatives			
No	4 (50.00)	1 (100.00)	-
Yes	4 (50.00)	0 (0.00)	

[a] Median age, in years, refers to age at diagnosis of the second cancer

4.2 Pathogenic variants in genes other than *BRCA1/2*

We found 23 pathogenic/likely-pathogenic variants in genes other than *BRCA1/2*. These variants had either <1% or n/a frequency in the general population (according to 1000Genomes, Esp6500 and Exac03 databases) and where tested for pathogenicity in dbSNP and ClinVar databases (Table 6).

Among the 23 variants, we found 1 deletion in *ERCC3* gene in 1 patient also carrying a pathogenic variant in *BRCA1*, and 3 other lesions (1 deletion in *FANCA* gene, 1 deletion in *BRIP1* and 1 nonsense variant in *ATM*) in 3 different patients also carrying a pathogenic variant in *BRCA2*.

The remaining 19 pathogenic/likely-pathogenic variants found in 17 *BRCA1/2* wild type patients included: 6 lesions in *PALB2* gene (3 deletions and 3 nonsense varaints), 2 in *ATM* (1 deletion and 1 insertion), 2 in *FANCL* (1 insertion and 1 nonsense variant), 1 deletion in *BRIP1*, 1 nonsense variant in *FANCM*, 1 deletion in *FANCI*, 1 deletion in *SLX4*, 1 nonsense variant in *MSH6*, 1 nonsense variant in *RAD51D*, 1 deletion in *PPM1D*, 1 deletion in *RECQL4*, and 1 deletion in *TSC2*. The *FANCL* insertion and 1 of the *PALB2* nonsense variants were both present in the same patient, as well as *BRIP1* and *SLX4* deletions in another patient.

All 17 *BRCA1/2* wild type patients had BC first; among these patients, 8 (47.1%) had BBC and 2 (11.8%) had a subsequent OC. Median age at the onset was 43 years for first BC, 57 for subsequent BC and 52.5 for subsequent OC (Table 1). None of these patients had cancers other than BC or OC.

Table 6: Pathogenic/likely-pathogenic variants in genes other than *BRCA1/2* detected in 21 unrelated patients

Sample ID	Cancer (age)[a]	*BRCA* status[b]	Gene	chr	start	end	ref	alt	Mutation type	HGVS[c]	depth	VAF[d]	IARC class	dbSNP/ClinVar[e]	esp6500[f]	1000g[f]	exac03[f]
A243	ILC (26y), IDC (45y)	-	*PALB2*	16	23641307	23641308	AT	-	frameshift deletion	NM_024675: exon 5, c.2167_2168del p.Met723fs	596	0.44	class 5	rs587776416/Pathogenic	NA	NA	0,00007
A284	IDC (39y)	+	*ERCC3*	2	128030511	128030511	T	-	frameshift deletion	NM_000122: exon 11, c.1757delA p.Gln586fs	1031	0.44	class 4	-	0,00048	NA	0,00017
A414	IDC (30y)	+	*FANCA*	16	89862330	89862333	GTGA	-	frameshift deletion	NM_000135: exon 11, c.987_990del p.Thr329fs	387	0.46	class 5	rs772359099/Pathogenic	NA	NA	0,00003
A479	IDC (37y)	-	*FANCL*	2	58386928	58386928	-	TAAT	frameshift insertion	NM_018062: exon 14, c.1096_1099dupATTA p.Thr367fs	892	0.25	class 4	rs759217526/Pathogenic/VUS	0,00248	NA	0,00283
			PALB2	16	23646416	23646416	A	T	nonsense	NM_024675: exon 4, c.1451T>A p.Leu484Ter	667	0.50	class 5	rs786203714/Pathogenic	NA	NA	NA
A482	IDC (37y), IDC (37y)	+	*BRIP1*	17	59761412	59761415	TCTT	-	frameshift deletion	NM_032043: exon 20, c.2992_2995del p.Lys998fs	611	0.43	class 4	rs786203717/Pathogenic	NA	NA	NA
A485	IDC (43y), OSC (51y)	-	*PPM1D*	17	58740695	58740695	T	-	frameshift deletion	NM_003620: exon 6, c.1600delT p.Phe534fs	350	0.11	class 4	-	NA	NA	NA
A502	IDC (59y), IDC (59y)	-	*PALB2*	16	23641307	23641308	AT	-	frameshift deletion	NM_024675: exon 5, c.2167_2168del p.Met723fs	592	0.45	class 5	rs587776416/Pathogenic	NA	NA	0,00007
A519	IDC (32y)	-	*FANCM*	14	45667921	45667921	C	T	nonsense	NM_020937: exon 22, c.5791C>T p.Arg1931Ter (p.Gly1906fs)	531	0.47	class 5	rs144567652	0,00085	0,00060	0,00088
A520	ILC (48y)	-	*PALB2*	16	23641218	23641218	G	A	nonsense	NM_024675: exon 5, c.2257C>T p.Arg753Ter	459	0,6	class 5	rs180177110/Pathogenic	NA	NA	0,00003
A526	IDC (40y), IDC (40y)	-	*RECQL4*	8	145740367	145740367	A	-	frameshift deletion	NM_004260: exon 9, c.1573delT p.Cys525fs	141	0.34	class 4	rs386833845/Likely pathogenic	NA	NA	0,00023
A530	ILC (62y), ILC (62y)	-	*PALB2*	16	23647332	23647332	G	A	nonsense	NM_024675: exon 4, c.535C>T p.Gln179Ter	787	0.49	class 4	-	NA	NA	NA
A531	IDC (38y)	-	*MSH6*	2	48027775	48027775	A	T	nonsense	NM_000179: exon 4, c.2653A>T p.Lys885Ter	849	0.45	class 5	rs587782593/Pathogenic	NA	NA	NA
A532	DCIS (47y), OSC (54y)	-	*RAD51D*	17	33430317	33430317	G	A	nonsense	NM_002878: exon 8, c.694C>T p.Arg232Ter	381	0.50	class 5	rs587780104/Pathogenic	NA	NA	0,00001
A544	DCIS (55y), DCIS (55y)	-	*PALB2*	16	23646724	23646727	AAGA	-	frameshift deletion	NM_024675: exon 4, c.1140_1143del p.Ser380fs	434	0.46	class 4	-	NA	NA	NA

A554	IDC (59y), IDC (77y)	-	*ATM*	11	108153550	108153554	CTTAT	-	frameshift deletion	NM_000051: exon 25, c.3690_3694del p.Asn1230fs	382	0.46	class 4	-	NA	NA	NA
A579	IDC (38y)	-	*FANCI*	15	89824444	89824456	AAGTTGTTCTTCT	-	frameshift deletion	NM_001113378: exon 15, c.1425_1437del p.Gln475fs	302	0.47	class 4	-	NA	NA	NA
A633	DCIS (38y), IDC (45y)	+	*ATM*	11	108143570	108143570	C	A	nonsense	NM_000051: exon 22, c.3275C>A p.Ser1092Ter	193	0.48	class 4	-	NA	NA	NA
A695	IDC (74y)	-	*FANCL*	2	58453913	58453913	G	A	nonsense	NM_018062: exon 4, c.223C>T p.Gln75Ter	204	0.42	class 4	-	NA	NA	NA
A767	IMC (63y), IDC (72y)	-	*ATM*	11	108213997	108213997	-	CTGTC	frameshift insertion	NM_000051: exon 57, c.8318_8322dupCTGTC p.Thr2773fs	816	0.27	class 4	-	NA	NA	NA
A790	IDC (29y), IDC (36y)	-	*TSC2*	16	2127626	2127627	AG	-	frameshift deletion	NM_000548: exon 26, c.2865_2866del p.Gln955fs	845	0.46	class 4	-	NA	NA	NA
A806	DCIS (26y)	-	*SLX4*	16	3639996	3640020	GCAGCACAGCTTCGCTTCTTGGTGG	-	frameshift deletion	NM_032444: exon 12, c.3619_3643del p.Pro1207fs	446	0.33	class 4	-	NA	NA	NA
			BRIP1	17	59763197	59763197	T	-	frameshift deletion	NM_032043: exon 19, c.2905delA p.Asn969fs	372	0.47	class 4	-	NA	NA	NA

[a] Tumor histotype: DCIS (ductal carcinoma in situ), IDC (invasive ductal carcinoma), ILC (invasive lobular carcinoma), IMC (invasive mucinous carcinoma), OSC (ovarian serous carcinoma)
[b] *BRCA* mutational status: + (patient with pathogenic variants in *BRCA1/2* genes), - (wild type patient for *BRCA1/2* genes)
[c] Mutation nomenclature according to the *Human Genome Variation Society* (HGVS)
[d] Variant Frequency
[e] Mutation classification according to *Single Nucleotide Polymorphism Database* (dbSNP) and *Clinical Variant* (ClinVar)
[f] Population frequencies present in Esp6500, 1000Genomes and Exac03 databases

Figures 11A and 11B show the BC/OC family history of 2 patients with *PALB2* and *TSC2* pathogenic variants, respectively.

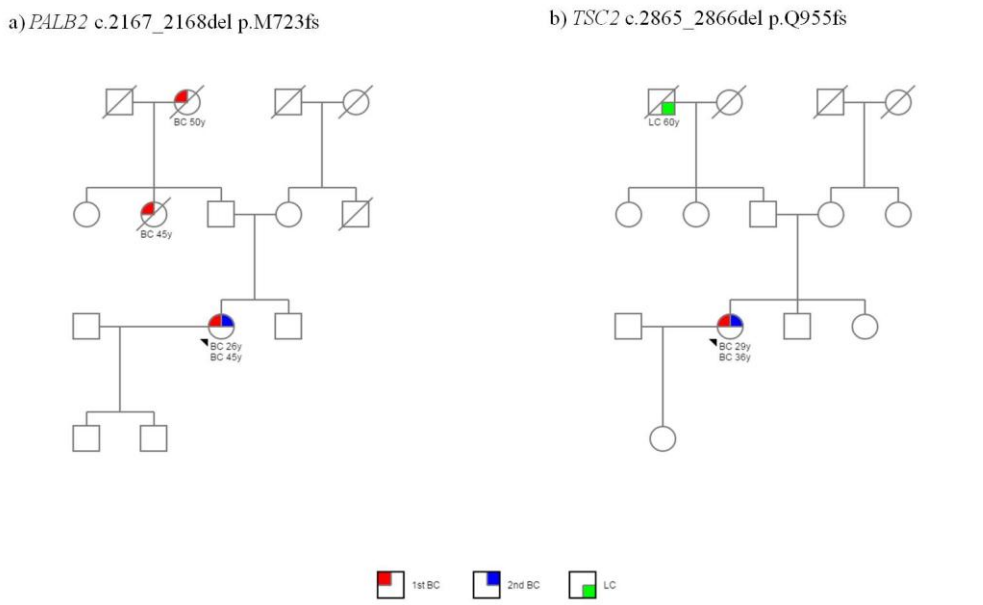


Figure 11: Pedigrees of two patients with mutations in genes other than *BRCA1/2*. a) Pedigree of patient A243 with c.2167_2168del p.M723fs mutation in *PALB2* gene. b) Pedigree of patient A790 with c.2865_2866del p.Q955fs mutation in *TSC2* gene. The probands are indicated by arrowheads. Cancer type and age at cancer diagnosis are indicated in the legend: 1st BC, first breast cancer; 2nd BC, second breast cancer; LC, lung cancer. Symbols: squares, males; circles, females; quadrant shading, cancer affected; slash through square or circle, deceased.

4.3 Other identified variants

Out of 181 patients with no pathogenic variants, 162 (89.5%) had BC first; among these patients, 32 (19.8%) had BBC and 4 (2.5%) had a subsequent OC. Median age was 42 years at onset of first BC, 58.5 for subsequent BC, and 68.5 for subsequent OC (Table 1). The remaining 19 (10.5%) had OC first; among these patients, 5 (26.3%) had a subsequent BC. Median age was 47 years at onset of first OC, and 55 for subsequent BC (Table 2).
In patients without pathogenic variants we identified 1,026 variants with <1% or n/a population frequency: among these, 379 (36.9%) were synonymous variants, 631 (61.5%) missense variants, 6 (0.6%) in frame deletions, 2 (0.2%) in frame insertions, and 8 (0.8%) splicing variants.

Globally, we found 674 unique variants in 92 genes, with an average of 6 variants per patient.
Among the 1,026 rare variants, we focused on the 631 identified missense variants by predicting their functional impact with PolyPhen-2 HVar and SIFT. The results allowed us to categorize the 181 patients into 3 groups: patients (70 subjects) with at least 1 variant classified as damaging by both PolyPhen-2 Hvar and SIFT tools; patients (26) with variants discordantly classified; patients (85) with variants classified as benign. No statistically significant differences were found between the three groups (Tables 7 and 8).

Table 7: Clinical features and personal/family cancer history of BC patients without pathogenic variants

BREAST CANCER (BC)	**Probably benign by PolyPhen/SIFT** *N (%)*	**PolyPhen/SIFT non concordant** *N (%)*	**Probably damaging by PolyPhen/SIFT** *N (%)*	***P***
N. of patients	77	23	62	
Age at diagnosis, years				
Median Age [Min-Max]	41 [26-79]	48 [26-60]	42 [25-79]	0.421
Missing	0	0	0	
Histotype				
In situ carcinoma	9 (12.50)	4 (18.18)	3 (5.00)	0.158
Invasive ductal carcinoma	43 (59.72)	16 (72.73)	46 (76.67)	
Invasive lobular carcinoma	10 (13.89)	2 (9.09)	5 (8.33)	
Other invasive histotypes	10 (13.89)	0 (0.00)	6 (10.00)	
Missing	5	1	2	
Grading				
Well-differentiated	8 (13.33)	0 (0.00)	9 (17.65)	0.304
Moderately differentiated	29 (48.33)	13 (65.00)	23 (45.10)	
Poorly differentiated	23 (38.33)	7 (35.00)	19 (37.25)	
Missing	17	3	11	
Stage				
0	9 (14.75)	4 (22.22)	3 (6.00)	0.213
I	36 (59.02)	6 (33.33)	30 (60.00)	
II	11 (18.03)	5 (27.78)	13 (26.00)	
III-IV	5 (8.20)	3 (16.67)	4 (8.00)	
Missing	16	5	12	
Tumor invasiveness				
In situ	9 (12.50)	4 (18.18)	3 (5.00)	0.128

Invasive	63 (87.50)	18 (81.82)	57 (95.00)	
Missing	5	1	2	
Ki-67				
High (≥14)	31 (62.00)	8 (57.14)	33 (66.00)	0.811
Low (<14)	19 (38.00)	6 (42.86)	17 (34.00)	
Missing	27	9	12	
St Gallen subtype				
Luminal A	11 (25.58)	4 (30.77)	11 (23.91)	0.589
Luminal B1	18 (41.86)	7 (53.85)	16 (34.78)	
Luminal B2	5 (11.63)	2 (15.38)	10 (21.74)	
Her2 positive	5 (11.63)	0 (0.00)	2 (4.35)	
Triple negative	4 (9.30)	0 (0.00)	7 (15.22)	
Missing	34	10	16	
Second BC				
No	59 (76.62)	20 (86.96)	51 (82.26)	0.504
Yes	18 (23.38)	3 (13.04)	11 (17.74)	
Median Age [Min-Max] [a]	62 [36-82]	60 [57-75]	55 [32-65]	0.303
Second OC				
No	76 (98.70)	22 (95.65)	60 (96.77)	0.485
Yes	1 (1.30)	1 (4.35)	2 (3.23)	
Median Age [Min-Max] [a]	65	55	74.5 [72-77]	-
Other tumors				
No	69 (89.61)	20 (86.96)	54 (87.10)	0.842
Yes	8 (10.39)	3 (13.04)	8 (12.90)	
BC/OC in I degree relatives				
No	30 (38.96)	7 (30.43)	17 (27.42)	0.340
Yes	47 (61.04)	16 (69.57)	45 (72.58)	
BC/OC in I/II degree relatives				
No	14 (18.18)	2 (8.70)	10 (16.13)	0.616
Yes	63 (81.82)	21 (91.30)	52 (83.87)	
Other cancers in I degree relatives				
No	51 (66.23)	14 (60.87)	35 (56.45)	0.497
Yes	26 (33.77)	9 (39.13)	27 (43.55)	
Other cancers in I/II degree relatives				
No	25 (32.47)	7 (30.43)	20 (32.26)	0.983
Yes	52 (67.53)	16 (69.57)	42 (67.74)	

[a] Median age, in years, refers to age at second cancer diagnosis

Table 8: Clinical features and personal/family cancer history of OC patients without pathogenic variants

OVARIAN CANCER (OC)	**Probably benign by PolyPhen/SIFT** *N (%)*	**PolyPhen/SIFT non concordant** *N (%)*	**Probably damaging by PolyPhen/SIFT** *N (%)*	***P***
N. of patients	8	3	8	
Age at diagnosis, years				
Median Age [Min-Max]	46.5 [28-58]	61 [34-66]	48 [36-81]	0.421
Missing	0	0	0	
Histotype				
Serous carcinoma	6 (75.00)	2 (66.67)	3 (37.50)	0.371
Other malignant histotypes	2 (25.00)	1 (33.33)	2 (25.00)	
Borderline tumors	0 (0.00)	0 (0.00)	3 (37.50)	
Missing	0	0	0	
Grading				
Well-differentiated	1 (12.50)	1 (33.33)	0 (0.00)	0.833
Moderately differentiated	1 (12.50)	0 (0.00)	0 (0.00)	
Poorly differentiated	6 (75.00)	2 (66.67)	5 (100.00)	
Missing	0	0	3	
Stage				
0	0 (0.00)	0 (0.00)	0 (0.00)	0.088
I	4 (66.67)	2 (66.67)	0 (0.00)	
II	0 (0.00)	0 (0.00)	1 (20.00)	
III-IV	2 (33.33)	1 (33.33)	4 (80.00)	
Missing	2	0	3	
Tumor invasiveness				
Borderline	0 (0.00)	0 (0.00)	3 (37.50)	0.166
Invasive	8 (100.00)	3 (100.00)	5 (62.50)	
Missing	0	0	0	
Second BC				
No	7 (87.50)	3 (100.00)	4 (50.00)	0.181
Yes	1 (12.50)	0 (0.00)	4 (50.00)	
Median Age [Min-Max] [a]	62	-	54.5 [45-81]	-
Other tumors				
No	8 (100.00)	3 (100.00)	8 (100.00)	-
Yes	0 (0.00)	0 (0.00)	0 (0.00)	

BC/OC in I degree relatives				
No	5 (62.50)	1 (33.33)	5 (62.50)	0.689
Yes	3 (37.50)	2 (66.67)	3 (37.50)	
BC/OC in I/II degree relatives				
No	2 (25.00)	1 (33.33)	5 (62.50)	0.344
Yes	6 (75.00)	2 (66.67)	3 (37.50)	
Other cancers in I degree relatives				
No	4 (50.00)	2 (66.67)	4 (50.00)	1.000
Yes	4 (50.00)	1 (33.33)	4 (50.00)	
Other cancers in I/II degree relatives				
No	2 (25.00)	2 (66.67)	3 (37.50)	0.580
Yes	6 (75.00)	1 (33.33)	5 (62.50)	

[a] Median age, in years, refers to age at second cancer diagnosis.

5. Discussion

In most clinical settings, current genetic screenings to assess BC/OC risk are based on the analysis of *BRCA1* and *BRCA2* genes only, despite emerging evidence of a high number of genes eligible for testing [Easton DF *et al.* 2015]. Given the number of genes the mutations of which can have a role in the development of a broad spectrum of tumors, we performed BC/OC genetic testing by using a multi-gene panel including 94 genes involved in the main hereditary cancer syndromes. To our knowledge, this is the first large study on BC/OC Italian patients based on multi-gene panel sequencing and one of the largest studies on BC/OC predisposition, including both a great number of tested genes and of recruited patients [Walsh T *et al.* 2010; Kurian AW *et al.* 2014; Desmond A *et al.* 2015; Gracia-Aznarez FJ *et al.* 2013; Kraus C *et al.* 2017].

By the above approach, we could detect a total of 81 pathogenic/likely-pathogenic variants in 74/255 (29.0%) patients: 32 (39.5%) in *BRCA1*, 26 (32.1%) in *BRCA2*, and 23 (28.4%) in other genes. The 23 pathogenic variants in genes other than *BRCA1/2* were present in 21 patients, 17 of whom were negative for *BRCA1/2* alterations.

The 57 patients with *BRCA1/2* pathogenic variants have now been included in a surveillance protocol according to the FONCaM guidelines [Collegio Italiano dei Senologi 2016] and the genetic test has been extended to the consenting relatives.

The BC characteristics of *BRCA1/2*-mutated patients were similar to those described in the literature [Templeton AJ *et al.* 2016; Wang W *et al.* 2016], with a significantly higher number of poorly differentiated (P=0.005) and triple-negative cancers (P=0.005), and of cancers with a higher expression (P=0.003) of Ki-67 compared to the other patients (Table 1); of relevance, these three parameters are well-known signs of malignancy aggressiveness.

Moreover, compared to the other patients, *BRCA1/2*-mutated subjects developed BC at a younger age, especially second BC (P=0.041), and showed a higher family history of BC/OC, especially for I-degree relatives of OC patients (P=0.039). These findings are expected on the basis of the high penetrance of pathogenic variants in *BRCA1/2* genes (Tables 1 and 2).

Thirteen patients had alterations in *ATM*, *BRIP1*, *PALB2*, *PPM1D* and *RAD51D* genes, which are known to be associated with an increased risk of BC [Apostolou P and Fostira F 2013; Ruark E *et al.* 2013]. Although these genes are considered moderately penetrant, guidelines for the clinical management of mutation carriers are still unavailable. At any rate, we

considered mutation carriers and their families eligible for further evaluation; accordingly, we are currently combining data on our case series with those of other institutions to gain insights on cancer risk development associated with moderately penetrant genes.

PALB2, the most frequently mutated gene besides *BRCA1* and *BRCA2* in our case series, is worth mentioning briefly. Antoniou and colleagues [Antoniou AC *et al.* 2014] reported that *PALB2* is the most important BC predisposition gene after *BRCA1* and *BRCA2*. We found 6 *BRCA1/2*-mutations negative patients with a pathogenic variant in *PALB2* gene, 4 (66.7%) of whom with BBC. These observations further highlight both the high risk of BC associated with *PALB2* pathogenic variants and the importance of adding *PALB2* gene to standard genetic tests for patients with suspected hereditary BC syndrome.

Two patients were carriers of frameshift mutations in *BRIP1* gene, the truncating mutations of which have recently been excluded from having a role in BC risk [Easton DF *et al.* 2016]. This last finding had no impact on patient assigned category, since each of the two patients proved to carry a deleterious mutation in another relevant gene (*BRCA2* and *SLX4*, respectively).

We also found 1 patient with a pathogenic variant in *MSH6*, a gene associated with Lynch syndrome (LS). If this colorectal cancer syndrome is directly related with BC predisposition is currently matter of debate [Win AK *et al.* 2013]. In our case, the *MSH6*-mutation carrier and her family will undergo a surveillance protocol including the screening for both BC, since this is the only cancer type present in the family, and colorectal cancer according to LS guidelines [Umar A *et al.* 2004], since *MSH6* mutations are well-known to increase colorectal cancer risk. In other words, to optimize surveillance, we considered both disease family history and acquired knowledge on genotype-phenotype associations.

Six patients had mutations in *FANCA*, *FANCI*, *FANCL*, *FANCM* and *SLX4* genes, which are known to be associated with Fanconi anemia (FA). FA is a recessive genetic disorder characterized by multiple congenital abnormalities, bone marrow failure and susceptibility to cancer, occurring when both alleles of one FA gene are mutated. Monoallelic mutations of some FA genes have been associated with BC risk [Barroso E *et al.* 2009; Peterlongo P *et al.* 2015], while biallelic mutations in *BRCA2* have been associated with FA [Meyer S *et al.* 2014]. Although further studies are required to give a clear overview, these observations indicate that biallelic mutations of FA genes result in FA, while monoallelic mutations are likely to increase the risk of BC.

Finally, we found 3 pathogenic/likely-pathogenic variants in *ERCC3*, *RECQL4* and *TSC2* genes, encoding transcription factors and tumor suppressors. Although mutations in these genes were not clearly associated with BC, their role in BC predisposition cannot be excluded due to the gene role in the major cancer pathways. Moreover, mutations in *ERCC3* and *RECQL*, a homologue gene of *RECQL4*, have recently been identified in families with multiple BC cases [Vijai J *et al.* 2016; Cybulski C *et al.* 2015]. Of note, the pathogenicity of the identified variants is based on guidelines [Richards S *et al.* 2015] and refers to their potential role in cancer development, not to their causality of BC. Therefore, in our patients, BC predisposition might be associated with unknown variants in genes not included in the panel. On the whole, due to lack of clear-cut information on cancer risk, the management of patients with *ERCC3*, *RECQL4* and *TSC2* mutations and of their families remains problematic.

By comparing *BRCA1/2*-mutation positive patients with carriers of pathogenic variants in genes other than *BRCA1/2*, we observed a much higher percentage of BBCs in patients without *BRCA1/2* mutations (47.1%) than in patients with *BRCA1* (26.1%) and *BRCA2* (25.0%) (P=0.036) mutations. This suggests a high penetrance and a high risk of BC for mutations other than *BRCA1/2*, despite the older age at onset of carrier patients (Table 1). On the contrary, the pathogenic variants in genes other than *BRCA1/2* did not appear to be associated with OC risk, since all carriers had BC, and 2 patients only developed OC as a second tumor.

Another feature of mutation carriers in genes other than *BRCA1/2* was the lower family history of BC/OC in I- and II-degree relatives (P=0.039) compared to both *BRCA1/2*-mutated patients and patients without pathogenic variants (Table 1). Although this observation has to be confirmed by larger studies, these carriers might have a more heterogeneous family history characterized by different types of malignancies in addition to BC/OC.

No clear pathogenic variants were identified in 181/255 (71.0%) subjects of our cohort; globally, in these patients we found 1,026 rare variants of uncertain significance. NGS-based studies lead to the identification of many non-easily classifiable variants. Although several techniques can now be used to investigate their pathogenicity [Thusberg J and Vihinen M 2009], efficient and accurate classification methods are still needed to translate theoretical information to clinical practice. The bioinformatic tools for the prediction of pathogenicity used in this study seemed inadequate to classify many variants and to identify higher risk patients. Besides bioinformatic predictions, some of the identified variants may actually increase BC and OC risk. However, risk assessment of candidate variants is made difficult by

the limited number of mutation carriers and by the possible interference of different genetic and environmental factors. The multifactorial nature of BC/OC and the presence of predisposing mutations in genes never included in panels that are currently used for genetic testing are likely to further increase the complexity of the scenario.

On the whole, the impact on cancer predisposition of many variants identified by NGS-based studies remains one of the biggest challenges in genetics and precision medicine.

References

Adank MA, Jonker MA, Kluijt I, van Mil SE, Oldenburg RA, Mooi WJ, Hogervorst FB, van den Ouweland AM, Gille JJ, Schmidt MK, van der Vaart AW, Meijers-Heijboer H, Waisfisz Q. *CHEK2**1100delC homozygosity is associated with a high breast cancer risk in women. J Med Genet. 2011;48:860-863.

Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods. 2010;7:248-249.

AIOM-AIRTUM. I numeri del cancro in Italia 2017. Available on the website www.aiom.it

Antoniou AC, Casadei S, Heikkinen T, Barrowdale D, Pylkäs K, Roberts J, Lee A, Subramanian D, De Leeneer K, Fostira F, Tomiak E, Neuhausen SL, Teo ZL, Khan S, Aittomäki K *et al.* Breast-cancer risk in families with mutations in *PALB2*. N Engl J Med. 2014;371:497-506.

Antoniou A, Pharoah PD, Narod S, Risch HA, Eyfjord JE, Hopper JL, Loman N, Olsson H, Johannsson O, Borg A, Pasini B, Radice P, Manoukian S, Eccles DM, Tang N *et al.* Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet. 2003;72:1117-1130.

Apostolou P, Fostira F. Hereditary breast cancer: the era of new susceptibility genes. Biomed Res Int. 2013;2013:747318.

Barroso E, Pita G, Arias JI, Menendez P, Zamora P, Blanco M, Benitez J, Ribas G. The Fanconi anemia family of genes and its correlation with breast cancer susceptibility and breast cancer features. Breast Cancer Res Treat. 2009;118:655-660.

Bartkova J, Tommiska J, Oplustilova L, Aaltonen K, Tamminen A, Heikkinen T, Mistrik M, Aittomäki K, Blomqvist C, Heikkilä P, Lukas J, Nevanlinna H, Bartek J. Aberrations of the MRE11-RAD50-NBS1 DNA damage sensor complex in human breast cancer: MRE11 as a candidate familial cancer-predisposing gene. Mol Oncol. 2008;2:296-316.

Bell DW, Varley JM, Szydlo TE, Kang DH, Wahrer DC, Shannon KE, Lubratovich M, Verselis SJ, Isselbacher KJ, Fraumeni JF, Birch JM, Li FP, Garber JE, Haber DA. Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science. 1999;286:2528-2531.

Berwick M, Satagopan JM, Ben-Porat L, Carlson A, Mah K, Henry R, Diotti R, Milton K, Pujara K, Landers T, Dev Batish S, Morales J, Schindler D, Hanenberg H, Hromas R *et al.* Genetic heterogeneity among Fanconi anemia heterozygotes and risk of cancer. Cancer Res. 2007;67:9591-9596.

Birch JM, Alston RD, McNally RJ, Evans DG, Kelsey AM, Harris M, Eden OB, Varley JM. Relative frequency and morphology of cancers in carriers of germline *TP53* mutations. Oncogene. 2001;20:4621-4628.

Bogdanova N, Feshchenko S, Schürmann P, Waltes R, Wieland B, Hillemanns P, Rogov YI, Dammann O, Bremer M, Karstens JH, Sohn C, Varon R, Dörk T. Nijmegen Breakage Syndrome mutations and risk of breast cancer. Int J Cancer. 2008;122:802-806.

Bogdanova N, Togo AV, Ratajska M, Kluźniak W, Takhirova Z, Tarp T, Prokofyeva D, Bermisheva M, Yanus GA, Gorodnova TV, Sokolenko AP, Kuźniacka A, Podolak A, Stukan M, Wokołorczyk D *et al.* Prevalence of the *BLM* nonsense mutation, p.Q548X, in ovarian cancer patients from Central and Eastern Europe. Fam Cancer. 2015;14:145-149.

Bonadona V, Bonaïti B, Olschwang S, Grandjouan S, Huiart L, Longy M, Guimbaud R, Buecher B, Bignon YJ, Caron O, Colas C, Noguès C, Lejeune-Dumoulin S, Olivier-Faivre L, Polycarpe-Osaer F *et al.* Cancer risks associated with germline mutations in *MLH1*, *MSH2*, and *MSH6* genes in Lynch syndrome. JAMA. 2011;305:2304-2310.

Breast Cancer Linkage Consortium. Cancer risks in *BRCA2* mutation carriers. J Natl Cancer Inst. 1999;91:1310-1316.

Brierley JD, Gospodarowicz MK, Christian Wittekind C (editors). TNM classification of malignant tumours. Union for International Cancer Control (UICC) 8th edition 2017.

Buisson R, Dion-Côté AM, Coulombe Y, Launay H, Cai H, Stasiak AZ, Stasiak A, Xia B, Masson JY. Cooperation of breast cancer proteins PALB2 and piccolo BRCA2 in stimulating homologous recombination. Nat Struct Mol Biol. 2010;17:1247-1254.

Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490:61-70.

Caputo S, Benboudjema L, Sinilnikova O, Rouleau E, Béroud C, Lidereau R; French BRCA GGC Consortium. Description and analysis of genetic variants in French hereditary breast and ovarian cancer families recorded in the UMD-BRCA1/BRCA2 databases. Nucleic Acids Res. 2012; 40:D992-D1002.

Catucci I, Milgrom R, Kushnir A, Laitman Y, Paluch-Shimon S, Volorio S, Ficarazzi F, Bernard L, Radice P, Friedman E, Peterlongo P. Germline mutations in

BRIP1 and PALB2 in Jewish high cancer risk families. Fam Cancer. 2012;11:483-491.

Chehab NH, Malikzay A, Appel M, Halazonetis TD. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev. 2000;14:278-288.

Chen S, Parmigiani G. Meta-analysis of BRCA1 and BRCA2 penetrance. J Clin Oncol. 2007;25:1329-1333.

Cho MY, Kim HS, Eng C, Kim DS, Kang SJ, Eom M, Yi SY, Bronner MP. First report of ovarian dysgerminoma in Cowden syndrome with germline PTEN mutation and PTEN-related 10q loss of tumor heterozygosity. Am J Surg Pathol. 2008;32:1258-1264.

Collegio Italiano dei Senologi. Carcinoma mammario eredo-familiare – Linee guida 2016. Available at: http://senologia.it/wp-content/uploads/Carcinoma-eredo-familiare-def.pdf

Cybulski C, Carrot-Zhang J, Kluźniak W, Rivera B, Kashyap A, Wokołorczyk D, Giroux S, Nadaf J, Hamel N, Zhang S, Huzarski T, Gronwald J, Byrski T, Szwiec M, Jakubowska A *et al.* Germline RECQL mutations are associated with breast cancer susceptibility. Nat Genet. 2015;47:643-646.

Cybulski C, Górski B, Huzarski T, Masojć B, Mierzejewski M, Debniak T, Teodorczyk U, Byrski T, Gronwald J, Matyjasik J, Zlowocka E, Lenner M, Grabowska E, Nej K, Castaneda J *et al.* CHEK2 is a multiorgan cancer susceptibility gene. Am J Hum Genet. 2004;75:1131-1135.

Cybulski C, Huzarski T, Górski B, Masojć B, Mierzejewski M, Debniak T, Gliniewicz B, Matyjasik J, Złowocka E, Kurzawski G, Sikorski A, Posmyk M, Szwiec M, Czajka R, Narod SA *et al.* A novel founder *CHEK2* mutation is associated with increased prostate cancer risk. Cancer Res. 2004;64:2677-2679.

Cybulski C, Wokołorczyk D, Huzarski T, Byrski T, Gronwald J, Górski B, Debniak T, Masojć B, Jakubowska A, Gliniewicz B, Sikorski A, Stawicka M, Godlewski D, Kwias Z, Antczak A *et al.* A large germline deletion in the Chek2 kinase gene is associated with an increased risk of prostate cancer. J Med Genet. 2006;43:863-866.

Cybulski C, Wokołorczyk D, Jakubowska A, Huzarski T, Byrski T, Gronwald J, Masojć B, Deebniak T, Górski B, Blecharz P, Narod SA, Lubiński J. Risk of breast cancer in women with a *CHEK2* mutation with and without a family history of breast cancer. J Clin Oncol. 2011;29:3747-3752.

Damiola F, Pertesi M, Oliver J, Le Calvez-Kelm F, Voegele C, Young EL, Robinot N, Forey N, Durand G, Vallée MP, Tao K, Roane TC, Williams GJ, Hopper JL, Southey MC *et al.* Rare key functional domain missense substitutions in *MRE11A*, *RAD50*, and *NBN* contribute to breast cancer susceptibility: results from a Breast

Cancer Family Registry case-control mutation-screening study. Breast Cancer Res. 2014;16:R58.

de Voer RM, Hahn MM, Mensenkamp AR, Hoischen A, Gilissen C, Henkes A, Spruijt L, van Zelst-Stams WA, Kets CM, Verwiel ET, Nagtegaal ID, Schackert HK, van Kessel AG, Hoogerbrugge N, Ligtenberg MJ *et al.* Deleterious Germline BLM Mutations and the Risk for Early-onset Colorectal Cancer. Sci Rep. 2015;5:14060.

Desmond A, Kurian AW, Gabree M, Mills MA, Anderson MJ, Kobayashi Y, Horick N, Yang S, Shannon KM, Tung N, Ford JM, Lincoln SE, Ellisen LW. Clinical Actionability of Multigene Panel Testing for Hereditary Breast and Ovarian Cancer Risk Assessment. JAMA Oncol. 2015;1:943-951.

Desrichard A, Bidet Y, Uhrhammer N, Bignon YJ. CHEK2 contribution to hereditary breast cancer in non-BRCA families. Breast Cancer Res. 2011;13:R119.

Dong X, Wang L, Taniguchi K, Wang X, Cunningham JM, McDonnell SK, Qian C, Marks AF, Slager SL, Peterson BJ, Smith DI, Cheville JC, Blute ML, Jacobsen SJ, Schaid DJ *et al.* Mutations in CHEK2 associated with prostate cancer risk. Am J Hum Genet. 2003;72:270-280.

Easton DF, Lesueur F, Decker B, Michailidou K, Li J, Allen J, Luccarini C, Pooley KA, Shah M, Bolla MK, Wang Q, Dennis J, Ahmad J Thompson ER, Damiola F, Pertesi M *et al.* No evidence that protein truncating variants in BRIP1 are associated with breast cancer risk: implications for gene panel testing. J Med Genet. 2016;53:298-309.

Easton DF, Pharoah PD, Antoniou AC, Tischkowitz M, Tavtigian SV, Nathanson KL, Devilee P, Meindl A, Couch FJ, Southey M, Goldgar DE, Evans DG, Chenevix-Trench G, Rahman N, Robson M *et al.* Gene-Panel Sequencing and the Prediction of Breast-Cancer Risk. N Engl J Med. 2015;372:2243-2257.

Economopoulou P, Dimitriadis G, Psyrri A. Beyond BRCA: new hereditary breast cancer susceptibility genes. Cancer Treat Rev. 2015;41:1-8.

Eng C. PTEN Hamartoma Tumor Syndrome. GeneReviews. 2001 [updated 2016].

Engel C, Loeffler M, Steinke V, Rahner N, Holinski-Feder E, Dietmaier W, Schackert HK, Goergens H, von Knebel Doeberitz M, Goecke TO, Schmiegel W, Buettner R, Moeslein G, Letteboer TG, Gómez García E *et al.* Risks of less common cancers in proven mutation carriers with lynch syndrome. J Clin Oncol. 2012;30:4409-4415.

Evans DG, Birch JM, Narod SA. Is CHEK2 a cause of the Li-Fraumeni syndrome? J Med Genet. 2008;45:63-64.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr

Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, Gavin A, Visser O, Bray F. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. Eur J Cancer. 2018.

Fitzgibbons PL, Page DL, Weaver D, Thor AD, Allred DC, Clark GM, Ruby SG, O'Malley F, Simpson JF, Connolly JL, Hayes DF, Edge SB, Lichter A, Schnitt SJ. Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. Arch Pathol Lab Med. 2000;124:966-978.

Fokkema IF, Taschner PE, Schaafsma GC, Celli J, Laros JF, den Dunnen JT. LOVD v.2.0: the next generation in gene variant databases. Hum Mutat. 2011;32:557-563.

Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, Mortimer P, Swaisland H, Lau A, O'Connor MJ, Ashworth A, Carmichael J, Kaye SB, Schellens JH, de Bono JS. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med. 2009;361:123-134.

Ford D, Easton DF, Bishop DT, Narod SA, Goldgar DE. Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. Lancet. 1994;343:692-695.

Foulkes WD, Shuen AY. In brief: BRCA1 and BRCA2. J Pathol. 2013;230:347-349.

Gage M, Wattendorf D, Henry LR. Translational advances regarding hereditary breast cancer syndromes. J Surg Oncol. 2012;105:444-451.

Gatti R, Perlman S. Ataxia-Telangiectasia. GeneReviews. 1999 [updated 2016].

Ghimenti C, Sensi E, Presciuttini S, Brunetti IM, Conte P, Bevilacqua G, Caligo MA. Germline mutations of the BRCA1-associated ring domain (*BARD1*) gene in breast and breast/ovarian families negative for BRCA1 and BRCA2 alterations. Genes Chromosomes Cancer. 2002;33:235-242.

Ghiorzo P, Pensotti V, Fornarini G, Sciallero S, Battistuzzi L, Belli F, Bonelli L, Borgonovo G, Bruno W, Gozza A, Gargiulo S, Mastracci L, Nasti S, Palmieri G, Papadia F *et al.* Contribution of germline mutations in the BRCA and PALB2 genes to pancreatic cancer in Italy. Fam Cancer. 2012;11:41-47.

Giordano SH, Buzdar AU, Hortobagyi GN. Breast cancer in men. Ann Intern Med. 2002;137:678-687.

Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Akinyemiju TF, Al Lami FH, Alam T, Alizadeh-Navaei R, Allen C, Alsharif U, Alvis-Guzman N,

Amini E, Anderson BO, Aremu O, Artaman A, Asgedom SW, Assadi R *et al.* Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 29 Cancer Groups, 1990 to 2016: A Systematic Analysis for the Global Burden of Disease Study. JAMA Oncol. 2018.

Goldgar DE, Healey S, Dowty JG, Da Silva L, Chen X, Spurdle AB, Terry MB, Daly MJ, Buys SM, Southey MC, Andrulis I, John EM; BCFR; kConFab, Khanna KK *et al.* Rare variants in the ATM gene and risk of breast cancer. Breast Cancer Res. 2011;13:R73.

Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, Senn HJ; Panel members. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol. 2013;24:2206-2223.

Gracia-Aznarez FJ, Fernandez V, Pita G, Peterlongo P, Dominguez O, de la Hoya M, Duran M, Osorio A, Moreno L, Gonzalez-Neira A, Rosa-Rosa JM, Sinilnikova O, Mazoyer S, Hopper J, Lazaro C *et al.* Whole exome sequencing suggests much of non-*BRCA1/BRCA2* familial breast cancer is due to moderate and low penetrance susceptibility alleles. PLoS One. 2013;8:e55681.

Harkness EF, Barrow E, Newton K, Green K, Clancy T, Lalloo F, Hill J, Evans DG. Lynch syndrome caused by *MLH1* mutations is associated with an increased risk of breast cancer: a cohort study. J Med Genet. 2015;52:553-556.

Harris CC. Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. J Natl Cancer Inst. 1996;88:1442-1455.

Hedenfalk IA, Ringnér M, Trent JM, Borg A. Gene expression in inherited breast cancer. Adv Cancer Res. 2002;84:1-34.

Hilbers FS, Wijnen JT, Hoogerbrugge N, Oosterwijk JC, Collee MJ, Peterlongo P, Radice P, Manoukian S, Feroce I, Capra F, Couch FJ, Wang X, Guidugli L, Offit K, Shah S *et al.* Rare variants in XRCC2 as breast cancer susceptibility alleles. J Med Genet. 2012;49:618-620.

Hollestelle A, Wasielewski M, Martens JW, Schutte M. Discovering moderate-risk breast cancer susceptibility genes. Curr Opin Genet Dev. 2010;20:268-276.

Iqbal J, Ragone A, Lubinski J, Lynch HT, Moller P, Ghadirian P, Foulkes WD, Armel S, Eisen A, Neuhausen SL, Senter L, Singer CF, Ainsworth P, Kim-Sing C, Tung N *et al.* The incidence of pancreatic cancer in BRCA1 and BRCA2 mutation carriers. Br J Cancer. 2012;107:2005-2009.

Jayson GC, Kohn EC, Kitchener HC, Ledermann JA. Ovarian cancer. Lancet. 2014;384:1376-1388.

Kaurah P, Huntsman DG. Hereditary Diffuse Gastric Cancer. GeneReviews. 2002 [updated 2018].

Kiiski JI, Pelttari LM, Khan S, Freysteinsdottir ES, Reynisdottir I, Hart SN, Shimelis H, Vilske S, Kallioniemi A, Schleutker J, Leminen A, Bützow R, Blomqvist C, Barkardottir RB, Couch FJ *et al.* Exome sequencing identifies FANCM as a susceptibility gene for triple-negative breast cancer. Proc Natl Acad Sci USA 2014;111:15172-15177.

Kote-Jarai Z, Leongamornlert D, Saunders E, Tymrakiewicz M, Castro E, Mahmud N, Guy M, Edwards S, O'Brien L, Sawyer E, Hall A, Wilkinson R, Dadaev T, Goh C, Easton D *et al.* BRCA2 is a moderate penetrance gene contributing to young-onset prostate cancer: implications for genetic testing in prostate cancer patients. Br J Cancer. 2011;105:1230-1234.

Kraus C, Hoyer J, Vasileiou G, Wunderle M, Lux MP, Fasching PA, Krumbiegel M, Uebe S, Reuter M, Beckmann MW, Reis A. Gene panel sequencing in familial breast/ ovarian cancer patients identifies multiple novel mutations also in genes others than *BRCA1/2*. Int J Cancer. 2017;140:95-102.

Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009;4:1073-1081.

Kurian AW, Antoniou AC, Domchek SM. Refining Breast Cancer Risk Stratification: Additional Genes, Additional Information. Am Soc Clin Oncol Educ Book. 2016;35:44-56.

Kurian AW, Hare EE, Mills MA, Kingham KE, McPherson L, Whittemore AS, McGuire V, Ladabaum U, Kobayashi Y, Lincoln SE, Cargill M, Ford JM. Clinical evaluation of a multiple-gene sequencing panel for hereditary cancer risk assessment. J Clin Oncol. 2014;32:2001-2009.

Kwong A, Chen JW, Shin VY. A new paradigm of genetic testing for hereditary breast/ovarian cancers. Hong Kong Med J. 2016;22:171-177.

Lakhani SR, Ellis IO, Schnitt SJ, Tan PH, van de Vijver MJ. WHO Classification of Tumours of the Breast. Fourth Edition. IARC press 2012.

Lauper JM, Krause A, Vaughan TL, Monnat RJ Jr. Spectrum and risk of neoplasia in Werner syndrome: a systematic review. PLoS One. 2013;8:e59709.

Lee JS, Collins KM, Brown AL, Lee CH, Chung JH. hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response. Nature. 2000;404:201-204.

Leongamornlert D, Mahmud N, Tymrakiewicz M, Saunders E, Dadaev T, Castro E, Goh C, Govindasami K, Guy M, O'Brien L, Sawyer E, Hall A, Wilkinson R,

Easton D; UKGPCS Collaborators *et al.* Germline BRCA1 mutations increase prostate cancer risk. Br J Cancer. 2012;106:1697-1701.

Li FP, Fraumeni JF Jr. Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome? Ann Intern Med. 1969;71:747-752.

Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25:1754-1760.

Lim W, Olschwang S, Keller JJ, Westerman AM, Menko FH, Boardman LA, Scott RJ, Trimbath J, Giardiello FM, Gruber SB, Gille JJ, Offerhaus GJ, de Rooij FW, Wilson JH, Spigelman AD *et al.* Relative frequency and morphology of cancers in STK11 mutation carriers. Gastroenterology. 2004;126:1788-1794.

Lin WY, Camp NJ, Cannon-Albright LA, Allen-Brady K, Balasubramanian S, Reed MW, Hopper JL, Apicella C, Giles GG, Southey MC, Milne RL, Arias-Pérez JI, Menéndez-Rodríguez P, Benítez J, Grundmann M *et al.* A role for *XRCC2* gene polymorphisms in breast cancer risk and survival. J Med Genet. 2011;48:477-484.

Loveday C, Turnbull C, Ramsay E, Hughes D, Ruark E, Frankum JR, Bowden G, Kalmyrzaev B, Warren-Perry M, Snape K, Adlard JW, Barwell J, Berg J, Brady AF, Brewer C *et al.* Germline mutations in *RAD51D* confer susceptibility to ovarian cancer. Nat Genet. 2011;43:879-882.

Loveday C, Turnbull C, Ruark E, Xicola RM, Ramsay E, Hughes D, Warren-Perry M, Snape K; Breast Cancer Susceptibility Collaboration (UK), Eccles D, Evans DG, Gore M, Renwick A, Seal S, Antoniou AC *et al.* Germline *RAD51C* mutations confer susceptibility to ovarian cancer. Nat Genet. 2012;44:475-476.

Madanikia SA, Bergner A, Ye X, Blakeley JO. Increased risk of breast cancer in women with *NF1*. Am J Med Genet A. 2012;158A:3056-3060.

Marabelli M, Cheng SC, Parmigiani G. Penetrance of ATM Gene Mutations in Breast Cancer: A Meta-Analysis of Different Measures of Risk. Genet Epidemiol. 2016;40:425-431.

Masciari S, Dillon DA, Rath M, Robson M, Weitzel JN, Balmana J, Gruber SB, Ford JM, Euhus D, Lebensohn A, Telli M, Pochebit SM, Lypas G, Garber JE. Breast cancer phenotype in women with TP53 germline mutations: a Li-Fraumeni syndrome consortium effort. Breast Cancer Res Treat. 2012;133:1125-1130.

Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science. 1998;282:1893-1897.

Mavaddat N, Peock S, Frost D, Ellis S, Platte R, Fineberg E, Evans DG, Izatt L, Eeles RA, Adlard J, Davidson R, Eccles D, Cole T, Cook J, Brewer C *et al.* Cancer

risks for BRCA1 and BRCA2 mutation carriers: results from prospective analysis of EMBRACE. J Natl Cancer Inst. 2013;105:812-822.

McGarrity TJ, Amos CI, Baker MJ. Peutz-Jeghers Syndrome. GeneReviews. 2001 [updated 2016].

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010;20:1297-1303.

Mehta PA, Tolar J. Fanconi Anemia. GeneReviews. 2002 [updated 2018].

Meijers-Heijboer H, van den Ouweland A, Klijn J, Wasielewski M, de Snoo A, Oldenburg R, Hollestelle A, Houben M, Crepin E, van Veghel-Plandsoen M, Elstrodt F, van Duijn C, Bartels C, Meijers C, Schutte M *et al.* Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. Nat Genet. 2002;31:55-59.

Meijers-Heijboer H, Wijnen J, Vasen H, Wasielewski M, Wagner A, Hollestelle A, Elstrodt F, van den Bos R, de Snoo A, Fat GT, Brekelmans C, Jagmohan S, Franken P, Verkuijlen P, van den Ouweland A *et al.* The CHEK2 1100delC mutation identifies families with a hereditary breast and colorectal cancer phenotype. Am J Hum Genet. 2003;72:1308-1314.

Mester J, Eng C. Cowden syndrome: recognizing and managing a not-so-rare hereditary cancer syndrome. J Surg Oncol. 2015;111:125-130

Meyer S, Tischkowitz M, Chandler K, Gillespie A, Birch JM, Evans DG. Fanconi anaemia, BRCA2 mutations and childhood cancer: a developmental perspective from clinical and epidemiological observations with implications for genetic counselling. J Med Genet. 2014;51:71-75.

Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, Bell R, Rosenthal J, Hussey C, Tran T, McClure M *et al.* A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science. 1994;266:66-71.

Moran A, O'Hara C, Khan S, Shack L, Woodward E, Maher ER, Lalloo F, Evans DG. Risk of cancer other than breast or ovarian in individuals with *BRCA1* and *BRCA2* mutations. Fam Cancer. 2012;11:235-242.

National Cancer Institute. Surveillance, Epidemiology, and End Results (SEER) database available at https://seer.cancer.gov/

Ngeow J, Sesock K, Eng C. Breast cancer risk and clinical implications for germline PTEN mutation carriers. Breast Cancer Res Treat. 2017;165:1-8.

Nielsen FC, van Overeem Hansen T, Sørensen CS. Hereditary breast and ovarian cancer: new genes in confined pathways. Nat Rev Cancer. 2016; 16: 599-612.

Park DJ, Lesueur F, Nguyen-Dumont T, Pertesi M, Odefrey F, Hammet F, Neuhausen SL, John EM, Andrulis IL, Terry MB, Daly M, Buys S, Le Calvez-Kelm F, Lonie A, Pope BJ *et al.* Rare mutations in XRCC2 increase the risk of breast cancer. Am J Hum Genet. 2012;90:734-739.

Pennington KP, Walsh T, Harrell MI, Lee MK, Pennil CC, Rendi MH, Thornton A, Norquist BM, Casadei S, Nord AS, Agnew KJ, Pritchard CC, Scroggins S, Garcia RL, King MC *et al.* Germline and somatic mutations in homologous recombination genes predict platinum response and survival in ovarian, fallopian tube, and peritoneal carcinomas. Clin Cancer Res. 2014;20:764-775.

Peterlongo P, Catucci I, Colombo M, Caleca L, Mucaki E, Bogliolo M, Marin M, Damiola F, Bernard L, Pensotti V, Volorio S, Dall'Olio V, Meindl A, Bartram C, Sutter C *et al.* *FANCM* c.5791C>T nonsense mutation (rs144567652) induces exon skipping, affects DNA repair activity and is a familial breast cancer risk factor. Hum Mol Genet. 2015;24:5345-5355.

Pezzolesi MG, Zbuk KM, Waite KA, Eng C. Comparative genomic and functional analyses reveal a novel cis-acting PTEN regulatory element as a highly conserved functional E-box motif deleted in Cowden syndrome. Hum Mol Genet. 2007;16:1058-1071.

Plichta JK, Griffin M, Thakuria J, Hughes KS. What's New in Genetic Testing for Cancer Susceptibility? Oncology. 2016;15:787-99.

Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FB, Hoogerbrugge N, Spurdle AB, Tavtigian SV; IARC Unclassified Genetic Variants Working Group. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. Hum Mutat. 2008;29:1282-1291.

Prat A, Adamo B, Cheang MC, Anders CK, Carey LA, Perou CM. Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. Oncologist. 2013;18:123-133.

Prat J, Ribé A, Gallardo A. Hereditary ovarian cancer. Hum Pathol. 2005;36:861-870.

Rafnar T, Gudbjartsson DF, Sulem P, Jonasdottir A, Sigurdsson A, Jonasdottir A, Besenbacher S, Lundin P, Stacey SN, Gudmundsson J, Magnusson OT, le Roux L, Orlygsdottir G, Helgadottir HT, Johannsdottir H *et al.* Mutations in BRIP1 confer high risk of ovarian cancer. Nat Genet. 2011;43:1104-1107.

Ramus SJ, Song H, Dicks E, Tyrer JP, Rosenthal AN, Intermaggio MP, Fraser L, Gentry-Maharaj A, Hayward J, Philpott S, Anderson C, Edlund CK, Conti D, Harrington P, Barrowdale D *et al.* Germline Mutations in the BRIP1, BARD1, PALB2, and NBN Genes in Women With Ovarian Cancer. J Natl Cancer Inst. 2015;107.

Ratajska M, Antoszewska E, Piskorz A, Brozek I, Borg Å, Kusmierek H, Biernat W, Limon J. Cancer predisposing BARD1 mutations in breast-ovarian cancer families. Breast Cancer Res Treat. 2012;131:89-97.

Renwick A, Thompson D, Seal S, Kelly P, Chagtai T, Ahmed M, North B, Jayatilake H, Barfoot R, Spanova K, McGuffog L, Evans DG, Eccles D; Breast Cancer Susceptibility Collaboration (UK), Easton DF *et al.* ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. Nat Genet. 2006;38:873-875.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17:405-424.

Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer. 2011;12:68-78.

Ruark E, Snape K, Humburg P, Loveday C, Bajrami I, Brough R, Rodrigues DN, Renwick A, Seal S, Ramsay E, Duarte Sdel V, Rivas MA, Warren-Perry M, Zachariou A, Campion-Flora A *et al.* Mosaic PPM1D mutations are associated with predisposition to breast and ovarian cancer. Nature. 2013;493:406-410.

Ruddy KJ, Winer EP. Male breast cancer: risk factors, biology, diagnosis, treatment, and survivorship. Ann Oncol. 2013;24:1434-1443.

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR *et al.* A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science. 1995;268:1749-1753.

Schneider K, Zelley K, Nichols KE, Garber J. Li-Fraumeni Syndrome. GeneReviews. 1999 [updated 2013].

Seal S, Thompson D, Renwick A, Elliott A, Kelly P, Barfoot R, Chagtai T, Jayatilake H, Ahmed M, Spanova K, North B, McGuffog L, Evans DG, Eccles D; Breast Cancer Susceptibility Collaboration (UK) *et al.* Truncating mutations in the Fanconi anemia J gene *BRIP1* are low-penetrance breast cancer susceptibility alleles. Nat Genet. 2006;38:1239-1241.

Seminog OO, Goldacre MJ. Age-specific risk of breast cancer in women with neurofibromatosis type 1. Br J Cancer. 2015;112:1546-1548.

Silvestri V, Rizzolo P, Zelli V, Valentini V, Zanna I, Bianchi S, Tibiletti MG, Varesco L, Russo A, Tommasi S, Coppa A, Capalbo C, Calistri D, Viel A, Cortesi L *et al.* A possible role of *FANCM* mutations in male breast cancer susceptibility: Results from a multicenter study in Italy. Breast. 2018;38:92-97.

Solyom S, Aressy B, Pylkäs K, Patterson-Fortin J, Hartikainen JM, Kallioniemi A, Kauppila S, Nikkilä J, Kosma VM, Mannermaa A, Greenberg RA, Winqvist R. Breast cancer-associated Abraxas mutation disrupts nuclear localization and DNA damage response functions. Sci Transl Med. 2012;4:122ra23.

Song H, Dicks E, Ramus SJ, Tyrer JP, Intermaggio MP, Hayward J, Edlund CK, Conti D, Harrington P, Fraser L, Philpott S, Anderson C, Rosenthal A, Gentry-Maharaj A, Bowtell DD *et al.* Contribution of Germline Mutations in the *RAD51B*, *RAD51C*, and *RAD51D* Genes to Ovarian Cancer in the Population. J Clin Oncol. 2015;33:2901-2907.

Stoffel EM, Koeppe E, Everett J, Ulintz P, Kiel M, Osborne J, Williams L, Hanson K, Gruber SB, Rozek LS. Germline genetic features of young individuals with colorectal cancer. Gastroenterology. 2018;154:897-905.

Szabo C, Masiello A, Ryan JF, Brody LC. The breast cancer information core: database design, structure, and scope. Hum Mutat. 2000;16:123-131.

Tai YC, Domchek S, Parmigiani G, Chen S. Breast cancer risk among male *BRCA1* and *BRCA2* mutation carriers. J Natl Cancer Inst. 2007;99:1811-1814.

Tan MH, Mester JL, Ngeow J, Rybicki LA, Orloff MS, Eng C. Lifetime cancer risks in individuals with germline *PTEN* mutations. Clin Cancer Res. 2012;18:400-407.

Tedaldi G, Danesi R, Zampiga V, Tebaldi M, Bedei L, Zoli W, Amadori D, Falcini F, Calistri D. First evidence of a large *CHEK2* duplication involved in cancer predisposition in an Italian family with hereditary breast cancer. BMC Cancer. 2014;14:478.

Templeton AJ, Gonzalez LD, Vera-Badillo FE, Tibau A, Goldstein R, Šeruga B, Srikanthan A, Pandiella A, Amir E, Ocana A. Interaction between Hormonal Receptor Status, Age and Survival in Patients with *BRCA1/2* Germline Mutations: A Systematic Review and Meta-Regression. PLoS One. 2016;11:e0154789.

ten Broeke SW, Brohet RM, Tops CM, van der Klift HM, Velthuizen ME, Bernstein I, Capellá Munar G, Gomez Garcia E, Hoogerbrugge N, Letteboer TG, Menko FH, Lindblom A, Mensenkamp AR, Moller P, van Os TA *et al.* Lynch syndrome

caused by germline *PMS2* mutations: delineating the cancer risk. J Clin Oncol. 2015;33:319-325.

Teodorczyk U, Cybulski C, Wokołorczyk D, Jakubowska A, Starzyńska T, Lawniczak M, Domagała P, Ferenc K, Marlicz K, Banaszkiewicz Z, Wiśniowski R, Narod SA, Lubiński J. The risk of gastric cancer in carriers of *CHEK2* mutations. Fam Cancer. 2013;12:473-478.

Thompson D, Duedal S, Kirner J, McGuffog L, Last J, Reiman A, Byrd P, Taylor M, Easton DF. Cancer risks and mortality in heterozygous *ATM* mutation carriers. J Natl Cancer Inst. 2005;97:813-822.

Thompson D, Easton DF; Breast Cancer Linkage Consortium. Cancer Incidence in *BRCA1* mutation carriers. J Natl Cancer Inst. 2002;94:1358-1365.

Thusberg J, Vihinen M. Pathogenic or not? And if so, then how? Studying the effects of missense mutations using bioinformatics methods. Hum Mutat. 2009;30:703-714.

Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65:87-108.

Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT *et al.* Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 2004;96:261-268.

Vahteristo P, Tamminen A, Karvinen P, Eerola H, Eklund C, Aaltonen LA, Blomqvist C, Aittomäki K, Nevanlinna H. p53, CHK2, and CHK1 genes in Finnish families with Li-Fraumeni syndrome: further evidence of CHK2 in inherited cancer predisposition. Cancer Res. 2001;61:5718-22.

van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L *et al.* A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med. 2002;347:1999-2009.

van der Post RS, Vogelaar IP, Carneiro F, Guilford P, Huntsman D, Hoogerbrugge N, Caldas C, Schreiber KE, Hardwick RH, Ausems MG, Bardram L, Benusiglio PR, Bisseling TM, Blair V, Bleiker E *et al.* Hereditary diffuse gastric cancer: updated clinical guidelines with an emphasis on germline *CDH1* mutation carriers. J Med Genet. 2015;52:361-374.

van Os NJ, Roeleveld N, Weemaes CM, Jongmans MC, Janssens GO, Taylor AM, Hoogerbrugge N, Willemsen MA. Health risks for ataxia-telangiectasia mutated heterozygotes: a systematic review, meta-analysis and evidence-based guideline. Clin Genet. 2016;90:105-117.

van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. Cell Mol Life Sci. 2008;65:3756-88.

Vijai J, Topka S, Villano D, Ravichandran V, Maxwell KN, Maria A, Thomas T, Gaddam P, Lincoln A, Kazzaz S, Wenz B, Carmi S, Schrader KA, Hart SN, Lipkin SM *et al.* A Recurrent *ERCC3* Truncating Mutation Confers Moderate Risk for Breast Cancer. Cancer Discov. 2016;6:1267-1275.

Walsh MD, Buchanan DD, Cummings MC, Pearson SA, Arnold ST, Clendenning M, Walters R, McKeone DM, Spurdle AB, Hopper JL, Jenkins MA, Phillips KD, Suthers GK, George J, Goldblatt J *et al.* Lynch syndrome-associated breast cancers: clinicopathologic characteristics of a case series from the colon cancer family registry. Clin Cancer Res. 2010;16:2214-2224.

Walsh T, Casadei S, Coats KH, Swisher E, Stray SM, Higgins J, Roach KC, Mandell J, Lee MK, Ciernikova S, Foretova L, Soucek P, King MC. Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. JAMA. 2006;295:1379-1388.

Walsh T, Lee MK, Casadei S, Thornton AM, Stray SM, Pennil C, Nord AS, Mandell JB, Swisher EM, King MC. Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. Proc Natl Acad Sci USA. 2010; 107: 12629-12633.

Wang B, Matsuoka S, Ballif BA, Zhang D, Smogorzewska A, Gygi SP, Elledge SJ. Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. Science. 2007;316:1194-1198.

Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010;38:e164.

Wang W, Wu J, Zhang P, Fei X, Zong Y, Chen X, Huang O, He JR, Chen W, Li Y, Shen K, Zhu L. Prognostic and predictive value of Ki-67 in triple-negative breast cancer. Oncotarget. 2016;7:31079-31087.

Wang Y, Cortez D, Yazdi P, Neff N, Elledge SJ, Qin J. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. Genes Dev. 2000;14:927-939.

Weaver JM, Edwards PA. Targeted next-generation sequencing for routine clinical screening of mutations. Genome Med. 2011;3:58.

Weber-Lassalle N, Hauke J, Ramser J, Richters L, Groß E, Blümcke B, Gehrig A, Kahlert AK, Müller CR, Hackmann K, Honisch E, Weber-Lassalle K, Niederacher D, Borde J, Thiele H *et al.* *BRIP1* loss-of-function mutations confer

high risk for familial ovarian cancer, but not familial breast cancer. Breast Cancer Res. 2018;20:7.

Weischer M, Bojesen SE, Ellervik C, Tybjaerg-Hansen A, Nordestgaard BG. *CHEK2**1100delC genotyping for clinical assessment of breast cancer risk: meta-analyses of 26,000 patient cases and 27,000 controls. J Clin Oncol. 2008;26:542-548.

Win AK, Lindor NM, Jenkins MA. Risk of breast cancer in Lynch syndrome: a systematic review. Breast Cancer Res. 2013;15:R27.

Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G, Barfoot R, Hamoudi R, Patel S, Rices C, Biggs P *et al.* Identification of the breast cancer susceptibility gene BRCA2. Nature. 1995;378: 789-792.

Xia B, Sheng Q, Nakanishi K, Ohashi A, Wu J, Christ N, Liu X, Jasin M, Couch FJ, Livingston DM. Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. Mol Cell. 2006;22:719-729.

Xu X, Jin D, Durgan J, Hall A. LKB1 controls human bronchial epithelial morphogenesis through p114RhoGEF-dependent RhoA activation. Mol Cell Biol. 2013;33:2671-2682.

Yang S, Kuo C, Bisi JE, Kim MK. PML-dependent apoptosis after DNA damage is regulated by the checkpoint kinase hCds1/Chk2. Nat Cell Biol. 2002;4:865-870.

Zaki-Dizaji M, Akrami SM, Abolhassani H, Rezaei N, Aghamohammadi A. Ataxia telangiectasia syndrome: moonlighting ATM. Expert Rev Clin Immunol. 2017;13:1155-1172.

List of original manuscripts

Gianluca Tedaldi, Michela Tebaldi, Valentina Zampiga, Rita Danesi, Valentina Arcangeli, Mila Ravegnani, Ilaria Cangini, Francesca Pirini, Elisabetta Petracci, Andrea Rocca, Fabio Falcini, Dino Amadori, Daniele Calistri. Multiple-gene panel analysis in a case series of 255 women with hereditary breast and ovarian cancer. Oncotarget 2017.
Pdf of the paper has been included

SM Nielsen, DM Eccles, I Romero, F Al-Mulla, J Balmaña, M Biancolella, R Blok, MA Caligo, M Calvello, GL Capone, P Cavalli, TL Chan, KBM Claes, L Cortesi, FJ Couch, M de la Hoya, S De Toffol, O Diez, S M Domchek, R Eeles, A Efremidis, F Fostira, D Goldgar, M Hadjisavvas, TvOHansen, A Hirasawa, C Houdayer, P Kleiblova, S Krieger, C Lázaro, M Loizidou, S Manoukian, A R Mensenkamp, S Moghadasi, A N Monteiro, L Mori, A Morrow, N Naldi, HR Nielsen, OI Olopade, NS Pachter, EI Palmero, IS Pedersen, M Piane, M Puzzo, M Robson, M Rossing, MC Sini, A Solano, J Soukupova, Gianluca Tedaldi, M Teixeira, M Thomassen, MG Tibiletti, A Toland, T Törngren, E Vaccari, L Varesco, A Vega, Y Wallis, B Wappenschmidt, J Weitzel, A B Spurdle, A De Nicolo, EB Gómez-García. Genetic Testing and Clinical Management Practices for Variants in Non-BRCA1/2 Breast (and Breast and Ovarian) Cancer Susceptibility Genes: an International Survey by the ENIGMA Clinical Working Group.
Accepted in July 2018 by JCO Precision Oncology (pdf not available)

Part three

Additional contributions

During my PhD program I had the opportunity to collaborate to various projects dealing with NGS-based approaches to detect germline and somatic mutations in different types of cancer.

1. Study of predisposing mutations in patients with multiple primary tumors

The best characterized genetic syndrome which confers a high risk of developing several types of cancer, is the Li-Fraumeni syndrome (LFS) [Li FP and Fraumeni JF Jr. 1969; Li FP *et al.* 1988], a rare disorder predisposing to breast cancer at premenopausal age, sarcoma (especially soft tissue and bone sarcomas), brain tumors and adrenal cortical carcinomas [Nichols KE *et al.* 2001]. Germline mutations in *TP53* tumor suppressor gene (OMIM *191170) are known to be associated with the syndrome and 80% of LFS patients have been found to be mutation carriers [Malkin D 2011]. In order to explain the missing heritability, other genes have been investigated and proposed as possible candidates.
With the above reported cancer gene panel (part one-Material and Methods), we performed an NGS-based analysis on two patients, both diagnosed with multiple primary tumors. The first was a 44-year-old woman who developed, in order, a chondrosarcoma (15 years), a bilateral breast cancer (35 years), a bladder cancer (38 years) and an astrocytoma (42 years). The pedigree of the patient is reported in Figure 1. In this patient NGS analysis revealed a *TP53* missense mutation (c.524G>A p.Arg175His in exon 5) in a mutation hotspot frequently associated with cancer. In the literature, this mutation is reported to be responsible for aggressive tumors in LFS families and to be frequently associated with breast, brain, and soft tissue cancers [Pötzsch C *et al.* 2002].

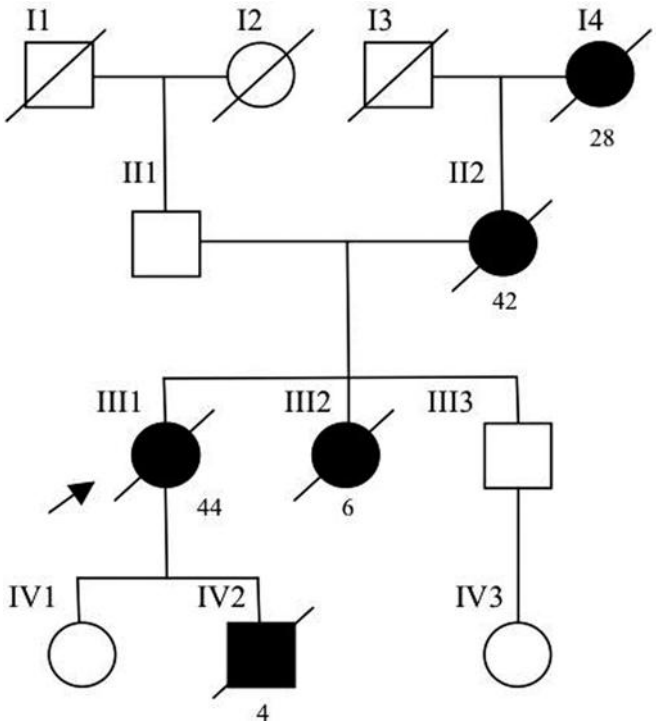


Fig. 1. Pedigree of the family with disease-associated *TP53* mutation. Circles represent females and squares represent males. Solid symbols represent cancer patients. Symbols with a slash indicate deceased individuals. The arrow points to the proband. I-4 breast cancer (28 years); II-2 bilateral breast cancer (42 years); III-1 chondrosarcoma (15 years), bilateral breast cancer (35 years), bladder cancer (37 years), astrocytoma (42 years); III-2 brain tumor (5 years); IV-1 Down syndrome; IV-2 rhabdomyosarcoma (4 years).

The second patient was a 47-year-old woman who developed, in order, a Hodgkin's lymphoma (20 years), a retroperitoneal lymphangioma (38 years), a bilateral breast carcinoma in situ (46 years) and a malignant perivascular epithelioid cell tumor (47 years). The pedigree of the patient is reported in Figure 2. In this case, NGS analysis revealed two novel deleterious mutations in two different genes, *PMS1* and *CDKN2A*. The frameshift mutation in the *PMS1* gene, c.1139dupA p.Y380_S381delinsX, causes an early termination of the PMS1 protein synthesis at codon 380. Only three germline mutations have been reported in *PMS1* gene [Wang Q *et al.* 1999; Liu T *et al.* 2001] which, although classified as a mismatch repair gene, has been associated with Lynch syndrome in very rare cases. In *CDKN2A*, NGS analysis detected the c.58delG p.V20X variant; this

nucleotide deletion in exon 1β causes an early termination of the p14(ARF) protein synthesis at codon 20. *CDKN2A* gene encodes two different tumor suppressor proteins by alternative splicing, p16(INK4), a cyclin-dependent kinase inhibitor, and p14(ARF), an inhibitor of MDM2 that stabilizes p53 protein [Robertson KD and Jones PA 1999]. Only few mutations of *CDKN2A* affecting p14(ARF) but not p16(INK4), have been reported so far in the literature [Randerson-Moor JA *et al.* 2001; Binni F *et al.* 2010].

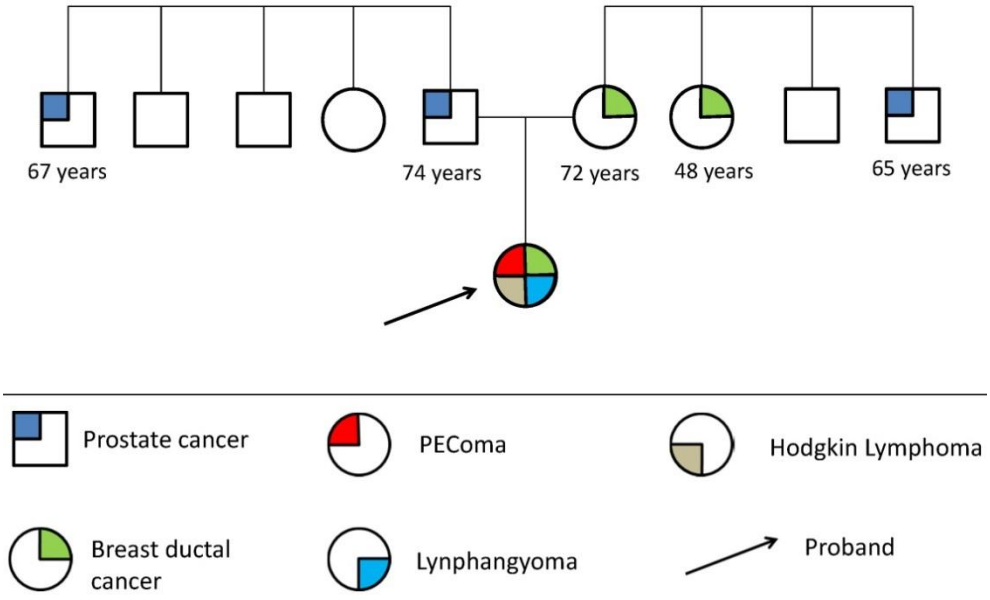


Fig. 2. Pedigree of the patient with *PMS1* and *CDKN2A* mutations

2. Preclinical evidence of multiple mechanisms underlying trastuzumab resistance in gastric cance

See: Arienti C *et al.* Oncotarget. 2016; 7:18424-18439

3. Morphological and genetic heterogeneity in multifocal lung adenocarcinoma

See: Bonanno L *et al.* Lung Cancer 2016; 96:52-55

4. Cell-free DNA as a diagnostic marker for cancer: current insights

See: Salvi S *et al.* OncoTargets and Therapy 2016; 9:6549-6559 (Review)

References

Binni F, Antigoni I, De Simone P, Majore S, Silipo V, Crisi A, Amantea A, Pacchiarini D, Castori M, De Bernardo C, Catricalà C, Grammatico P. Novel and recurrent p14 mutations in Italian familial melanoma. Clin Genet. 2010;77:581-586.

Li FP, Fraumeni JF Jr. Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome? Ann Intern Med. 1969;71:747-752.

Li FP, Fraumeni JF Jr, Mulvihill JJ, Blattner WA, Dreyfus MG, Tucker MA, Miller RW. A cancer family syndrome in twenty-four kindreds. Cancer Res. 1988;48:5358-5362.

Liu T, Yan H, Kuismanen S, Percesepe A, Bisgaard ML, Pedroni M, Benatti P, Kinzler KW, Vogelstein B, Ponz de Leon M, Peltomäki P, Lindblom A. The role of hPMS1 and hPMS2 in predisposing to colorectal cancer. Cancer Res. 2001;61:7798-7802.

Malkin D. Li-fraumeni syndrome. Genes Cancer. 2011;2:475-484.

Nichols KE, Malkin D, Garber JE, Fraumeni JF Jr, Li FP. Germ-line p53 mutations predispose to a wide spectrum of early-onset cancers. Cancer Epidemiol Biomarkers Prev. 2001;10:83-87.

Pötzsch C, Voigtländer T, Lübbert M. p53 Germline mutation in a patient with Li-Fraumeni Syndrome and three metachronous malignancies. J Cancer Res Clin Oncol. 2002;128:456-460.

Randerson-Moor JA, Harland M, Williams S, Cuthbert-Heavens D, Sheridan E, Aveyard J, Sibley K, Whitaker L, Knowles M, Bishop JN, Bishop DT. A germline deletion of p14(ARF) but not CDKN2A in a melanoma-neural system tumour syndrome family. Hum Mol Genet. 2001;10:55-62.

Robertson KD, Jones PA. Tissue-specific alternative splicing in the human INK4a/ARF cell cycle regulatory locus. Oncogene. 1999;18:3810-3820.

Wang Q, Lasset C, Desseigne F, Saurin JC, Maugard C, Navarro C, Ruano E, Descos L, Trillet- Lenoir V, Bosset JF, Puisieux A. Prevalence of germline mutations of hMLH1, hMSH2, hPMS1, hPMS2, and hMSH6 genes in 75 French kindreds with nonpolyposis colorectal cancer. Hum Genet. 1999;105:79-85.

List of original manuscripts

Valentina Zampiga, Rita Danesi, Gianluca Tedaldi, Michela Tebaldi, Ilaria Cangini, Francesca Pirini, Cristina Pittureri, Elena Amaducci, Luciano Guidi, Marina Faedi, Dino Amadori, Fabio Falcini, Daniele Calistri. Multiple primary tumours in a family with Li-Fraumeni syndrome with a *TP53* germline mutation identified by Next-Generation Sequencing. Int J Biol Markers. 2016;31:e461-e465.
Pdf of the paper has been included

Marco Cassone, Leila Baghernajad Salehi, Gianluca Tedaldi, Michela Tebaldi, Valentina Zampiga, Michela Biancolella, Barbara Testa, Daniele Calistri, Gerarda Mastrogiorgio, Silvia Lanciotti, Maria Rosaria D'Apice, Giuseppe Novelli, Federica Sangiuolo. Coexistence of Two Novel Mutations in *CDKN2A* and *PMS1* Genes in a Single Patient Identifies a New and Severe Cancer Predisposition Syndrome. Oncomedicine. 2017;2:88-92.
Pdf of the paper has been included

Chiara Arienti, Michele Zanoni, Sara Pignatta, Alberto Del Rio, Silvia Carloni, Michela Tebaldi, Gianluca Tedaldi, Anna Tesei. Preclinical evidence of multiple mechanisms underlying trastuzumab resistance in gastric cancer. Oncotarget. 2016;7:18424-18439.
Pdf of the paper has been included

Laura Bonanno, Fiorella Calabrese, Giorgia Nardo, Daniele Calistri, Michela Tebaldi, Gianluca Tedaldi, Valentina Polo, Stefania Vuljan, Adolfo Favaretto, Pierfranco Conte, Alberto Amadori, Federico Rea, Stefano Indraccolo. Morphological and genetic heterogeneity in multifocal lung adenocarcinoma: The case of a never-smoker woman. Lung Cancer. 2016;96:52-55.
Pdf of the paper has been included

Samanta Salvi, Giorgia Gurioli, Ugo De Giorgi, Vincenza Conteduca, Gianluca Tedaldi, Daniele Calistri, Valentina Casadio. Cell-free DNA as a diagnostic marker for cancer: current insights. Onco Targets Ther. 2016;9:6549-6559.
Pdf of the paper has been included

Acknowledgements

First of all I would like to thank Prof. G. Nadia Ranzani, who gave me the opportunity to pursue my PhD under her supervision. She taught me to think as a researcher and I will always be grateful to her for this. A big thank you also to her PhD students, Monica, Raefa and Alessia, who helped me with the courses, the exams and the thesis.

An equal weight of gratitude goes to Dr. Daniele Calistri, who always supported me since I was a bachelor student. He introduced me to the world of the hereditary tumors and I hope we will work together for a long time.

Another big thank you is for my colleagues of the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori of Meldola, in particular to Michela, Sara, Samanta, and Livia, to the Diagnostics group and to our medical geneticists.

A heartfelt thank you goes to Prof. Carla Oliveira of the Instituto de Investigação e Inovação em Saúde (i3s) of Porto, who welcomed me in her laboratory, and to the members of the ERiC group, in particular Patricia, Joana, Anabela, Sara T., Sara R., Barbara, Carla P., Rita, Ana and Marlene. My stay in Portugal has been one of the best experiences of my life.

Finally I would like to thank my family and my wife Gloria, who have always supported me, especially in the difficult moments. An affectionate thought goes to Loretta, who unfortunately left us last year, with her example she taught me to never give up.

www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 29), pp: 47064-47075

Research Paper

Multiple-gene panel analysis in a case series of 255 women with hereditary breast and ovarian cancer

Gianluca Tedaldi[1], Michela Tebaldi[1], Valentina Zampiga[1], Rita Danesi[2], Valentina Arcangeli[3], Mila Ravegnani[2], Ilaria Cangini[1], Francesca Pirini[1], Elisabetta Petracci[4], Andrea Rocca[5], Fabio Falcini[2], Dino Amadori[5] and Daniele Calistri[1]

[1]Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

[2]Romagna Cancer Registry, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

[3]Department of Medical Oncology, Ospedale Infermi, Rimini, Italy

[4]Unit of Biostatistics and Clinical Trials, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

[5]Department of Medical Oncology, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

Correspondence to: *Daniele Calistri,* ***email:*** *daniele.calistri@irst.emr.it*

Keywords: *hereditary breast and ovarian cancer, multiple-gene panel, next-generation sequencing, bilateral breast cancer, cancer predisposition*

Received: *October 19, 2016* ***Accepted:*** *March 14, 2017* ***Published:*** *April 03, 2017*

ABSTRACT

As new genes predisposing to breast (BC) and ovarian cancer (OC) are constantly emerging, the use of panels of genes analyzed by Next-Generation Sequencing (NGS) is increasing in clinical diagnostics. The identification of a large number of new germline mutations allows for deeper knowledge of cancer predisposition, although raising many questions about patient management.

BC and OC patients recruited by our counseling service between 2012-2015 were included in this study. DNA was extracted from peripheral blood and a panel of 94 genes involved in hereditary tumors was analyzed by NGS. Patient clinical features of BC and OC and cancer family history were collected and compared to the patient genetic profile.

A total of 255 women were analyzed, 57 of whom had a pathogenic mutation in *BRCA1/2* genes, and 17 carried pathogenic mutations in other genes, such as *PALB2*, *ATM*, *BRIP1*, *RAD51D*, *MSH6*, *PPM1D*, *RECQL4*, *ERCC3*, *TSC2*, *SLX4* and other Fanconi anemia genes.

Patients with a pathogenic mutation in genes other than *BRCA1* and *BRCA2* showed no significant difference from the *BRCA1/2*-mutated carriers with respect to age at diagnosis and clinical features, suggesting that mutations in other genes could pose a high risk of cancer development.

These patients had a much higher percentage of bilateral breast cancer (BBC) and a lower rate of OC than *BRCA*-mutated patients and patients with no pathogenic mutations: as a consequence, the surveillance protocol should be customized to the patient genetic characteristics.

INTRODUCTION

Breast cancer (BC) is the second most common cancer worldwide, and the most frequent cancer in women overall with about 1.7 million new cases diagnosed in 2012. BC is the second cause of cancer-related death in women in economically developed countries and the fifth worldwide [1].

Ovarian cancer (OC), is the fourth commonest cause of female cancer death in the developed world, also called "the silent killer" given the high mortality rate often due to late diagnosis [2].

About 10-30% of BCs and OCs shows a familial aggregation, but it is estimated that only 5-10% is hereditary, namely due to a genetic mutation which is transmitted to offspring [3, 4].

The main genes involved in hereditary breast and ovarian cancer (HBOC) are *BRCA1* [5], with 65% and 39% risk of developing BC and OC by the age of 70, respectively, and *BRCA2* [6], with 45% and 11% risk of developing BC and OC, respectively [7].

To date, many other genes have been associated to BC risk, such as *PALB2*, *TP53*, *ATM*, *BRIP1*, *CHEK2*, *CDH1*, *PTEN*, *STK11* [8, 9].

In the last few years the advent of Next-Generation Sequencing (NGS) has enabled the analysis of a greater number of genes with the advantage of lower costs and a wider access to molecular tests for patients with suspected genetic syndromes [10–13].

The discovery of new genes determining susceptibility to disease is crucial in oncology, as genetic transmission is more difficult to identify due to the frequent incomplete penetrance and the influence of the environment on genetics [14].

RESULTS

We performed an NGS analysis of a panel of 94 genes involved in the main hereditary cancer syndromes (Supplementary Table 1) in a case series of 255 women.

The patient cohort included 227 (89.0%) patients with initial BC (median age 41 years) and 28 (11.0%) with initial OC (median age 49.5 years). BC and OC patient tumor characteristics are summarized in Tables 1 and 2, respectively.

Of the 227 BC patients, 52 (22.9%) had bilateral breast cancer (BBC), 8 (3.5%) had subsequent OC and 22 (9.7%) had other malignancies (reported as "Second BC", "Second OC" and "Other tumors", respectively, in Table 1).

Of the 28 OC patients, 7 (25.0%) had subsequent BC (reported as "Second BC" in Table 2). None (0.0%) presented other malignancies (reported as "Other tumors" in Table 2).

The molecular analysis of the 255 patients showed a mean target coverage of 399,7X and a 95.5% mean percentage of target covered >50X.

We focused at first on the *BRCA* mutation status of patients.

According to the databases and guidelines (see Materials and Methods), 57 (22.4%) patients had a pathogenic/likely-pathogenic mutation in *BRCA* genes, in particular 31 (12.2%) had a *BRCA1* mutation, 25 (9.8%) had a *BRCA2* mutation and 1 (0.4%) had pathogenic mutations in both *BRCA1* and *BRCA2* (Supplementary Table 2).

We then observed the mutations in the remaining 92 genes of the panel.

The analysis revealed 23 pathogenic/likely-pathogenic mutations in 14 genes in 21/255 (8.2%) patients (Supplementary Table 3). Out of these 21 patients, 4 were also *BRCA*-positive and 17 *BRCA*-negative.

We finally analyzed the 181 (71.0%) patients with pathogenic mutations in neither *BRCA1/2* nor other genes, showing 23,882 exonic and splicing (± 5bp) variants.

The frequencies present in 1000Genomes, Esp6500 and Exac03 databases were used to exclude polymorphic variants.

Among the remaining 1,026 variants with frequency <1% or n/a, we worked on the missense variants with PolyPhen-2 HVar and SIFT to assess their possible role in cancer development.

BRCA mutations and patient characteristics

We identified 32 pathogenic/likely-pathogenic mutations in *BRCA1* gene and 26 in *BRCA2* gene (Supplementary Table 2).

Most of the 31 patients with a *BRCA1* pathogenic/likely-pathogenic mutation had BC: 23 (74.2%) had initial BC, 6 (26.1%) of whom BBC, and 1 (4.3%) subsequent OC. The remaining 8 (25.8%) had initial OC, 2 (25.0%) of whom had subsequent BC.

Also most of the 25 patients with a *BRCA2* pathogenic/likely-pathogenic mutation had BC: 24 (96.0%) had initial BC, 6 (25.0%) of whom BBC, and 1 (4.2%) had subsequent OC. Only 1 (4.0%) patient had initial OC.

The only patient with pathogenic mutations in both *BRCA1* and *BRCA2* had BC.

The clinical features of the 31 *BRCA1*-mutated patients were compared with those of the 25 *BRCA2*-mutated patients with no statistically significant differences, except for the grading of BC with a higher number of poorly differentiated tumors in *BRCA1*-mutated patients (Supplementary Tables 4 and 5). The two groups were thus treated as one group including the single patient with both *BRCA1* and *BRCA2* genes mutated (Tables 1 and 2).

Median age at the onset of BC was 39 years for initial BC and 46 for subsequent BC. Median age at the onset of OC was 50 years.

The number of triple-negative BCs was significantly higher in *BRCA*-positive patients (34.3%) than in *BRCA*-negative patients (11.0%).

The BC/OC family history in I- and II-degree relatives was significantly higher in *BRCA*-mutated patients and *BRCA*-wild type patients with BC than in patients with mutations in extra-*BRCA* genes (P=0.039, Table 1).

Table 1: Clinical features and personal/family cancer history of BC patients

BREAST CANCER (BC)	All patients	Patients with *BRCA1/2* mutations	Patients with extra-*BRCA* mutations	Patients with no pathogenic mutations	*P*
	N (%)	*N (%)*	*N (%)*	*N (%)*	
N. of patients	227	48	17	162	
Age at diagnosis, years					
Median Age [Min-Max]	41 [25–79]	39 [25–70]	43 [26–74]	42 [25–79]	0.140
Missing	0	0	0	0	
Histotype					
In situ carcinoma	22 (10.14)	3 (6.52)	3 (17.65)	16 (10.39)	0.810
Invasive ductal carcinoma	148 (68.20)	33 (71.74)	10 (58.82)	105 (68.18)	
Invasive lobular carcinoma	26 (11.98)	6 (13.04)	3 (17.65)	17 (11.04)	
Other invasive histotypes	21 (9.68)	4 (8.70)	1 (5.88)	16 (10.39)	
Missing	10	2	0	8	
Grading					
Well-differentiated	18 (9.68)	0 (0.00)	1 (6.67)	17 (12.98)	0.005
Moderately differentiated	85 (45.70)	13 (32.50)	7 (46.67)	65 (49.62)	
Poorly differentiated	83 (44.62)	27 (67.50)	7 (46.67)	49 (37.40)	
Missing	41	8	2	31	
Stage					
0	22 (12.50)	3 (8.82)	3 (23.08)	16 (12.40)	0.375
I	92 (52.27)	15 (44.12)	5 (38.46)	72 (55.81)	
II	45 (25.57)	13 (38.24)	3 (23.08)	29 (22.48)	
III-IV	17 (9.66)	3 (8.82)	2 (15.38)	12 (9.30)	
Missing	51	14	4	33	
Tumor invasiveness					
In situ	22 (10.09)	3 (6.38)	3 (17.65)	16 (10.39)	0.420
Invasive	196 (89.91)	44 (93.62)	14 (82.35)	138 (89.61)	
Missing	9	1	0	8	
Ki-67					
High (≥14)	115 (70.55)	37 (90.24)	6 (75.00)	72 (63.16)	0.003
Low (<14)	48 (29.45)	4 (9.76)	2 (25.00)	42 (36.84)	
Missing	64	7	9	48	
St Gallen subtype					
Luminal A	29 (20.14)	1 (2.86)	2 (28.57)	26 (25.49)	0.005
Luminal B1	56 (38.89)	13 (37.14)	2 (28.57)	41 (40.20)	
Luminal B2	26 (18.06)	8 (22.86)	1 (14.29)	17 (16.67)	
Her2 positive	9 (6.25)	1 (2.86)	1 (14.29)	7 (6.86)	
Triple negative	24 (16.67)	12 (34.29)	1 (14.29)	11 (10.78)	
Missing	83	13	10	60	

(*Continued*)

BREAST CANCER (BC)	All patients	Patients with *BRCA1/2* mutations	Patients with extra-*BRCA* mutations	Patients with no pathogenic mutations	*P*
	N (%)	*N (%)*	*N (%)*	*N (%)*	
Second BC					
No	175 (77.09)	36 (75.00)	9 (52.94)	130 (80.25)	0.036
Yes	52 (22.91)	12 (25.00)	8 (47.06)	32 (19.75)	
Median Age [Min-Max] [a]	55 [32–82]	46 [37–70]	57 [36–77]	58.5 [32–82]	0.041
Second OC					
No	219 (96.48)	46 (95.83)	15 (88.24)	158 (97.53)	0.103
Yes	8 (3.52)	2 (4.17)	2 (11.76)	4 (2.47)	
Median Age [Min-Max][a]	66.5 [51–77]	69 [68–70]	52.5 [51–54]	68.5 [55–77]	0.135
Other tumors					
No	205 (90.31)	45 (93.75)	17 (100.00)	143 (88.27)	0.254
Yes	22 (9.69)	3 (6.25)	0 (0.00)	19 (11.73)	
BC/OC in I-degree relatives					
No	81 (35.68)	17 (35.42)	10 (58.82)	54 (33.33)	0.113
Yes	146 (64.32)	31 (64.58)	7 (41.18)	108 (66.67)	
BC/OC in I/II-degree relatives					
No	43 (18.94)	10 (20.83)	7 (41.18)	26 (16.05)	0.039
Yes	184 (81.06)	38 (79.17)	10 (58.82)	136 (83.95)	
Other cancers in I-degree relatives					
No	144 (63.44)	33 (68.75)	11 (64.71)	100 (61.73)	0.670
Yes	83 (36.56)	15 (31.25)	6 (35.29)	62 (38.27)	
Other cancers in I/II-degree relatives					
No	81 (35.68)	22 (45.83)	7 (41.18)	52 (32.10)	0.193
Yes	146 (64.32)	26 (54.17)	10 (58.82)	110 (67.90)	

[a] Median age, in years, refers to age at second cancer diagnosis

Also *BRCA*-mutated patients with OC had a higher BC/OC family history in I-degree relatives than *BRCA*-wild type patients (P=0.039, Table 2).

Extra-*BRCA* mutations and patient characteristics

Among the 23 pathogenic/likely-pathogenic mutations identified (Supplementary Table 3), 1 deletion in *ERCC3* gene was found in 1 patient with a pathogenic mutation in *BRCA1*, and 3 mutations (1 deletion in *FANCA* gene, 1 deletion in *BRIP1* gene and 1 nonsense mutation in *ATM*) were found in 3 patients with a pathogenic mutation in *BRCA2*.

The remaining 19 pathogenic/likely-pathogenic mutations found in 17 *BRCA1/2* wild-type patients included 6 mutations in *PALB2* (3 deletions and 3 nonsense mutations), 2 in *ATM* (1 deletion and 1 insertion), 2 in *FANCL* (1 insertion and 1 nonsense mutation), 1 deletion in *BRIP1*, 1 nonsense mutation in *FANCM*, 1 deletion in *FANCI*, 1 deletion in *SLX4*, 1 nonsense mutation in *MSH6*, 1 nonsense mutation in *RAD51D*, 1 deletion in *PPM1D*, 1 deletion in *RECQL4*, and 1 deletion in *TSC2*.

Table 2: Clinical features and personal/family cancer history of OC patients

OVARIAN CANCER (OC)	All patients	Patients with *BRCA1/2* mutations	Patients with extra-*BRCA* mutations	Patients with no pathogenic mutations	*P*
	N (%)	*N (%)*	*N (%)*	*N (%)*	
N. of patients	28	9	0	19	
Age at diagnosis, years					
Median Age [Min-Max]	49.5 [28–81]	50 [38–68]	-	47 [28–81]	0.640
Missing	0	0	-	0	
Histotype					
Serous carcinoma	18 (64.29)	7 (77.78)	-	11 (57.89)	0.700
Other malignant histotypes	7 (25.00)	2 (22.22)	-	5 (26.32)	
Borderline tumors	3 (10.71)	0 (0.00)	-	3 (15.79)	
Missing	0	0	-	0	
Grading					
Well-differentiated	2 (8.00)	0 (0.00)	-	2 (12.50)	0.772
Moderately differentiated	2 (8.00)	1 (11.11)	-	1 (6.25)	
Poorly differentiated	21 (84.00)	8 (88.89)	-	13 (81.25)	
Missing	3	0	-	3	
Stage					
0	0 (0.00)	0 (0.00)	-	0 (0.00)	0.343
I	7 (31.82)	1 (12.50)	-	6 (42.86)	
II	2 (9.09)	1 (12.50)	-	1 (7.14)	
III-IV	13 (59.09)	6 (75.00)	-	7 (50.00)	
Missing	6	1	-	5	
Tumor invasiveness					
Borderline	3 (10.71)	0 (0.00)	-	3 (15.79)	0.530
Invasive	25 (89.29)	9 (100.00)	-	16 (84.21)	
Missing	0	0	-	0	
Second BC					
No	21 (75.0)	7 (77.78)	-	14 (73.68)	1.000
Yes	7 (25.0)	2 (22.22)	-	5 (26.32)	
Median Age [Min-Max][a]	55 [45–81]	58.5 [53–64]	-	55 [45–81]	1.000
Other tumors					
No	28 (100.00)	9 (100.00)	-	19 (100.00)	-
Yes	0 (0.00)	0 (0.00)	-	0 (0.00)	
BC/OC in I-degree relatives					
No	12 (42.86)	1 (11.11)	-	11 (57.89)	0.039
Yes	16 (57.14)	8 (88.89)	-	8 (42.11)	

(*Continued*)

OVARIAN CANCER (OC)	All patients	Patients with *BRCA1/2* mutations	Patients with extra-*BRCA* mutations	Patients with no pathogenic mutations	*P*
	N (%)	*N (%)*	*N (%)*	*N (%)*	
BC/OC in I/II-degree relatives					
No	9 (32.14)	1 (11.11)	-	8 (42.11)	0.195
Yes	19 (67.86)	8 (88.89)	-	11 (57.89)	
Other cancers in I-degree relatives					
No	16 (57.14)	6 (66.67)	-	10 (52.63)	0.687
Yes	12 (42.86)	3 (33.33)	-	9 (47.37)	
Other cancers in I/II-degree relatives					
No	12 (42.86)	5 (55.56)	-	7 (36.84)	0.432
Yes	16 (57.14)	4 (44.44)	-	12 (63.16)	

[a] Median age, in years, refers to age at second cancer diagnosis

The *FANCL* insertion and 1 of the *PALB2* nonsense mutations were both present in 1 patient; the *BRIP1* deletion and the *SLX4* deletion were both present in another patient.

All 23 variants had either <1% or n/a frequency in the population (1000Genomes, Esp6500 and Exac03 databases) and where checked in dbSNP and ClinVar databases (Supplementary Table 3 and Materials and Methods).

All 17 (100.0%) patients had initial BC, 8 (47.1%) of whom had BBC and 2 (11.8%) had subsequent OC. Median age at the onset of BC was 43 years for initial BC, 57 for subsequent BC and 52.5 for subsequent OC (Table 1). None of these patients had cancers other than BC or OC.

The family history of these patients included BC/OC and other types of cancer, as shown in the pedigrees of 2 patients with *PALB2* and *TSC2* mutations (Figure 1A and 1B).

Characteristics of patients with no pathogenic mutations

Out of 181 patients with no pathogenic mutations, 162 (89.5%) had initial BC, of whom 32 (19.8%) had BBC and 4 (2.5%) had subsequent OC. Median age was 42 years at onset of initial BC, 58.5 for subsequent BC, and 68.5 for subsequent OC (Table 1).

The remaining 19 (10.5%) had initial OC, 5 (26.3%) of whom had subsequent BC. Median age was 47 years at onset of initial OC, and 55 for subsequent BC (Table 2).

Among these 181 patients we identified 1,026 variants with <1% or n/a population frequency: 379 (36.9%) were synonymous mutations, 631 (61.5%) missense mutations, 6 (0.6%) nonframeshift deletions, 2 (0.2%) nonframeshift insertions, and 8 (0.8%) splicing mutations, with a total of 674 unique variants in 92 genes, and an average of 6 variants per patient.

Among the 1,026 rare variants identified, we worked on the 631 missense variants using functional effect prediction tools PolyPhen-2 HVar and SIFT, dividing the 181 patients into 3 categories: the first group (70 patients) with at least 1 mutation classified as damaging by both PolyPhen-2 Hvar and SIFT, the second group (26 patients) with mutations discordantly classified, and the third group (85 patients) with mutations classified as benign. No statistically significant differences were found between the three groups (Supplementary Tables 6 and 7).

DISCUSSION

Current clinical genetic tests for BC and OC risks have been based on the analysis of *BRCA1* and *BRCA2* genes only, despite new evidence of a higher number of genes eligible for testing [15].

Given the considerable amount of genes whose mutations have a role in determining a broad spectrum of tumors, we used a gene panel including almost all the genes involved in the main hereditary cancer syndromes.

To our knowledge, this is the first large Italian study on the sequencing of a multiple-gene panel for cancer predisposition and one of the widest genetic studies on

HBOC for both the number of genes analyzed and the number of recruited patients [10, 12, 13, 16, 17].

We detected a total of 81 pathogenic/likely-pathogenic mutations in 74/255 (29.0%) patients, 32 (39.5%) in *BRCA1*, 26 (32.1%) in *BRCA2* and 23 (28.4%) in other genes. The 23 mutations in the other genes were present in 21 patients, 17 of whom were negative for *BRCA* genes; some of these genes were not clearly correlated to BC.

The 57 patients with *BRCA1/2* pathogenic mutations have already been included in a surveillance protocol according to the F.O.N.Ca.M. (Forza Operativa Nazionale sul Carcinoma Mammario) guidelines [18] and the genetic test has been performed on their consenting relatives.

The BC characteristics of *BRCA*-mutated patients corresponded to what is described in literature [19, 20], with a significant higher number of poorly differentiated tumors (P=0.005), a significant number of triple-negative cancers (P=0.005) and higher Ki-67 expression (P=0.003) than in other patients (Table 1), which are all signs of the greater aggressiveness of the malignancy.

BRCA-mutated patients, compared to other patients, developed BC at a younger age, especially second BC (P=0.041), and had a higher family history of BC/OC, especially for I-degree relatives of OC patients (P=0.039), which are both predictable results given the higher penetrance of mutations in *BRCA1/2* genes (Tables 1 and 2).

Thirteen patients had alterations in *ATM*, *BRIP1*, *PALB2*, *PPM1D* and *RAD51D* genes, which are known to be associated with an increased risk of BC, even if they are considered moderate penetrant genes [8]. Guidelines for the clinical management of mutation carriers are still unavailable.

Thanks to the discovery of these mutations, these patients and their families are eligible for further studies on the development of malignancies in mutation carriers over time, which combine our case series with those of other institutes with the same type of patients.

PALB2, the most frequent mutated gene after *BRCA1* and *BRCA2* in our case series, is worth mentioning. As recently reported by Antoniou and colleagues [21], *PALB2* gene has been proven the most important BC predisposition gene after *BRCA1* and *BRCA2*.

We found 6 patients negative for *BRCA1/2* mutations with a pathogenic mutation in *PALB2* gene, 4 (66.7%) of whom had BBC. These data further highlighted both the high risk of BC associated with these mutations and the

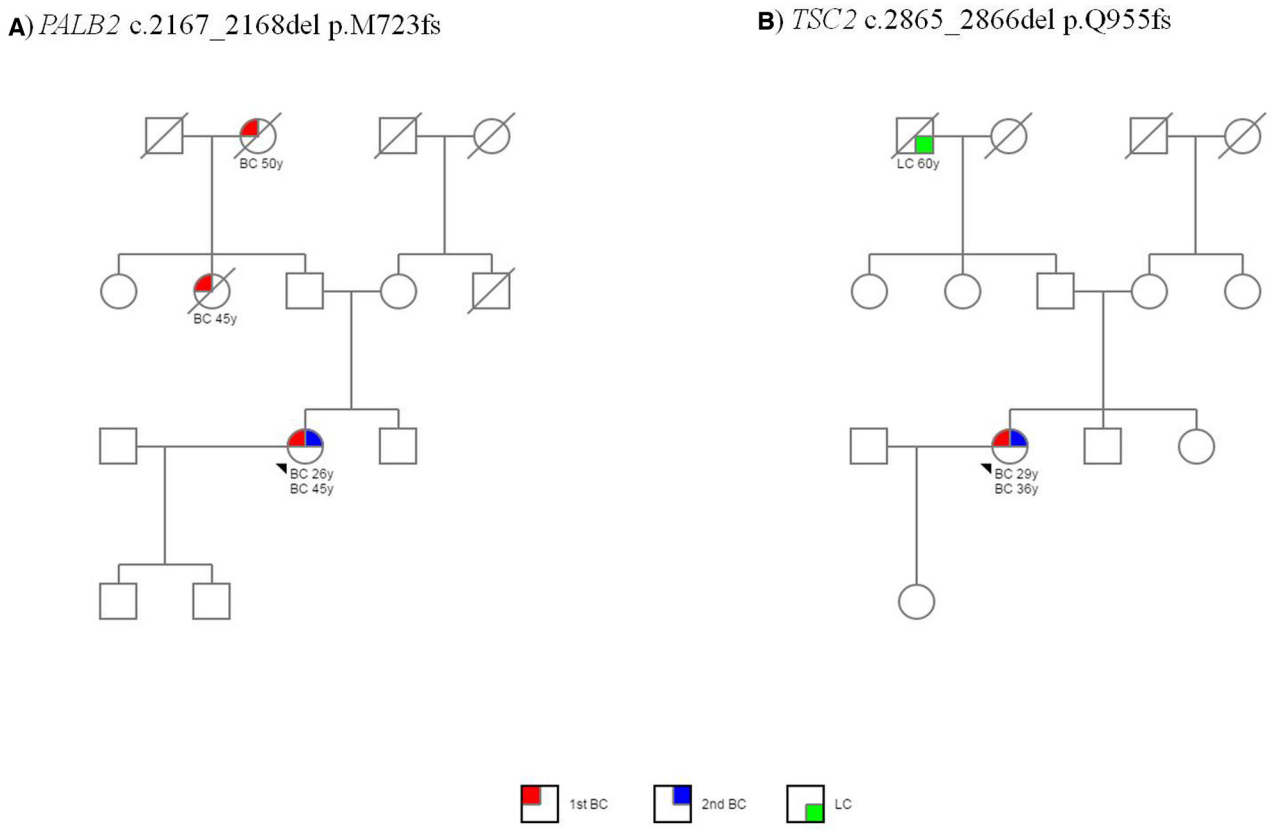


Figure 1: Pedigrees of two patients with a mutation in extra-*BRCA* genes. (**A**) Pedigree of patient A243 with c.2167_2168del p.M723fs mutation in *PALB*2 gene. (**B**) Pedigree of patient A790 with c.2865_2866del p.Q955fs mutation in *TSC2* gene. The probands are indicated by arrowheads. Cancer type and age at cancer diagnosis are indicated in the legend: 1st BC, first breast cancer; 2nd BC, second breast cancer; LC, lung cancer. Symbols: squares, males; circles, females; quadrant shading, cancer affected; slash through square or circle, deceased.

importance of introducing the *PALB2* gene in standard genetic analysis protocols for patients with suspected hereditary BC syndrome.

Two patients (A482 and A806) were carriers of frameshift mutations in *BRIP1* gene, whose truncating mutations have been recently excluded from having a role in BC risk [22]. This had no effect on their assigned category, as each patient had another deleterious mutation (*BRCA2* and *SLX4* respectively).

We also found 1 patient with a pathogenic mutation in *MSH6* gene, associated to Lynch syndrome, a colorectal cancer syndrome whose correlation with BC is still debated [23]: this finding will allow for appropriate genetic counseling and the extension of the genetic test to the relatives. The surveillance protocol for these patients must take the cancer family history and the cancer risk given by the mutation into account. In the case of *MSH6* mutation, the family will undergo a surveillance protocol including screening for BC, as it is the only cancer type present in the family, and screening for colon cancer, according to the Lynch syndrome guidelines [24], as the risk for colon cancer in *MSH6* mutation carriers cannot be ignored.

Six patients had mutations in *FANCA*, *FANCI*, *FANCL*, *FANCM* and *SLX4* genes, which are involved in Fanconi anemia (FA). FA is a recessive genetic disorder characterized by multiple congenital abnormalities, bone marrow failure and susceptibility to cancer, occurring when both the alleles of one of the FA genes are mutated. Monoallelic mutations of some FA genes have been associated to BC risk [25, 26], and biallelic mutations in *BRCA2* have been associated to FA [27]. These observations suggest that biallelic mutations of these genes may result in FA and that monoallelic mutations can pose a risk of BC. Further studies are necessary to confirm such association and to assess the actual risk for the patients.

Finally, we found 3 pathogenic/likely-pathogenic mutations in *ERCC3*, *RECQL4* and *TSC2* genes, encoding transcription factors and tumor suppressors.

Although mutations in these genes are not clearly associated with BC, a role in the predisposition to BC cannot be excluded since they are involved in the major cancer pathways.

Specific mutations in *ERCC3* and *RECQL*, a homologue of *RECQL4*, have also been identified in families with multiple BC cases [28, 29].

The management of these patients still remains problematic. Only further studies on larger case series will determine the factual cancer risk for the mutation carriers.

It is important to underline that the pathogenicity of the identified variants based on the guidelines [30] refers to their potential role in cancer development, not to their causality of BC, as there might be other variants in genes not analyzed in the present study.

We detected a much higher percentage (47.1%) of BBCs in patients with pathogenic mutations in non-*BRCA* genes than in *BRCA1*- (26.1%) and *BRCA2*-positive patients (25.0%) (P=0.036), despite their older age at onset (Table 1). This suggests a high penetrance and a high risk of BC for the carriers; the pathogenic mutations in genes other than *BRCA1/2* do not appear to be linked to OC, since all these patients have BC, only 2 of whom developed OC as second tumor.

These results underscore the importance of a multigenic approach for identifying the genetic cause in a greater number of cases than with a targeted analysis on *BRCA1/2* genes. It also allows accurate patient monitoring for developing surveillance programs customized to their genetic characteristics.

Another remarkable feature is the lower family history of BC/OC in I- and II-degree relatives (P=0.039) than for both the *BRCA*-mutated patients and the patients with no pathogenic mutations (Table 1). Although this result should be verified in larger studies, we hypothesize that it might be due to the fact that these patients have a heterogeneous cancer family history, which includes other types of cancer.

No clear pathogenic mutation was identified in 181/255 (71.0%) patients. We thus studied the 1,026 rare variants identified in order to assess whether they could contribute to cancer risk.

NGS-based studies lead to the identification of many non-easily classifiable variants. Several techniques can now be used to determine pathogenicity of mutations [31], yet quick, efficient and accurate methods for classifying variants are needed for translating the information to clinical practice.

The bioinformatic tools for the prediction of pathogenicity used in this study seemed irrelevant for discriminating higher risk from lower risk patients. This may be due to the fact that the bioinformatic prediction method used in the present work is based only on two different tools, which can be insufficient to highlight clinicopathological differences among the patients. Moreover, the multifactorial nature of the disease and the possible presence of alterations in genes other than those analyzed in this study could explain this result. Some of the identified variants, however, may increase BC and OC risk, whose determination is difficult due to the limited number of carriers and the interference of other genetic and environmental factors.

The interpretation of the potential role in disease development of the great number of variants identified by NGS-based studies remains one of the major future challenges.

MATERIALS AND METHODS

Ethics statement

Investigation was conducted in accordance with ethical standards, the Declaration of Helsinki and national

and international guidelines. It was also approved by the authors' institutional review board.

Patients and samples

Patients referring to genetic counseling at the Cancer Prevention Unit of the Morgagni-Pierantoni Hospital (Forlì-Italy) in the years 2012-2015 with a history of BC and/or OC were included in the study.

The 255 patients were selected according to the F.O.N.Ca.M guidelines [18], based on the age at BC/OC onset and on the number of cancer cases in I- and II-degree relatives.

The study was performed in accordance with the Good Clinical Practice and the Declaration of Helsinki, and approved by the IRST Ethics Committee (CE IRST IRCCS-AVR, protocol 2207/2012).

Information about age at diagnosis, histotype, grading, stage, tumor invasiveness and receptor status was collected. BC subtype classification, based on receptor status, was established according to the St Gallen guidelines [32].

Information about a second BC and/or OC or other malignancies and the cancer family history in I- and II-degree relatives was also collected.

After obtaining informed consent from patients, we collected peripheral blood samples.

Genomic DNA was extracted from blood using the QIAamp DNA mini kit (Qiagen) and quantified using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

Sequencing

Sequencing libraries were created using 50 ng of genomic DNA and the enrichment protocol Trusight Cancer (Illumina) for simultaneous sequencing of a panel of 94 genes (Supplementary Table 1).

The panel covers a total of 355 kb and includes the entire coding regions of the 94 genes and the flanking introns (50bp upstream and downstream each exon).

The sequencing was performed using the MiSeq platform (Illumina) with MiSeq Reagent Kit v2 configured 2x150 cycles, according to the manufacturer's instructions.

The Trusight Cancer kit had been previously validated in our laboratory on a case series of 50 cases with known *BRCA1/2* mutations identified by Sanger sequencing.

Data analysis and variant calling

Raw de-multiplexed reads from the MiSeq sequencer were aligned to the reference human genome (UCSC-Build37/hg19) using the Burrows–Wheeler algorithm [33], running in paired-end mode. To ensure good call quality and to reduce the number of false positives, samples underwent Base Quality Score Recalibration (BQSR), using the Genome Analysis Toolkit GATK, version 3.2.2 [34]. After BQSR, sequences around regions with insertions and deletions (indels) were realigned locally with GATK. MarkDuplicates [35] was used to remove duplicate read-pairs arisen as artifacts during either polymerase chain reaction amplification or sequencing. For variant analysis Unified Genotyper of GATK was used to search for SNVs and indels. Genomic and functional annotations of detected variants were made by Annovar [36]. Coverage statistics was performed by DepthOfCoverage utility of GATK. BASH and R custom scripts were used to obtain the list of low coverage (<50X) regions per sample. The regions under this threshold were considered not evaluable. The potential impact of amino acid changes (MAPP P value) was assessed with PolyPhen-2 HVAR [37] and SIFT [38].

BRCA1/2 analysis

BRCA1/2 regions covered <50X were amplified by standard polymerase chain reaction (PCR). PCR products were sequenced using the BigDye terminator v.3.1 cycle sequencing kit (Thermo Fisher Scientific) on an ABI-3130 Genetic analyzer (Applied Biosystems).

To complete the analysis on the *BRCA1/2* genes and identify gross deletions/insertions not detectable by sequencing, we performed the Multiplex Ligation-dependent Probe Amplification (MLPA) with BRCA1-P002 and BRCA2-P045 kits (MRC Holland). MLPA results were analyzed with Coffalyser software (MRC Holland).

Confirmation of mutations

All the mutations of classes 3-5 identified in *BRCA1* and *BRCA2* genes were confirmed by Sanger sequencing with the same protocol used for the uncovered regions.

All the deleterious mutations (classes 4-5) identified in the other genes were confirmed by a second NGS analysis.

Variant classification

Genetic variants identified in this work were divided into 5 classes according to the IARC recommendations [39].

The classification of *BRCA1/2* variants was performed using the main *BRCA* mutation databases: Breast Cancer Information Core (BIC) [40], *BRCA* Share (formerly Universal Mutation Database) [41] and Leiden Open Variation Database (LOVD) [42].

Sequence variants in the remaining 92 genes were classified using dbSNP [43] and ClinVar [44] databases.

Variants not present in any of these databases were classified on the basis of their characteristics. Only mutations introducing a premature stop codon (frameshift and nonsense) and gross deletions were considered pathogenic/likely-pathogenic and classified in accordance

with the guidelines of the American College of Medical Genetics (ACMG) [30].

Statistical analysis

Patient characteristics and sequencing results were tabulated, with descriptive statistics including median and range for continuous data, and natural frequencies and percentages for categorical data. Proportions were compared using either the Pearson Chi-square test or the Fisher Exact test, as appropriate. The Wilcoxon-Mann Whitney or the Kruskal-Wallis test, as appropriate, were used for the continuous variables.

All P values were two-tailed. Analyses were performed using STATA Release 14.0.

Abbreviations

BC: breast cancer; OC: ovarian cancer; NGS: Next-Generation Sequencing; BBC: bilateral breast cancer; HBOC: hereditary breast and ovarian cancer; F.O.N.Ca.M.: Forza Operativa Nazionale sul Carcinoma Mammario; FA: Fanconi anemia; BQSR: Base Quality Score Recalibration; PCR: polymerase chain reaction; MLPA: Multiplex Ligation-dependent Probe Amplification; BIC: Breast Cancer Information Core; LOVD: Leiden Open Variation Database; ACMG: American College of Medical Genetics.

Authors' contributions

DC, GT, MT and VZ designed the study. RD, VA and MR recruited patients and collected clinical data. IC and FP performed NGS. GT and VZ analyzed the NGS data. MT performed the bioinformatic analysis of the results. EP performed the statistical analysis of the results. GT, MT, EP and DC drafted the manuscript. AR, FF and DA revised the manuscript. All authors read and approved the final version.

ACKNOWLEDGMENTS

The authors are grateful to all the patients enrolled in this study. We would like to thank Rosa Vattiato of the Romagna Cancer Registry for collection of clinical information of patients and Veronica Zanoni for editing the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FUNDING

This work was funded by the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS.

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015; 65: 87-108.
2. Jayson GC, Kohn EC, Kitchener HC, Ledermann JA. Ovarian cancer. Lancet. 2014; 384: 1376-88.
3. Gage M, Wattendorf D, Henry LR. Translational advances regarding hereditary breast cancer syndromes. J Surg Oncol. 2012; 105: 444-51.
4. Prat J, Ribé A, Gallardo A. Hereditary ovarian cancer. Hum Pathol. 2005; 36: 861-70.
5. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, Bell R, Rosenthal J, Hussey C, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science. 1994; 266: 66-71.
6. Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G, Barfoot R, Hamoudi R, Patel S, et al. Identification of the breast cancer susceptibility gene BRCA2. Nature. 1995; 378: 789-92.
7. Antoniou A, Pharoah PD, Narod S, Risch HA, Eyfjord JE, Hopper JL, Loman N, Olsson H, Johannsson O, Borg A, Pasini B, Radice P, Manoukian S, et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet. 2003; 72: 1117-30.
8. Apostolou P, Fostira F. Hereditary breast cancer: the era of new susceptibility genes. Biomed Res Int. 2013; 2013: 747318.
9. Nielsen FC, van Overeem Hansen T, Sørensen CS. Hereditary breast and ovarian cancer: new genes in confined pathways. Nat Rev Cancer. 2016; 16: 599-612.
10. Walsh T, Lee MK, Casadei S, Thornton AM, Stray SM, Pennil C, Nord AS, Mandell JB, Swisher EM, King MC. Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. Proc Natl Acad Sci U S A. 2010; 107: 12629-33.
11. Weaver JM, Edwards PA. Targeted next-generation sequencing for routine clinical screening of mutations. Genome Med. 2011; 3: 58.
12. Kurian AW, Hare EE, Mills MA, Kingham KE, McPherson L, Whittemore AS, McGuire V, Ladabaum U, Kobayashi Y, Lincoln SE, Cargill M, Ford JM. Clinical evaluation of a multiple-gene sequencing panel for hereditary cancer risk assessment. J Clin Oncol. 2014; 32: 2001-9.
13. Desmond A, Kurian AW, Gabree M, Mills MA, Anderson MJ, Kobayashi Y, Horick N, Yang S, Shannon KM, Tung N, Ford JM, Lincoln SE, Ellisen LW. Clinical Actionability of Multigene Panel Testing for Hereditary Breast and Ovarian Cancer Risk Assessment. JAMA Oncol. 2015; 1: 943-51.

14. Kurian AW, Antoniou AC, Domchek SM. Refining Breast Cancer Risk Stratification: Additional Genes, Additional Information. Am Soc Clin Oncol Educ Book. 2016; 35: 44-56.

15. Easton DF, Pharoah PD, Antoniou AC, Tischkowitz M, Tavtigian SV, Nathanson KL, Devilee P, Meindl A, Couch FJ, Southey M, Goldgar DE, Evans DG, Chenevix-Trench G, et al. Gene-Panel Sequencing and the Prediction of Breast-Cancer Risk. N Engl J Med. 2015; 372: 2243-57.

16. Gracia-Aznarez FJ, Fernandez V, Pita G, Peterlongo P, Dominguez O, de la Hoya M, Duran M, Osorio A, Moreno L, Gonzalez-Neira A, Rosa-Rosa JM, Sinilnikova O, Mazoyer S et al. Whole exome sequencing suggests much of non-BRCA1/BRCA2 familial breast cancer is due to moderate and low penetrance susceptibility alleles. PLoS One. 2013; 8: e55681.

17. Kraus C, Hoyer J, Vasileiou G, Wunderle M, Lux MP, Fasching PA, Krumbiegel M, Uebe S, Reuter M, Beckmann MW, Reis A. Gene panel sequencing in familial breast/ovarian cancer patients identifies multiple novel mutations also in genes others than BRCA1/2. Int J Cancer. 2017; 140: 95-102.

18. http://senologia.it/wp-content/uploads/Carcinoma-eredo-familiare-def.pdf

19. Templeton AJ, Gonzalez LD, Vera-Badillo FE, Tibau A, Goldstein R, Šeruga B, Srikanthan A, Pandiella A, Amir E, Ocana A. Interaction between Hormonal Receptor Status, Age and Survival in Patients with BRCA1/2 Germline Mutations: A Systematic Review and Meta-Regression. PLoS One. 2016; 11: e0154789.

20. Wang W, Wu J, Zhang P, Fei X, Zong Y, Chen X, Huang O, He JR, Chen W, Li Y, Shen K, Zhu L. Prognostic and predictive value of Ki-67 in triple-negative breast cancer. Oncotarget. 2016; 7:31079-31087. doi: 10.18632/oncotarget.9075.

21. Antoniou AC, Casadei S, Heikkinen T, Barrowdale D, Pylkas K, Roberts J, Lee A, Subramanian D, De Leeneer K, Fostira F, Tomiak E, Neuhausen SL, Teo ZL, et al. Breast-cancer risk in families with mutations in PALB2. N Engl J Med. 2014; 371: 497–506.

22. Easton DF, Lesueur F, Decker B, Michailidou K, Li J, Allen J, Luccarini C, Pooley KA, Shah M, Bolla MK, Wang Q, Dennis J, Ahmad J et al. No evidence that protein truncating variants in BRIP1 are associated with breast cancer risk: implications for gene panel testing. J Med Genet. 2016; 53: 298-309.

23. Win AK, Lindor NM, Jenkins MA. Risk of breast cancer in Lynch syndrome: a systematic review. Breast Cancer Res. 2013; 15: R27.

24. Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 2004; 96: 261-8.

25. Barroso E, Pita G, Arias JI, Menendez P, Zamora P, Blanco M, Benitez J, Ribas G. The Fanconi anemia family of genes and its correlation with breast cancer susceptibility and breast cancer features. Breast Cancer Res Treat. 2009; 118: 655-60.

26. Peterlongo P, Catucci I, Colombo M, Caleca L, Mucaki E, Bogliolo M, Marin M, Damiola F, Bernard L, Pensotti V, Volorio S, Dall'Olio V, Meindl A, et al. FANCM c.5791C>T nonsense mutation (rs144567652) induces exon skipping, affects DNA repair activity and is a familial breast cancer risk factor. Hum Mol Genet. 2015; 24: 5345-55.

27. Meyer S, Tischkowitz M, Chandler K, Gillespie A, Birch JM, Evans DG. Fanconi anaemia, BRCA2 mutations and childhood cancer: a developmental perspective from clinical and epidemiological observations with implications for genetic counselling. J Med Genet. 2014; 51: 71-5.

28. Vijai J, Topka S, Villano D, Ravichandran V, Maxwell KN, Maria A, Thomas T, Gaddam P, Lincoln A, Kazzaz S, Wenz B, Carmi S, Schrader KA, et al. A Recurrent ERCC3 Truncating Mutation Confers Moderate Risk for Breast Cancer. Cancer Discov. 2016; 6: 1267-1275.

29. Cybulski C, Carrot-Zhang J, Kluźniak W, Rivera B, Kashyap A, Wokołorczyk D, Giroux S, Nadaf J, Hamel N, Zhang S, Huzarski T, Gronwald J, Byrski T, et al. Germline RECQL mutations are associated with breast cancer susceptibility. Nat Genet. 2015; 47: 643-6.

30. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015; 17: 405-24.

31. Thusberg J, Vihinen M. Pathogenic or not? And if so, then how? Studying the effects of missense mutations using bioinformatics methods. Hum Mutat. 2009; 30: 703-14.

32. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, Senn HJ; Panel members. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol. 2013; 24: 2206-23.

33. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009; 25: 1754-60.

34. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010; 20: 1297-303.

35. http://broadinstitute.github.io/picard/

36. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010; 38: e164.
37. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods. 2010; 7: 248-9.
38. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009; 4: 1073-81.
39. Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FB, Hoogerbrugge N, Spurdle AB, Tavtigian SV; IARC Unclassified Genetic Variants Working Group. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. Hum Mutat. 2008; 29: 1282-91.
40. Szabo C, Masiello A, Ryan JF, Brody LC. The breast cancer information core: database design, structure, and scope. Hum Mutat. 2000; 16: 123-31.
41. Caputo S, Benboudjema L, Sinilnikova O, Rouleau E, Béroud C, Lidereau R; French BRCA GGC Consortium. Description and analysis of genetic variants in French hereditary breast and ovarian cancer families recorded in the UMD-BRCA1/BRCA2 databases. Nucleic Acids Res. 2012; 40(Database issue): D992-1002.
42. Fokkema IF, Taschner PE, Schaafsma GC, Celli J, Laros JF, den Dunnen JT. LOVD v.2.0: the next generation in gene variant databases. Hum Mutat. 2011; 32: 557-63.
43. https://www.ncbi.nlm.nih.gov/projects/SNP/
44. https://www.ncbi.nlm.nih.gov/clinvar/

IJBM
eISSN 1724-6008

Int J Biol Markers 2016; 31(4): e461-e465
DOI: 10.5301/jbm.5000227

CASE REPORT

Multiple primary tumors in a family with Li-Fraumeni syndrome with a TP53 germline mutation identified by next-generation sequencing

Valentina Zampiga[1], Rita Danesi[2], Gianluca Tedaldi[1], Michela Tebaldi[1], Ilaria Cangini[1], Francesca Pirini[1], Cristina Pittureri[3], Elena Amaducci[3], Luciano Guidi[4], Marina Faedi[5], Dino Amadori[5], Fabio Falcini[2], Daniele Calistri[1]

[1] Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola (Forlì-Cesena) - Italy
[2] Romagna Cancer Registry, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola (Forlì-Cesena) - Italy
[3] Palliative Care Unit, Savignano sul Rubicone Hospice, Savignano sul Rubicone (Forlì-Cesena) - Italy
[4] General Practitioner, AUSL Romagna, Longiano (Forlì-Cesena) - Italy
[5] Department of Medical Oncology, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola (Forlì-Cesena) - Italy

ABSTRACT

Li-Fraumeni syndrome (LFS) is an autosomal dominant disorder occurring at a young age that predisposes individuals to multiple forms of cancer and to a heterogeneous spectrum of malignancies. We describe the clinical history of a patient who had 5 primary malignant cancers and a familiar history consistent with LFS. We analyzed the genomic DNA of the proband and her relatives by next-generation sequencing (NGS) technology using an enrichment protocol for the simultaneous sequencing of 94 genes involved in hereditary cancers. Genetic analysis of the proband revealed a TP53 germline mutation in exon 5 determining a nucleotide alteration at codon 175 (R175H), a hot spot mutation site related to LFS and a reported pathogenic mutation. The proband daughter's and brother's DNA did not carry the TP53 mutation but they had some rare variants in common with the proband, in addition to other variants with a still unclear role. In conclusion, we identified a TP53 mutation in a patient with multiple primary tumors and a family history characterized by a severe susceptibility to cancer. The genetic analysis by targeted NGS led to the identification of the genetic background and to the exclusion of a cancer risk for the family members. Targeted NGS represents an efficient approach for the identification of mutations in families with a heterogeneous phenotype.

Keywords: Li-Fraumeni syndrome, Multiple primary tumors, Next-generation sequencing, R175H TP53 mutation

Introduction

Li-Fraumeni syndrome (LFS; OMIM no.151623) is a rare disorder associated with a high risk of developing several types of cancer; in particular, individuals with LFS have an increased risk of developing cancer at a younger age than people who are not affected (1, 2). LFS is often associated with breast cancer at premenopausal age, sarcoma (especially soft tissue and bone sarcomas), brain tumors and adrenal cortical carcinomas (3). Individuals with LFS have a 50%-56% risk of developing multiple primary tumors by the age of 30, which rises to 90%-100% by the age of 60 compared with the general population (4, 5). The estimated risk of developing a second cancer within 30 years of the diagnosis of the first cancer is 57% (6).

Germline mutations in the TP53 tumor suppressor gene (chromosome 17p13; OMIM no.191170) are the molecular basis of LFS and have been identified in 80% of patients with LFS (7). The p53 protein normally controls and regulates cell division and growth through action on the cell cycle and is involved in the repair or destruction of damaged DNA, thus preventing abnormal growth of cells. The TP53 gene consists of 11 exons: 75% of patients affected by LFS have mutations in exons 5-8, which encode the core DNA-binding region of the gene, and 25% show mutations in either exon 4 or 9 (8). Most of the mutations reported are missense mutations (75%), and only a small number of mutations result in premature stop codons and a truncated protein, such as nonsense mutations (9%) and frameshift mutations (6%). Carriers of TP53 mutations have a lifetime risk higher than 90% of developing a malignancy and a 20% risk of developing a tumor before the age of 20 (9).

Received: May 16, 2016
Accepted: July 12, 2016
Published online: August 5, 2016

Corresponding author:
Valentina Zampiga
Biosciences Laboratory
Istituto Scientifico Romagnolo per lo
Studio e la Cura dei Tumori (IRST) IRCCS
47014 Meldola (Forlì-Cesena), Italy
valentina.zampiga@irst.emr.it

At the moment, TP53 is the only gene clearly associated with LFS (7), but many other genes have been investigated as possible candidates. Further research is needed to expand the knowledge of the genetic basis of this syndrome in support of the family history and to enable efficient differential diagnosis and accurate treatment.

Case report

We describe the case of a family with LFS with a peculiar history of tumor development due to the presence of 5 different primary tumors in the same individual and the early development of breast and brain cancers. The study was performed in accordance with the principles of Good Clinical Practice and the ethical standards laid down in the Declaration of Helsinki, and was approved by the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) Ethics Committee (CE IRST IRCCS-AVR, protocol 2207/2012). The first family member, recruited by our counseling service at the Cancer Prevention Unit of Morgagni-Pierantoni Hospital in Forlì, was a 44-year-old woman (III-1, Fig. 1). She suffered, at the age of 15, of chondrosarcoma. At 35 years old, she was diagnosed with bilateral breast cancer (infiltrating and poorly differentiated ductal carcinoma). Three years later, she developed a superficial bladder cancer (low grade). At the age of 42, she developed a thalamomesencephalic lesion with relatively well-defined margins attributable to cancer of the glial cells that turned out to be an anaplastic astrocytoma grade 3. The patient died at the age of 44.

The patient had a daughter (IV-1) suffering from Down syndrome and a son (IV-2) who died at the age of 4 of a rhabdomyosarcoma with relapse. Her sister (III-2) died at 6 years of age because of a brain tumor in the third ventricle (highly differentiated tumor) and her mother (II-2) developed bilateral breast cancer at the age of 42. Her grandmother (I-4) died at 28 years old, probably of breast cancer.

After her informed consent was obtained, the peripheral blood of patient III-1 (proband) was collected. DNA extraction from leukocytes was performed using the QIAamp DNA mini kit (Qiagen). Genetic analysis of the proband was performed using Trusight Cancer (Illumina), an enrichment protocol for the simultaneous sequencing of 94 genes involved in the main hereditary cancer syndromes.

The panel covers a total of 255 kb, on the entire coding regions of 94 genes (AIP, ALK, APC, ATM, BAP1, BLM, BMPR1A, BRCA1, BRCA2, BRIP1, BUB1B, CDC73, CDH1, CDK4, CDKN1C, CDKN2A, CEBPA, CEP57, CHEK2, CYLD, DDB2, DICER1, DIS3L2, EGFR, EPCAM, ERCC2, ERCC3, ERCC4, ERCC5, EXT1, EXT2, EZH2, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM, FH, FLCN, GATA2, GPC3, HNF1A, HRAS, KIT, MAX, MEN1, MET, MLH1, MSH2, MSH6, MUTYH, NBN, NF1, NF2, NSD1, PALB2, PHOX2B, PMS1, PMS2, PRF1, PRKAR1A, PTCH1, PTEN, RAD51C, RAD51D, RB1, RECQL4, RET, RHBDF2, RUNX1, SBDS, SDHAF2, SDHB, SDHC, SDHD, SLX4, SMAD4, SMARCB1, STK11, SUFU, TMEM127, TP53, TSC1, TSC2, VHL, WRN, WT1, XPA, XPC). In addition, 100 kb of exon-intron boundaries (50 bp upstream and downstream of each exon) were added to the target regions.

The DNA libraries were prepared starting from 50 ng of genomic DNA and were sequenced on the MiSeq sequencer (Illumina) with MiSeq Reagent Kit v2 (2 × 150 cycles), according to the manufacturer's instructions.

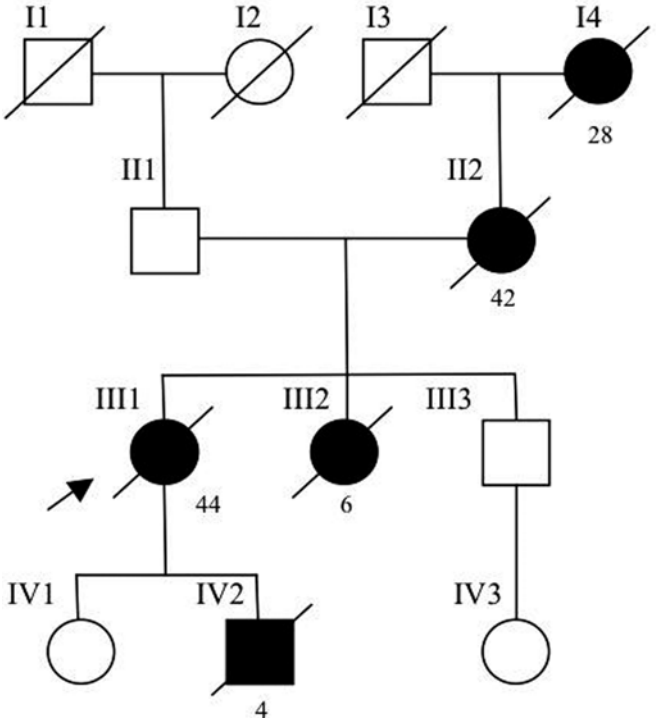


Fig. 1 - Pedigree of the family with disease-associated TP53 mutation. Circles represent females and squares represent males. Solid symbols represent cancer patients. Symbols with a slash indicate deceased individuals. The arrow points to the proband. I-4 breast cancer (28 y); II-2 bilateral breast cancer (42 y); III-1 chondrosarcoma (15 y), bilateral breast cancer (35 y), bladder cancer (37 y), astrocytoma (42 y); III-2 brain tumor (5 y); IV-1 Down syndrome; IV-2 rhabdomyosarcoma (4 y).

The reads obtained from sequencing were aligned against the human reference genome hg19 with BWA MEM (10). Genome Analysis Toolkit (GATK) version 3.2.2 (11) was used to recalibrate base qualities and realign aligned reads around indels. MarkDuplicates was used to remove duplicate read pairs that arise as artefacts during polymerase chain reaction amplification or sequencing. For variant analysis, GATK UnifiedGenotyper (version 3.5) was used to search for single-nucleotide variants (SNVs) and indels with a minimum fraction of 10% and a minimum base quality score of 15. Genomic and functional annotation of detected variants was done with ANNOVAR (12).

Coverage statistics was performed by the Depth of Coverage utility of GATK. BASH and R custom scripts were used to obtain the list of low coverage (50X) regions per sample. We evaluated the impact of amino acid changes on the 3 patients with predictor tools such as PolyPhen-2 HVAR and SIFT, that use naive Bayes classifiers. Variants were viewed manually on integrative genomics viewer (IGV) to eliminate strand bias and reduce false positive calls. The same next-generation sequencing (NGS) analysis protocol was extended to the brother (III-3) and daughter (IV-1) of the proband.

Confirmatory analysis on the identified TP53 mutation was performed by direct sequencing: the region of

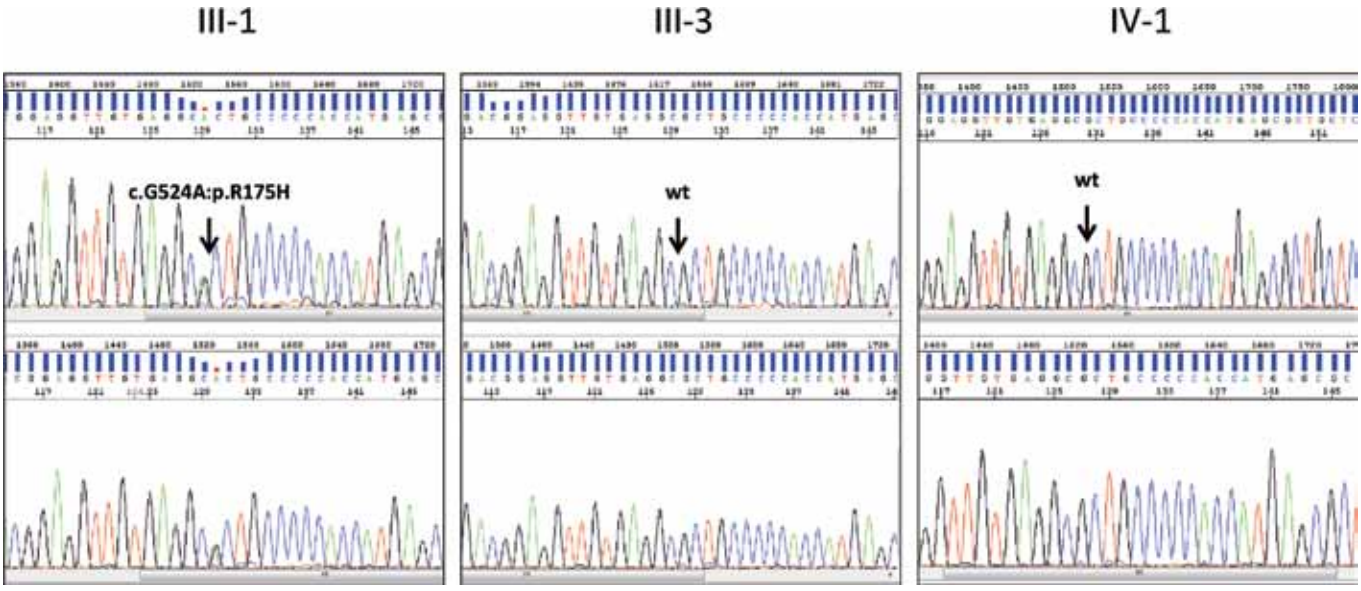


Fig. 2 - Direct sequencing of TP53 exon 5 showing the heterozygous mutation c.G524A:p.R175H in the proband III-1 (forward strand in the upper electropherogram and reverse strand in the lower electropherogram) compared to the wild-type sequence in her brother (III-3) and daughter (IV-1).

TP53 exon 5 was amplified by PCR using specific primers (5'-CTCTTCCTACAGTACTCCCCTGC and 5'-GCCCCAGCTGCTCACCATCGCTA) with Ex Taq DNA polymerase (Takara) and subjected to Sanger sequencing using the BigDye Terminator v3.1 (Life Technologies). The sequences were analyzed by capillary electrophoresis on the 3130 Genetic Analyzer (Life Technologies).

The bioinformatics analysis revealed the presence of 149 exonic variants and 19 splicing variants in the genomic DNA of the proband. To exclude polymorphisms, the identified variants were filtered based on a frequency in the population lower than 0.01 or unknown (Esp6500, 1000genomes and Exac03). The remaining 7 variants were 4 missense mutations in the TP53, BLM, PALB2 and SLX4 genes (c.G524A:p.R175H, c.G3427A:p.E1143K, c.A1001G:p.Y334C and c.C2009A:p.T670N, respectively), 2 synonymous mutations in the ATM and TSC2 genes, and 1 splicing variant in the EGFR gene.

The prediction of the pathogenicity of the 4 missense mutations was concordant for PolyPhen-2 HVAR and SIFT: the mutations in BLM, PALB2 and SLX4 were classified as benign and the mutation in TP53 was classified as deleterious.

The germline mutation in TP53 exon 5, c.G524A:p.R175H (rs28934578), is a hot spot codon commonly mutated in LFS families and was confirmed by a second independent analysis through direct sequencing (Fig. 2).

Even though the proband's brother (III-3) and the proband's daughter (IV-1) who suffers from Down syndrome were healthy at the time of the study, in order to assess their risk, they underwent the same NGS genetic testing.

Applying a frequency filter of the identified variants (<0.01 or NA), the cases III-3 and IV-1 were both found to be carriers of the mutations in ATM and BLM identified in the proband. The individual III-3 was also a carrier of the missense mutation in PALB2, while the individual IV-1 was a carrier of the splicing mutation in EGFR. In addition, case III-3 had a missense mutation in the FANCM gene (c.A974T:p.D325V) and 2 synonymous mutations in BRCA2 and ERCC5 (c.C741T:p.I247I and c.T1983C:p.I661I, respectively). Case IV-1 had 4 missense mutations in BRCA1, BRCA2, RAD51C and ERCC4 (c.A4039G:p.R1347G, c.G3581A:p.G1194D, c.G376A:p.A126T and c.C1135T:p.P379S, respectively), 2 synonymous mutations in MSH2 and FANCD2 (c.G2094A:p.E698E and c.G2181A:p.P727P, respectively), and a splicing mutation in EZH2 (c.247-9insT). Table I shows the genetic variants identified in each member of the family.

Prediction of the effect of the missense variants identified in the proband's relatives was concordant for PolyPhen-2 HVAR and SIFT only for the ERCC4 and BRCA2 mutations (classified as deleterious and benign, respectively). It was not concordant for the FANCM mutation (classified as deleterious by SIFT and possibly deleterious by PolyPhen), BRCA1 and RAD51C mutations (classified as deleterious by SIFT and benign by PolyPhen).

A second analysis in individuals III-3 and IV-1 by direct sequencing on TP53 exon 5 confirmed that they were not carriers of the R175H mutation in TP53 (Fig. 2).

Discussion

We have described the family history and genetic characterization of a 44-year-old female patient who developed 5 primary malignant tumors during her lifetime. The proband showed the germline mutation c.G524A:p.R175H in the TP53 gene, which lies in a hot spot codon commonly mutated in sporadic cancers. The protein with the R175H mutation failed to fold properly, resulting in a significant loss of transcriptional and tumor suppressive activity (13). In the literature, the reported mutation is indicated as responsible for the clinical history of aggressive tumors in LFS families (14) and is

TABLE I - Genetic variants identified in each member of the family

Id sample	Gene	Variant	Variant effect	Protein change	VAF (variant allele frequency)	Coverage	Annotation	Funtional prediction (HVAR, SIFT)
III-1 III-3	BLM, NM_000057	chr15:91346819-91346819	Nonsynonymous SNV	c.G3427A:p.E1143K	0.50; 0.51; 0.52	1037; 509; 542	rs140387675	B,T
IV-1	ATM, NM_000051	chr11:108122678-108122678	Synonymous SNV	c.A1722G:p.E574E	0.51; 0.49; 0.48	1358; 702; 729	rs372334891	-,-
III-1 III-3	PALB2, NM_024675	chr16:23646866-23646866	Nonsynonymous SNV	c.A1001G:p.Y334C	0.51; 0.47	1091; 485	rs200620434	B,T
III-1 IV-1	EGFR, NM_005228	chr7:55210990-55210990	Splicing	c.241-8C>G	0.55; 0.51	414; 200	rs138872748	-,-
III-1	TP53, NM_000546	chr17:7578406-7578406	Nonsynonymous SNV	c.G524A:p.R175H	0.50	383	rs28934578	D,D
	TSC2, NM_000548	chr16:2134492-2134492	Synonymous SNV	c.G4269A:p.L1423L	0.56	425	rs45438898	-,-
	SLX4, NM_032444	chr16:3645610-3645610	Nonsynonymous SNV	c.C2009A:p.T670N	0.51	972	-	B,T
III-3	ERCC5, NM_000123	chr13:103518045-103518045	Synonymous SNV	c.T1983C:p.I661I	0.50	311	-	-;-
	BRCA2, NM_000059	chr13:32905115-32905115	Synonymous SNV	c.C741T:p.I247I	0.44	553	rs276174892	-;-
	FANCM, NM_020937	chr14:45620655-45620655	Nonsynonymous SNV	c.A974T:p.D325V	0.45	658	-	D,P
IV-1	BRCA2, NM_000059	chr13:32912073-32912073	Nonsynonymous SNV	c.G3581A:p.G1194D	0.43	508	rs28897721	B,T
	ERCC4, NM_005236	chr16:14028081-14028081	Nonsynonymous SNV	c.C1135T:p.P379S	0.48	474	rs1799802	D,D
	BRCA1, NM_007294	chr17:41243509-41243509	Nonsynonymous SNV	c.A4039G:p.R1347G	0.49	441	rs28897689	B,D
	RAD51C, NM_058216	chr17:56772522-56772522	Nonsynonymous SNV	c.G376A:p.A126T	0.47	188	rs61758784	B,D
	MSH2, NM_000251	chr2:47703594-47703594	Synonymous SNV	c.G2094A:p.E698E	0.47	333	-	-;-
	FANCD2, NM_001018115	chr3:10107090-10107090	Synonymous SNV	c.G2181A:p.P727P	0.60	360	-	-;-
	EZH2, NM_004456	chr7:148529851-148529851	Splicing	c.247-9->T	0.43	217	-	-;-

List of the rare variants (frequency in the population less than 1%) identified both in the proband and the 2 relatives and in the individuals singularly.
HVAR: B = benign; P = potentially deleterious; D = probably deleterious; SIFT: T = tolerated; D = deleterious; SNV = single-nucleotide variant.

frequently associated with breast cancer, brain cancer and soft tissue cancer. All these findings are consistent with the type of cancers diagnosed in the family described. The development of such a large number of primary tumors, the association with the mutation in TP53, and the family history make this a model case of LFS, and therefore interesting to be thoroughly genetically characterized.

In order to clarify the causes of the genetic predisposition to multiple tumors in the proband and the segregation pathway in relatives, we performed a comprehensive investigation of the family genetic background by targeted NGS, expanding the analysis to 94 genes involved in multiple hereditary cancer syndromes.

Effect prediction of the mutations found in the proband in addition to the TP53 mutation was possible only for the BLM, PALB2 and SLX4 missense variants, and they were classified as benign. The mutations in BLM, PALB2, ATM and EGFR were also present in one or both of the proband's relatives who were in good health, and therefore do not seem to segregate with the syndrome.

The analysis of TP53 gene mutation in other family members allowed to exclude a cancer risk in the proband's

healthy brother (III-3) and consequently in his daughter (IV-3). Moreover, the Down syndrome case (IV-1) was not associated with the TP53 mutation identified. In addition, in cases III-3 and IV-1 we found 10 rare mutations that were not present in the proband and whose role in the cancer risk is unclear. The prediction of the effect of these alterations by bioinformatics tools is not always concordant and the lack of data on other members of the family prevents us from drawing any further conclusions. Unfortunately, it was not possible to analyze the sister (III-2) and the son (IV-2) of the proband for TP53 gene mutation but, given their personal history of cancer, they were also likely to be carriers of the mutation.

Analysis of the genetic background of the proband provided the identification of the causal mutation of the syndrome but also other genetic alterations, including mutations in BLM, PALB2, SLX4 and ATM. Many studies have reported an increased risk of breast cancer associated with mutations of these genes (15-18), which is consistent with the family history described here, where 3 individuals were affected by breast cancer (III-1, II-2 and I-4).

Conclusion

Although it was not possible to recover DNA from all family members, the genetic analysis of a broad spectrum of genes involved in hereditary cancers, combined with the family history, allowed to outline a more accurate diagnosis and to assess the risk of specific cancers. The analysis of Multi-gene panels is an important tool to gain insight into the mechanisms that lead to a high susceptibility to certain tumors and the interactions between causative mutations. Moreover, background mutations increase the knowledge of the connections between the genotype and the phenotype of the family.

Abbreviations

LFS	Li-Fraumeni syndrome
NGS	next-generation sequencing
PCR	polymerase chain reaction
SNV	single-nucleotide variant

Disclosures

Financial support: No grants or funding have been received for this study.
Conflict of interest: None of the authors has any financial interest related to this study to disclose.

References

1. Li FP, Fraumeni JF Jr. Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome? Ann Intern Med. 1969;71(4):747-752.
2. Li FP, Fraumeni JF Jr, Mulvihill JJ, et al. A cancer family syndrome in twenty-four kindreds. Cancer Res. 1988;48(18):5358-5362.
3. Nichols KE, Malkin D, Garber JE, Fraumeni JF Jr, Li FP. Germ-line p53 mutations predispose to a wide spectrum of early-onset cancers. Cancer Epidemiol Biomarkers Prev. 2001;10(2):83-87.
4. Lustbader ED, Williams WR, Bondy ML, Strom S, Strong LC. Segregation analysis of cancer in families of childhood soft-tissue-sarcoma patients. Am J Hum Genet. 1992;51(2):344-356.
5. Varley JM, Evans DG, Birch JM. Li-Fraumeni syndrome—a molecular and clinical review. Br J Cancer. 1997;76(1):1-14.
6. Hisada M, Garber JE, Fung CY, Fraumeni JF Jr, Li FP. Multiple primary cancers in families with Li-Fraumeni syndrome. J Natl Cancer Inst. 1998;90(8):606-611.
7. Malkin D. Li-fraumeni syndrome. Genes Cancer. 2011;2(4): 475-484.
8. Varley JM. Germline TP53 mutations and Li-Fraumeni syndrome. Hum Mutat. 2003;21(3):313-320.
9. Lammens CR, Aaronson NK, Wagner A, et al. Genetic testing in Li-Fraumeni syndrome: uptake and psychosocial consequences. J Clin Oncol. 2010;28(18):3008-3014.
10. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14): 1754-1760.
11. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010;20(9):1297-1303.
12. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010;38(16):e164.
13. Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science. 1994;265(5170):346-355.
14. Pötzsch C, Voigtländer T, Lübbert M. p53 Germline mutation in a patient with Li-Fraumeni Syndrome and three metachronous malignancies. J Cancer Res Clin Oncol. 2002;128(8):456-460.
15. Chen P, Liang J, Wang Z, et al. Association of common PALB2 polymorphisms with breast cancer risk: a case-control study. Clin Cancer Res. 2008;14(18):5931-5937.
16. Thompson D, Duedal S, Kirner J, et al. Cancer risks and mortality in heterozygous ATM mutation carriers. J Natl Cancer Inst. 2005;97(11):813-822.
17. Li J, Meeks H, Feng BJ, et al. Targeted massively parallel sequencing of a panel of putative breast cancer susceptibility genes in a large cohort of multiple-case breast and ovarian cancer families. J Med Genet. 2016;53(1):34-42.
18. Kurian AW, Hare EE, Mills MA, et al. Clinical evaluation of a multiple-gene sequencing panel for hereditary cancer risk assessment. J Clin Oncol. 2014;32(19):2001-2009.

IVYSPRING INTERNATIONAL PUBLISHER

Oncomedicine

2017; 2: 88-92. doi: 10.7150/oncm.19362

Research Paper

Coexistence of Two Novel Mutations in CDKN2A and PMS1 Genes in a Single Patient Identifies a New and Severe Cancer Predisposition Syndrome

Marco Cassone[1]✉, Leila Baghernajad Salehi[1], Gianluca Tedaldi[2], Michela Tebaldi[2], Valentina Zampiga[2], Michela Biancolella[1], Barbara Testa[1], Daniele Calistri[2], Gerarda Mastrogiorgio[1], Silvia Lanciotti[1], Maria Rosaria D'Apice[3], Giuseppe Novelli[1], Federica Sangiuolo[1]

1. Department of biomedicine and prevention, University Tor Vergata Rome, Rome, Italy;
2. Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola (Forlì-Cesena), Italy;
3. Medical Genetics Laboratory, Policlinico Tor Vergata, Rome, Italy.

✉ Corresponding author: m.cassone@umcg.nl; marcocassone@yahoo.it; Phone +393772867733

Received: 2017.01.26; Accepted: 2017.04.06; Published: 2017.05.04

Abstract

Introduction: Up to 10% of cancers occur through the inherited mutation of a group of genes called cancer predisposition genes [1]. Carriers of monoallelic mutations of these genes are associated with an increased susceptibility to cancer. Autosomal dominant cancer predisposition genes for common cancers have been well recognized for over decade. Each newly identified cancer predisposition gene has been associated with a distinctive autosomal dominant or recessive cancer syndrome [2]. The CDKN2A is the major known melanoma susceptibility gene and has been associated with Familial atypical multiple mole melanoma syndrome (FAMMM #155601), while PMS1 gene is involved in mismatch repair (MMR) process and its mutations have been associated with Lynch syndrome (LS) (HNPCC for hereditary non polyposis colorectal cancers) (OMIM #120435) in few cases [3].

Materials and methods: Genetic counseling, molecular analysis of 94 genes by using "IlluminaTruSight Cancer" panel.

Results: We have detected in a patient with a novel frameshift mutation within PMS1 gene, *PMS1c.1139dupA p.Y380_S381delinsX*, and in the same time a novel mutation within CDKN2a gene, *CDKN2a c.58delG p.V20X*. In this way, the coexistence of two germline mutations in two different genes, already associated to cancer predisposition syndrome, such as LS and FAMMM have been described.

Discussion: Our data frame this case as a new and severe cancer predisposition syndrome. An appropriate genetic counseling surely represents the key step for a correct test choice in panel genes era.

Key words: PMS1, CDKN2A, Lynch syndrome, cancer predisposition syndrome, familial melanoma.

Introduction

Up to 10% of cancers occur through the inherited mutation of a group of genes called 'cancer predisposition genes. Individuals who carry a mutant allele of these genes have an increased susceptibility to cancer.

A growing number of cancer susceptibility genes are being identified and the physiopathology of germline mutation-based cancer development is also being elucidated with accumulating clinical and molecular data. New technologies for DNA

sequencing allow us to analyze simultaneously multiple genes assembled in specific panels and associated with cancer predisposition, offering a useful tool in the common clinical practice of genetic counseling. The characterization of a genetic cause of cancer plays an extremely important role for the treatment, which may differs from the treatment of sporadic disease, and for periodic surveillance.

The first description of inherited cancers was established in "family G" pedigree with an impressive aggregation of colon and gynecological cancers in family members throughout four generations [3]. The family was reinvestigated from Lynch and Krush in 1971 and the predisposition appeared to be inherited in an autosomal dominant manner [4].

The syndrome was named "Lynch Syndrome" (LS) or HNPCC for hereditary non polyposis colorectal cancers (OMIM #120435), one of the most common cancer syndrome, accounting for 1-3% of unselected colorectal carcinomas. Lynch syndrome is an autosomal dominant condition caused by heterozygous germline mutations in the DNA mismatch repair genes, the PMS1 is recognized as (MMR) genes and its mutations were associated with LS in a few cases PMS1 [5-6]. Rare cases have been reported of an inherited bi-allelic deficiency of MMR genes, associated with multiple café-au-lait spots, early onset CNS tumors, hematological malignancies, and early onset gastrointestinal neoplasia [7-8].

There are also a number of less frequent syndromes with distinctive cancer spectrums. Among them, Familial Atypical Multiple Mole Melanoma (FAMMM#155601) and hereditary melanoma are rare predisposition cancer syndrome in which CDKN2A mutations remain the most common gene association.

Materials and Methods

Clinical Data

Our patient has been referred to genetic counseling by physicians on the basis of her own clinical history described and listed in Table 1.

The patient is a female of 47 years old at the time of genetic visit, with a remarkable history of cancer disease: at age of 20 she was diagnosed with Hodgkin's lymphoma, treated with chemotherapy and radio-therapy, at the age of 38 she underwent to first surgical treatment for retroperitoneal lymphangioma, which is followed with a second surgical treatment for the same lesion recurrence one year later.

After three years patient was diagnosed with a retroperitoneal mass referred to malignant PEComa, finally at the age of 46 years old she was surgically treated for bilateral breast ductal in situ carcinoma (see Table 1).

Table 1. Clinical history of the proband

Patient's Age	Tumour	Treatment
20 years	Hodgkin Lymphoma	Chemiotherapy and radiotherapy
38 years	Retroperitoneal lymphangioma	Surgical excision
41 years	Recurrence of retroperitoneal lymphangioma	Surgical excision
46 years	Bilateral breast carcinoma in situ	Bilateral mastectomia
47 years	Malignant PEComa.	surgical
48 years	Recurrence of PEComa in the contest of Ilio-psoas muscle	surgical
49 years	Patient death	

Histological analysis of retroperitoneal mass revealed classical PEComas features, positive for HMB45 antibody and also for Melan-A, microophtalmia transcription factor (Mitf) and actin.

Patient's Family history does not show occurrence of any type of rare tumor at early age. Only two cases of breast cancer disease and two cases of prostate cancer disease were reported in first degree relatives: at the age of 72 and 48 years for the breast cancer and 65 and 74 for prostate cancer respectively, overlapping epidemiological incidence data, as showed on family pedigree.

Family pedigree (fig.1) is drawn in genetic counseling, not informative for cancer risk assessment and thus for selecting specific cancer gene test among known available ones. However some patient's characteristics were suggestive of hereditary disease and it has been considered an indication for genetic testing.

Recognition of the inherited nature of the tumors is important for genetic counseling of these patients and their families.

So we analyze proband's sample DNA from blood, using a panel for multiple sequencing of 94 genes whose mutations are recognized to be engaged in the germline DNA for predisposition to tumor development.

Genetics Analysis

After the patient had signed an informed consent, a sample of peripheral blood was collected. The DNA was extracted from leukocytes using the kit EZ1 DNA Blood 200 µl, and successively analyzed by Next-Generation Sequencing (NGS) using an enrichment protocol (Illumina Trusight Cancer) for the simultaneous sequencing of 94 genes involved in the main hereditary cancer syndromes.

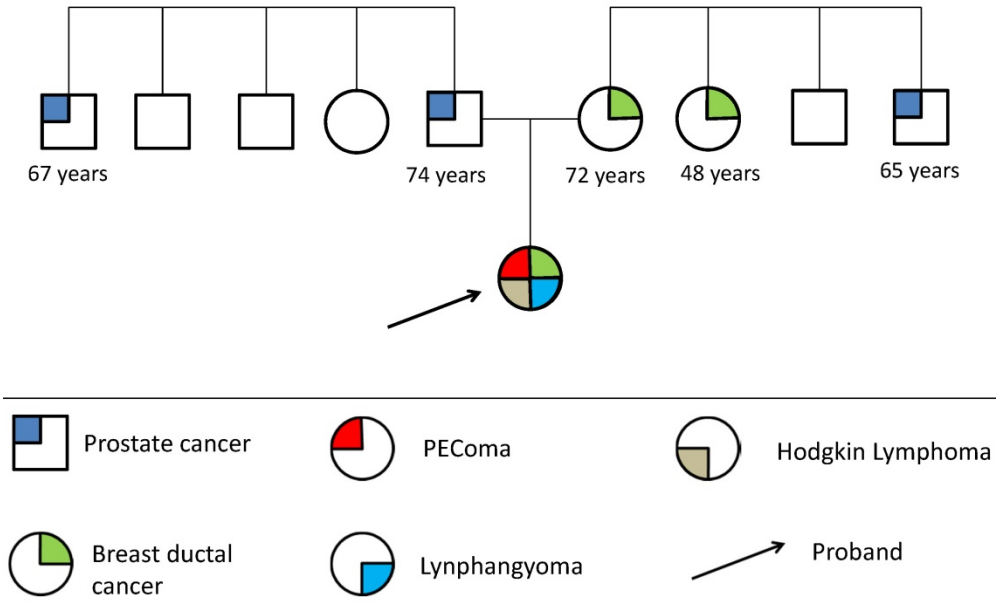


Figure 1. Familial pedigree

The panel covers the coding regions of 94 genes: *AIP*, *ALK*, *APC*, *ATM*, *BAP1*, *BLM*, *BMPR1A*, *BRCA1*, *BRCA2*, *BRIP1*, *BUB1B*, *CDC73*, *CDH1*, *CDK4*, *CDKN1C*, *CDKN2A*, *CEBPA*, *CEP57*, *CHEK2*, *CYLD*, *DDB2*, *DICER1*, *DIS3L2*, *EGFR*, *EPCAM*, *ERCC2*, *ERCC3*, *ERCC4*, *ERCC5*, *EXT1*, *EXT2*, *EZH2*, *FANCA*, *FANCB*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCL*, *FANCM*, *FH*, *FLCN*, *GATA2*, *GPC3*, *HNF1A*, *HRAS*, *KIT*, *MAX*, *MEN1*, *MET*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *NBN*, *NF1*, *NF2*, *NSD1*, *PALB2*, *PHOX2B*, *PMS1*, *PMS2*, *PRF1*, *PRKAR1A*, *PTCH1*, *PTEN*, *RAD51C*, *RAD51D*, *RB1*, *RECQL4*, *RET*, *RHBDF2*, *RUNX1*, *SBDS*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *SLX4*, *SMAD4*, *SMARCB1*, *STK11*, *SUFU*, *TMEM127*, *TP53*, *TSC1*, *TSC2*, *VHL*, *WRN*, *WT1*, *XPA*, *XPC*.

The DNA library was prepared starting from 50 ng of genomic DNA and was sequenced on the MiSeq sequencer (Illumina) with MiSeq Reagent Kit v2 (2x150 cycles), according to manufacturer's instructions. The sequencing reads were aligned with BWA MEM [9] against the human reference genome hg19. The resulting BAM files were processed with GATK version 3.2.2 [10] to recalibrate base qualities and realign aligned reads around indels and with MarkDuplicates to remove duplicate read-pairs, that arise as artifacts during polymerase chain reaction amplification or sequencing. To identify all variants in the samples we used Genome Analysis Toolkit Unified Genotyper (GATK, version 3.5) to search SNVs and InDel with minimum fraction of indel of 10% and minimum base quality score of 15. The genomic and functional annotation of detected variants was made by Annovar [11]. Variants were viewed manually on integrative genomics viewer (IGV) to eliminate strand bias and reduce false positive calls. Sequencing quality statistic were obtained using GATK's Depth Of Coverage tool. A second NGS analysis, following the same protocol, was performed to confirm the presence of the mutations identified.

Results

Molecular analysis by NGS reveals the presence of two deleterious germline mutations in two different genes responsible for different predisposition syndromes: Lynch syndrome (OMIM #120435) and Familial atypical multiple mole melanoma (FAMMM#155601).

For Lynch syndrome we have found a novel frameshift mutation in the *PMS1* gene, **c.1139dupA p.Y380_S381delinsX**, that consists in a duplication of a nucleotide (A1140) in the exon9 (NM_000534) and determines an early termination of the PMS1 protein synthesis at codon 380. Only three mutations have been reported in the literature in this gene [5-6] and, although its classification as MMR gene has been recognized for a long time, its mutations were associated with Lynch syndrome only in very few cases.

In *CDKN2A* gene, NGS data detected the presence of **c.58delG p.V20X**, that consists in a deletion of a nucleotide (G58) in the exon 1β (NM_058195) and determines an early termination of the p14(ARF) protein synthesis at codon 20.

These mutations are not present in the ExAC and gnomAD database, also COSMIC database does not report these mutations [12].

CDKN2A gene encodes two different tumor suppressor proteins by alternative splicing, p16(INK4), a cyclin-dependent kinase inhibitor, and p14(ARF), an inhibitor of the p53-destablization protein MDM2[13] is an E3 ubiquitin ligase and mediates p53 degradation.

Only few mutations of *CDKN2A* affecting p14(ARF) but not p16(INK4) have been described in literature [14-15].

Unfortunately segregation of these mutations in the father, in the mother and in the proband's child is not available because they did not give their consent to the genetic test and to obtain the surgical specimens as well after patient's death, for this we could not verify whether mutations were present at somatic level within the tumors samples of the patient.

The other 131 exonic variants identified in the patient were filtered on the basis of the frequency in the population lower than 0.01 or unknown (Esp6500, 1000genomes and Exac03) in order to exclude them as polymorphisms. The remaining 4 variants were 2 missense mutations in *TP53* (c.587G>A p.R196Q) and *WRN* (c.3101A>T p.Y1034F) genes and 2 synonymous mutations in *EGFR* (c.1509C>T p.G503G) and *DICER1* (c.G1935A p.P645P) genes. All these variants have been previously described and have been classified as benign or with uncertain significance due to the lack of information.

In conclusion the coexistence of two germline mutations in two different genes, already associated to cancer predisposition syndrome, such as LS and FAMMM have been described.

Discussion

About 5% of all cancer cases are due to high penetrance, dominant germline mutations. A much larger proportion is caused by a combination of weaker susceptibility genes and/or environmental and lifestyle factors. A number of high penetrance susceptibility genes have been identified, and recently, the clinical significance of genetic germline alterations in tumors has started to be appreciated. Scientific and technological advances in genomics are revolutionizing the approach to genetic cancer risk assessment, in fact NGS technologies surely made easier and less expensive the analysis of many cancer susceptibility genes simultaneously. It has to be noted that using multi gene panels could allow the discovery of some disease-causing mutations in different genes stimulating us to reassess the nosography, the clinical and the diagnostic approach to this type of genetic syndromes in patients with evident history of multiple cancer. This will allow us to redefine the cancer risk assessment for every know cancer predisposition syndrome basing the calculation risk for every not only on a single gene mutation but on multiple gene mutations eventually identified. It is likely that further genes associated with different phenotypes in monoallelic and biallelic mutation carriers will be recognized for cancer syndromes.

Routine sequencing patients diagnosed with cancer with multiple genes panels, drive us towards a revision of the clinical classification of the tumor syndromes, better elucidating the phenotypic variability on the basis of the presence of single and/or multiple gene mutations.

Our reported case is an example about the coexistence of different rare novel mutations in different cancer predisposition genes.

The clinical data show that our patient has many tumor manifestations with aggressive characteristics, that's why we it like severe cancer predisposition syndrome.

PMS1 is not considered a classical gene causative of Lynch Syndromes, and it is not evaluated in the common clinical practice, for this reason its implication in the susceptibility to tumor development could be underestimated.

Indeed, this report shows that the deployment of NGS in medical laboratories according genetic counseling, significantly increases the throughput, providing a more accurate molecular diagnosis.

Moreover introducing the molecular analysis of multiple genes in common clinical practice help us to evaluate both clinical and molecular variability of cancer predisposition genes and thus the frequency of this conditions in the general population and more specifically in the various ethnic groups for every know cancer predisposition genes.

Finally we reported for the first time the coexistence of two common genetic syndromes in the same patient: Hereditary Nonpolyposis Colorectal Cancer (HNPCC-LS) and Familial Atypical Multiple Mole Melanoma (FAMMM #155601) (Table 2).

Table 2. Known pathogenic mutations in PMS1

Gene	Mutation	Clinical Phenotype	n° cases	Literature
PMS1	p.Met394Thr ATG>ACG	4 CRC	1	Qing Wang, et al., 1998
	P.Gly501Arg GGA>AGA	Leukemia		
	p.Gln233X	HNPCC	1	Tao Liu, et al., 2001
	c.1139dupA	see Table 1	1	Cassone et al. 2017

Competing Interests

The authors have declared that no competing interest exists.

References

[1] Wang Q. Cancer predisposition genes: molecular mechanisms and clinical impact on personalized cancer care: examples of Lynch and HBOC syndromes. Acta Pharmacol Sin. 2016 Feb; 37(2):143-9.

[2] By Aldred Scott Warthin. 1913. Classics in oncology. Heredity with reference to carcinoma as shown by the study of the cases examined in the pathological laboratory of the University of Michigan, 1895-1913. CA Cancer J Clin. 1985;35(6):348-59.

[3] Garber JE, Offit K. Hereditary cancer predisposition syndromes. J Clin Oncol 2005; 23: 276–92.

[4] Lynch HT, Krush AJ. Carcinoma of the breast and ovary in three families. Surg Gynecol Obstet. 1971 Oct;133(4):644-8.

[5] Wang Q, Lasset C, Desseigne F, Saurin JC, Maugard C, Navarro C, Ruano E, Descos L, Trillet-Lenoir V, Bosset JF, Puisieux A. Prevalence of germline mutations of hMLH1, hMSH2, hPMS1, hPMS2, and hMSH6 genes in 75 French kindreds with non polyposis colorectal cancer 1998. Hum Genet. 1999;105(1-2):79-85.

[6] Liu T, Yan H, Kuismanen S, Percesepe A, Bisgaard ML, Pedroni M, Benatti P, Kinzler KW, Vogelstein B, Ponz de Leon M, Peltomäki P, Lindblom A. The role of hPMS1 and hPMS2 in predisposing to colorectal cancer. Cancer Res. 2001 Nov 1;61(21):7798-802.

[7] Aronson M, Gallinger S, Cohen Z, Cohen S, Dvir R, Elhasid R, Baris HN, Kariv R, Druker H, Chan H, Ling SC, Kortan P, Holter S, Semotiuk K, Malkin D, Farah R, Sayad A, Heald B, Kalady MF, Penney LS, Rideout AL, Rashid M, Hasadsri L, Pichurin P, Riegert-Johnson D, Campbell B, Bakry D, Al-Rimawi H, Alharbi QK, Alharbi M, Shamvil A, Tabori U, Durno C. Gastrointestinal Findings in the Largest Series of Patients With Hereditary Biallelic Mismatch Repair Deficiency Syndrome: Report from the International Consortium. Am J Gastroenterol. 2016 Feb;111(2):275-84.

[8] Hamilton SR, Liu B, Parsons RE, Papadopoulos N, Jen J, Powell SM, Krush AJ, Berk T, Cohen Z, Tetu B, et al. The molecular basis of Turcot's syndrome. N Engl J Med. 1995 Mar 30;332(13):839-47.

[9] Li C, Courtot M, Le Novère N, Laibe C. BioModels.net Web Services, a free and integrated toolkit for computational modelling software. Brief Bioinform. 2010 May;11(3):270-7.

[10] McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010 Sep;20(9):1297-303.

[11] Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010 Sep;38(16):e164.

[12] [Internet] http://exac.broadinstitute.org, http://gnomad.broadinstitute.org.

[13] Robertson KD, Jones PA. Tissue-specific alternative splicing in the human INK4a/ARF cell cycle regulatory locus. Oncogene. 1999 Jul 1;18(26):3810-20.

[14] Randerson-Moor JA1, Harland M, Williams S, Cuthbert-Heavens D, Sheridan E, Aveyard J, Sibley K, Whitaker L, Knowles M, Bishop JN, Bishop DT. A germline deletion of p14(ARF) but not CDKN2A in a melanoma-neural system tumour syndrome family. Hum Mol Genet. 2001 Jan 1;10(1):55-62.

[15] Binni F, Antigoni I, De Simone P, Majore S, Silipo V, Crisi A, Amantea A, Pacchiarini D, Castori M, De Bernardo C, Catricalà C, Grammatico P. Novel and recurrent p14 mutations in Italian familial melanoma. Clin Genet. 2010 Jun;77(6):581-6.

Preclinical evidence of multiple mechanisms underlying trastuzumab resistance in gastric cancer

Chiara Arienti[1], Michele Zanoni[1], Sara Pignatta[1], Alberto Del Rio[2,3], Silvia Carloni[1], Michela Tebaldi[1], Gianluca Tedaldi[1], Anna Tesei[1]

[1]Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

[2]Institute of Organic Synthesis and Photoreactivity (ISOF), National Research Council (CNR), Bologna, Italy

[3]Innovamol Srls, Modena, Italy

Correspondence to: *Chiara Arienti,* ***e-mail:*** *chiara.arienti@irst.emr.it*
Anna Tesei, ***e-mail:*** *anna.tesei@irst.emr.it*

Keywords: *trastuzumab resistance, gastric cancer, HER family receptors, HER signaling pathways, IQGAP1*

Received: *October 19, 2015* ***Accepted:*** *February 11, 2016* ***Published:*** *February 22, 2016*

ABSTRACT

HER2-positive advanced gastric cancer patients frequently develop resistance to trastuzumab through mechanisms still poorly understood. In breast cancer, other members of the HER-family are known to be involved in trastuzumab-resistance, as is overexpression of the scaffold protein IQGAP1. In the present work, we investigated acquired resistance to trastuzumab in gastric cancer experimental models. Trastuzumab-resistant (HR) subclones derived from 3 HER2-overexpressing gastric cancer cells were generated and characterized for alterations in HER2- signaling mechanisms by next-generation sequencing, immunohistochemical, western blot and qRT-PCR techniques, and molecular modeling analysis. All subclones showed a reduced growth rate with respect to parental cell lines but each had a different resistance mechanism. In NCI N87 HR cells, characterized by a marked increase in HER2-signaling pathways with respect to the parental cell line, trastuzumab sensitivity was restored when IQGAP1 expression was silenced. AKG HR subclone showed higher HER3 protein expression than the parental line. High nuclear HER4 levels were observed in KKP HR cells. In conclusion, our study revealed that high IQGAP1 expression leads to resistance to trastuzumab in gastric cancer. Furthermore, 2 new mutations of the HER2 gene that may be involved in acquired resistance were identified in AKG HR and KKP HR subclones.

INTRODUCTION

Gastric cancer is the fourth most common malignant disease and the second leading cause of cancer-related death worldwide [1]. In Europe, it is the fifth most common cancer among men and women, representing about 23% of all cancers. Depending on tumor characteristics and stage [2], treatment modalities include a combination of surgery, chemotherapy generally based upon a platinum-fluoropyrimidine doublet, and radiation therapy [3, 4]. Although fluorouracil (5-FU)-based regimens have proven feasible and effective in the treatment of solid tumors, their therapeutic effect is unsatisfactory in advanced gastric cancer, *i.e.*, 7-51% overall response rate and 6 to 12-month median survival [5–7]. Thus, various combination regimens have been developed.

Trastuzumab, a monoclonal antibody targeting human epidermal growth factor receptor 2 (HER2), induces antibody-dependent cellular cytotoxicity and inhibits HER2-mediated signaling by binding the extracellular domain of HER2. Amplification of the *HER2* gene is observed in 20%-30% of gastric and gastroesophageal junction cancer [8–12] and is indicative of a poor prognosis, as recently highlighted in the systematic meta-analysis by Jorgensen et al. [13]. In 2010, the phase III ToGA trial showed the superiority of trastuzumab plus chemotherapy (based on a cisplatin-

fluoropyrimidine doublet) in patients with HER2-positive metastatic gastric cancer over chemotherapy alone in terms of response rate, progression-free survival (PFS) and overall survival (OS) [14]. These results led to the approval of trastuzumab as the first molecular targeted therapy for gastric cancer. However, subsequent clinical trials (TYTAN8 and LOGiC9) failed to show a survival advantage with the use of another anti-HER2 treatment, lapatinib [15]. Overall, the efficacy of HER2-targeted agents has proven more limited and unsatisfactory than originally expected because the majority of patients with gastric cancer develop acquire resistance to treatment [16]. In particular, it has been observed that, whilst few patients with HER2-positive advanced gastric cancer exhibit primary resistance to trastuzumab, all acquire resistance after a relatively short period of time (median PFS 6.7 months) [17], as already observed in HER2-positive breast cancer patients. The identification of mechanisms underlying treatment resistance would thus enhance the benefit from HER2-targeted therapy in patients with HER2-positive gastric cancer.

The etiology of resistance to HER2-directed therapies has been widely investigated in breast cancer [18–22]. Several molecular mechanisms underlying acquired resistance to HER-2 inhibitors have been described, including the activation of c-Src tyrosine kinase [20], HER3 upregulation [23], activating mutations in the p110a subunit of PI3K (PIK3CA) [24], and enhanced HER-ligand autocrine signaling [25]. It has also been proven that resistance to HER2-targeted therapy can trigger genetic alterations of receptor tyrosine kinases (RTKs), leading to the activation of downstream signaling targets and alternative pathways to compensate for HER-2 inhibition [26, 27]. Numerous studies have concluded that induction of the HER3 pathway is one of the reasons underlying this type of resistance [28–30]. Moreover, Mohd Nafi et al. observed that HER4 activation, cleavage and nuclear translocation influence sensitivity and resistance to trastuzumab in HER2-positive breast cancer [31].

A recent study reported that IQGAP1, a scaffold protein of 189-kDa ubiquitously expressed in all human tissues, governs HER2 expression, phosphorylation and signaling in breast cancer cell lines [32]. Moreover, IQGAP1 protein is overexpressed in squamous cell [33] and hepatocellular [34] carcinoma, astrocytoma [35], and aggressive forms of gastric cancer [36]. In particular, White et al. [37] showed that IQGAP1overexpression is correlated with trastuzumab-induced resistance in breast cancer cell lines. However, its involvement in resistance to trastuzumab in gastric cancer has never been investigated. In the present work we investigated mechanisms of resistance induced by trastuzumab in *in vitro* experimental gastric cancer cell lines rendered resistant to the antiproliferative effect of the drug.

RESULTS

Baseline expression and mutational status of HER2, -3 and -4 receptors in a panel of established human gastric cancer cell lines

Positivity to HER2, -3 and -4 proteins and their cellular localization in the human gastric cell lines NCI N87, AKG and KKP was assessed by immunohistochemistry (Figure 1A). HER2, -3 and -4 receptors were highly expressed in all 3 cell lines, albeit with a different diffusion pattern. In particular, HER2 was highly expressed in NCI N87 with a diffuse plasma membrane and cytosolic staining pattern. HER3 was also highly expressed in NCI N87 cells (~95% of positive cells) in both the plasma membrane and cytosol. In addition, HER3 was expressed in KKP and AKG cells, albeit to a lesser degree (~40% and ~30% of positive cells, respectively), whereas its staining pattern was mainly restricted to the cytosol. Finally, HER4 protein was mainly localized in the nuclei and cytoplasm of AKG cells.

We used next generation sequencing (NGS) to search for genomic alterations that might predispose to a different response to treatment with trastuzumab (average depth of 2779.73 and 99.7% of targets with a minimum coverage of 50). We first investigated whether the different cell lines harbored genetic alterations *of HER2, HER3 or HER4* genes and which, if any, were common to AKG, KKP and NCI N87 (Figure 1B). All 3 cell lines showed several alterations in the gene sequences investigated, only 12 of which are not annotated in the dbSNP and COSMIC databases. In particular, 4 of these were exonic variants (Tables 1 and 2). Notably, only 5 gene variants were common to all 3 lines, *i.e*. HER3 variants c.-211delCT and c. C2270A; and HER4 variants IVS17-60delAG, IVS17-102insG and IVS7-7delT (Figure 1B and Table 1).

A pairwise comparison of the 3 cell lines revealed that AKG and KKP cell lines shared the highest number of gene alterations, one in *HER2*, 2 in *HER3* and one in *HER4* (Table 1). Conversely, NCI N87 showed the highest number of genetic variants (13) relating to all 3 HER receptors (5 variants in *HER2*, 3 in *HER3* and 5 in *HER4*) that were not found in AKG or KKP (Table 2).

Generation of trastuzumab-resistant subclones

All of the cell lines were sensitive to trastuzumab, as confirmed by the clonogenic assay in which IC_{50} values were lower than the peak plasma concentration of the drug (Figure 2A). In particular, NCI N87, the cell line harboring the highest number of HER2 variants, was the most sensitive to the cytotoxic action of trastuzumab (IC_{50} value of 7 μg/ml), whereas the AKG cells (3 gene variants) were the most resistant (IC_{50} = 40 μg/ml). The

incorporation of BrdU after a 72-h treatment with the drug confirmed these data. In fact, after treatment with 100 μg/ml of trastuzumab, NCI N87 showed a lower incorporation of BrdU than that of untreated cells (35% and 42%, respectively), while no substantial change in cell proliferation was seen in AKG (Figure 2B).

We generated trastuzumab-resistant (HR) subclones derived from the above gastric cancer cell lines to investigate the mechanisms underlying acquired resistance to trastuzumab. Starting from the peak plasma concentration of 100 μg/ml, all cell lines were exposed to gradually increasing concentrations of trastuzumab for a period of 8-12 months. We thus obtained trastuzumab-resistant subclones that were capable of growing in culture medium containing a drug concentration of 250 μg/ml for the NCI N87 HR subclone and 400 μg/ml for the AKG HR and KKP HR subclones (Figure 2C).

The resistant phenotype was stable and all subclones showed IC_{50} values higher than the peak plasma concentration of the drug ranging from 120 μg/ml (AKG HR) to 200 μg/ml (KKP HR and NCI N87 HR) (Figure 2C). We also evaluated the relative resistance IC_{50} index (RR IC_{50}) of each subclone obtained (Figure 2D). Notably, the data revealed that the subclone with the highest RR IC_{50} value, NCI N87 HR, was obtained from the cell line with the highest number of genetic variants. In addition, trastuzumab was found to stimulate proliferation in all subclones, significantly so for NCI N87 HR cells ($p<0.05$) (Figure 2E).

We also observed changes in doubling times that were cell line-dependent (Figure 2F), *e.g.* AKG HR cells grew more rapidly, albeit not significantly, than those of the parental line. KKP and its subclone KKP HR showed similar growth, while NCI N87 HR grew significantly slower than its parental line ($p<0.05$).

The resistant subclone of NCI N87 displayed a different cell distribution in cell cycle phases compared to parental cells. In particular, an increase of cells in G0/G1 phase was observed in NCI-N87 HR (84.0%) compared to NCI N87 cells (69.63%), whereas a lower percentage of S-phase cells was found in the HR subclone than in the parental line (11.02% NCI N87 HR *vs*. 24.17% NCI N87) (Figure 2G).

Different HER2 signaling modulation in HR subclones

We quantified HER2 expression levels by flow cytometry and western blot analysis to verify whether its expression was modified in HR subclones (Figure 3). Flow cytometric analysis revealed an increase in HER2 membrane levels in NCI N87 HR cells with respect to parental cells (Figure 3A). Furthermore, immunohistochemistry analysis showed that HER2 was highly expressed in NCI N87 HR cells (~95% positive cells) which had both membrane and a cytoplasmic positivity (Figure 3B). We also detected a marked increase in p-HER2, AKT, p-AKT and MAPK protein

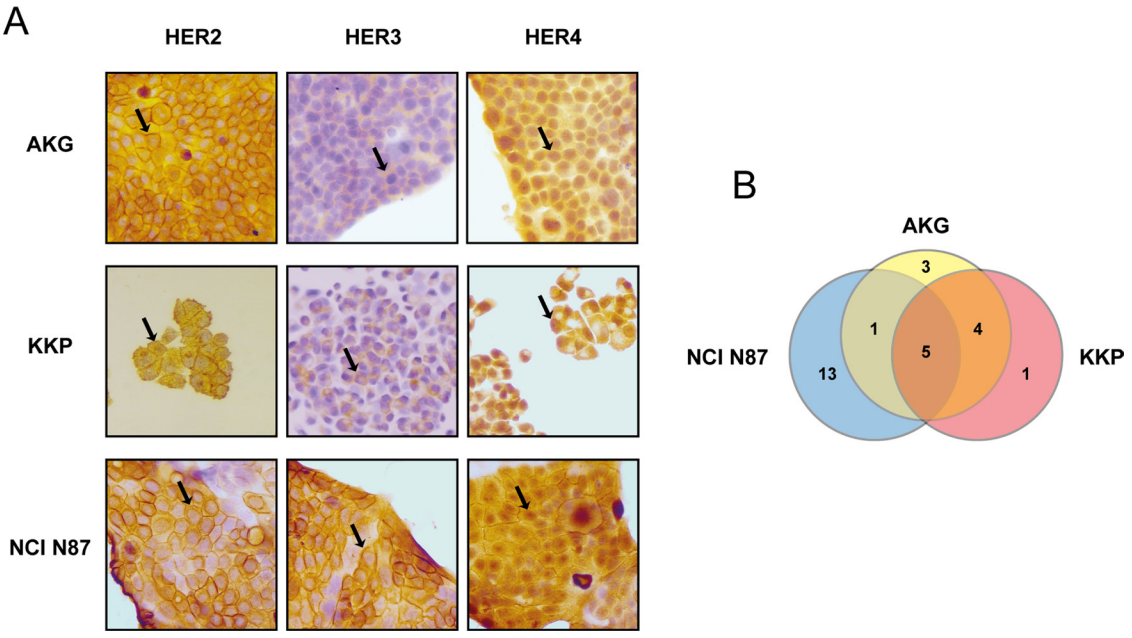


Figure 1: Baseline expression and mutational status of HER2, -3 and -4 receptors in a panel of established human gastric cancer cell lines. A. HER2, HER3 and HER4 staining by IHC in gastric cancer cell lines AKG, KKP and NCI N87. Sample reactivity was evaluated by light microscopy (×200 magnification) by two independent observers. Marker positivity was evaluated in a semiquantitative manner, as described in the *Materials and Methods* section. **B.** Venn-diagram comparison of gene variants across the three cell lines. The numbers refer to the number of somatic mutations affecting HER2, -3 and -4.

Table 1: Genetic variants shared in parental gastric cancer cell lines

Cell lines	gene	variant	localization	variant effect	protein change	annotation	Functional prediction score (HVAR, SIFT, MutationAssessor)
AKG KKP	HER2	c.C3508G	exonic	nonsynonymous SNV	p.P1170A	rs1058808	P, D, M
	HER3	c.-277T>C	upstream	-	-	rs7297175	-
		IVS2+8A>T	intronic	-	-	rs2271194	-
	HER4	IVS16-23insA	intronic	-	-	rs202070359	-
AKG NCIN87	HER4	IVS12-15T>C	intronic	-	-	rs4673628	-
AKG KKP NCIN87	HER3	c.-211delCT	UTR5	-	-	-	-
		c.C2270A	exonic; splicing	nonsynonymous SNV	p.T757K	-	D, D, N
	HER4	IVS17-60delAG	intronic	-	-	rs146953835	-
		IVS17-102insG	intronic	-	-	rs76332141	-
		IVS7-7delT	intronic	-	-	rs67894136	-

HVAR outputs: P, potentially deleterious; D, probably deleterious; SIFT outputs: D, deleterious; MutationAssessor outputs:, N, neutral; M, medium; - , unknown

levels in NCI N87 HR with respect to NCI N87 (Figure 3C). Protein levels of p-HER2 and of molecules involved in HER2 signaling were also analyzed by Western blot in the other cell lines. An increase in AKT expression was detected in KKP HR cells, while MAPK and p27 expression levels were significantly lower than those of the parental cell line. In AKG HR cell line, we observed an increase in mTOR and MAPK protein expression and a sharp decrease in AKT protein expression with respect to parental AKG cells.

Knockdown of IQGAP1 inhibits HER2-stimulated NCI-N87 HR cell growth

IQGAP1 gene and protein expression were analyzed in all cell lines to verify their involvement in trastuzumab-related resistance (Figure 4). A different modulation of the protein was observed in the 3 parental cell lines, AKG cells showing the highest IQGAP1 expression and KKP the lowest (Figure 4A). This expression pattern was confirmed by gene expression analysis (data not shown). We also observed an increase in IQGAP1 protein expression of HR-resistant subclones with respect to parental cells ($p<0.05$). However, trastuzumab-resistant NCI N87 cells were the only subclones to show an increase in IQGAP1 gene expression levels with respect to parental cells (expression value 2.5-fold higher than that of NCI N87).

The influence of IQGAP1 on trastuzumab resistance was evaluated by transfecting siRNAs against IQGAP1 into NCI N87 HR cells, the subclone with the highest drug resistance phenotype (also confirmed by its RR value). Gene silencing induced a total block in protein synthesis and a dramatic decrease (up to 90%) in mRNA expression (Figure 4B). IQGAP1-silenced NCI N87 HR cells exposed to different concentrations of trastuzumab for 144 h regained a certain degree of sensitivity to trastuzumab, reaching an IC_{50} value of 110 μg/ml. Furthermore, the RR decreased from 28.57 to 15.71, indicating an increase in sensitivity to trastuzumab (Figure 4C). These data were further supported by the results from a colony formation assay showing a reduction of about 55% in the number of colonies when IQGAP1-silenced NCI N87 HR cells were exposed to trastuzumab 100 μg/ml for 14 days.

Analysis of HER3 and HER4 protein expression in HR subclones

HER3 and HER4 expression levels in the plasma membrane were quantified by western blot analysis and immunohistochemistry to evaluate their role in the acquired resistance to trastuzumab (Figure 5). Protein expression detected by western blot revealed increased HER3 and decreased HER4 protein levels in AKG HR cells compared to parental cells. KKP HR subclone showed higher levels of HER4 than KKP cells. Finally, NCI N87 HR only showed significantly lower HER4 expression than parental cells (Figure 5A). HER3 was also highly expressed in about 95% of AKG HR cells, with cytoplasmic positivity. Furthermore,

Table 2: Genetic variants not shared by parental gastric cancer cell lines

Cell lines	gene	variant	localization	variant effect	protein change	annotation	Funtional prediction score (HVAR, SIFT, MutationAssessor)
AKG	HER2	IVS8-7T>C	intronic (7bp)	-	-	-	-
		c.T3182C	exonic	nonsynonymous SNV	p.L1061P	rs141142822	P, T, N
	HER3	c.-195delCA	UTR5	-	-	-	-
KKP	HER2	c.T2709G	exonic	nonsynonymous SNV	p.S903R	-	D, D, H
NCIN87	HER2	c.A2698C	exonic	nonsynonymous SNV	p.T900P	-	D, D, M
		c.C2692G	exonic	nonsynonymous SNV	p.R898G	-	D, D, L
		c.C2704A	exonic	nonsynonymous SNV	p.Q902K	-	D, D, N
		c.C2689G	exonic	nonsynonymous SNV	p.R897G	-	D, T, N
		c.A2705G	exonic	nonsynonymous SNV	p.Q902R	-	D, D, N
	HER3	c.A3355T	exonic	nonsynonymous SNV	p.S1119C	-	D, T, N
		c.G2606A	exonic	nonsynonymous SNV	p.S869N	rs143021252	B, T, N
	HER4	IVS27-7C>T	intronic	-	-	rs812826	-
		IVS24-7delCTTT	splicing	-	-	rs138150601	-
		IVS13-12A>T	intronic	-	-	rs78812564	-
		IVS25-53delC	intronic	-	-	rs142227938	-
		IVS21+81insA	intronic	-	-	rs141267844	-
		IVS16-18delT	intronic	-	-	-	-

HVAR outputs: B, benign; P, potentially deleterious; D, probably deleterious; SIFT outputs: T, tolerated; D, deleterious; MutationAssessor outputs: N, neutral; L, low; M, medium; H, high; - , unknown

immunohistochemistry analysis showed that HER4 was localized exclusively in the nuclei and cytoplasm of all trastuzumab-resistant subclones and, in particular, was highly expressed in KKP HR (Figure 5B).

Relationship between specific genetic variations and change in HER2 and HER3 receptor structure

We analyzed the exonic variants of target genes detected by NGS to investigate their role in the onset of resistance to trastuzumab (Figure 6). None of the cell lines showed exonic genetic variants for HER4 or IQGAP1 genes. In addition, no intronic variants were found in the IQGAP1 gene (data not shown). Once again, the cells showing the highest number of variants of all parental cells or subclones were NCI N87, which was also the most sensitive to trastuzumab. In particular, 5 mutations were located in a region in the predominantly α-helical C-terminal lobe between residues 898 and 906 of HER2 isoform 37 and between residues 897 and 902 of HER2 isoform 48. Its resistant subclone, NCI N87 HR, did not acquire new genetic variants. Conversely, this subclone showed the loss of 3 variants with respect to

the parental line. In particular, the genetic variants were located in clusters between residues 897 and 902 in the C-terminal lobe of HER2 isoform 48, and the mutation in position 759 belonging to the N-terminal lobe of HER3. Conversely, the trastuzumab-resistant subclones KKP HR and AKG HR acquired one and 2 genetic variants, respectively, compared to their parental cell lines, all located between residues 898 and 906 in a region in the predominantly α-helical C-terminal lobe of HER2 isoform 37 (Figure 6A). The crystal structure of the kinase domain of HER2 (HER2-KD) in complex with SYR127063 (PDB code 3PP0) is shown in Figure 6B.

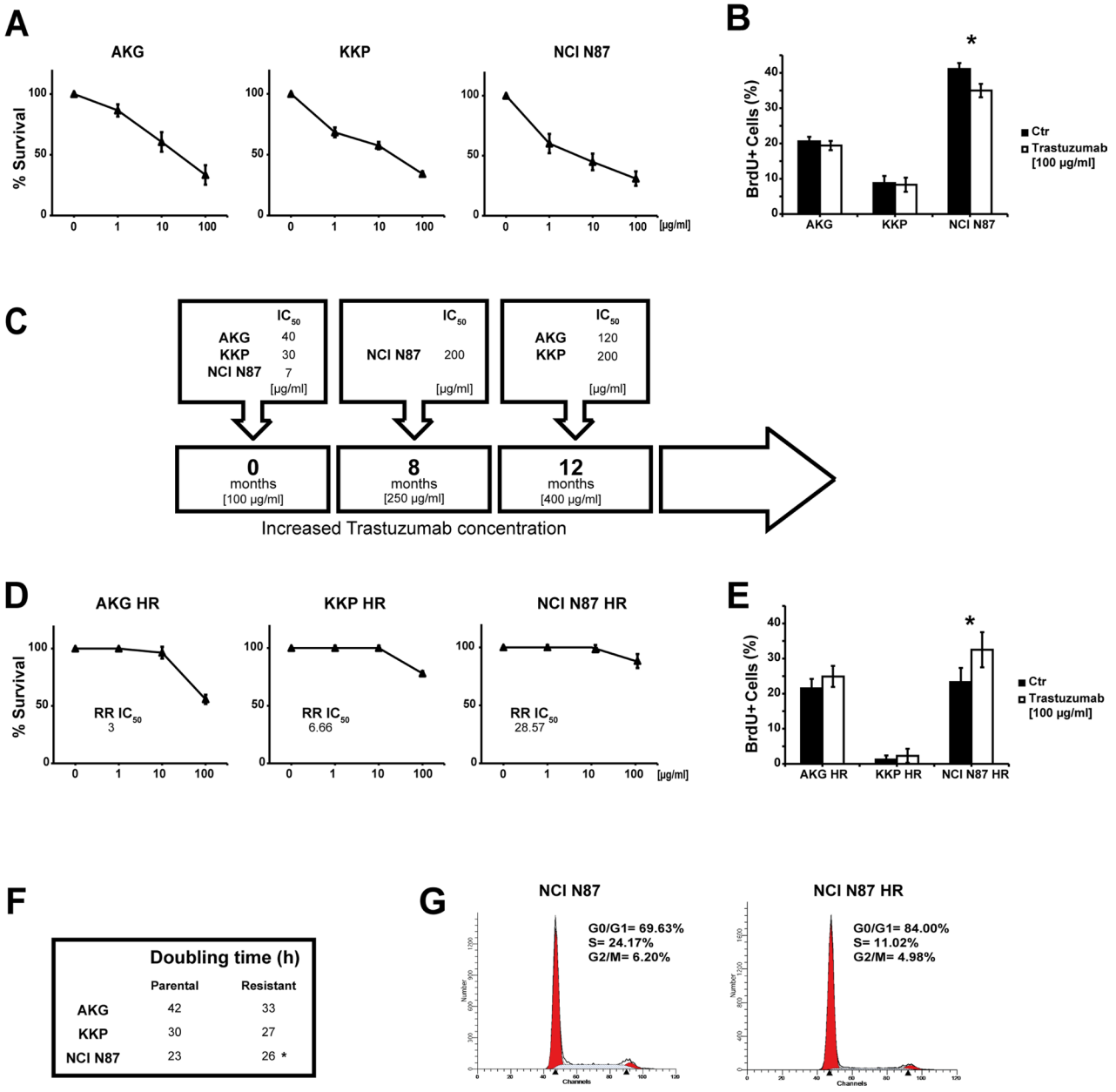


Figure 2: Induction of trastuzumab-resistance in gastric cancer cell lines. A. Trastuzumab sensitivity curves in parental cell lines evaluated by clonogenic assay. Each point indicates the mean of at least three experiments. The standard deviation never exceeded 5%. **B.** Percentage (median value) of BrdU-positive cells after trastuzumab treatment (100 µg/ml). Values are the mean ± SD of three independent experiments. * significance at $p<0.05$ by t-test. **C.** The induction timeline of trastuzumab resistance. Resistant cells were generated by continuous treatment with trastuzumab for more than 8 months. **D.** Trastuzumab sensitivity curves in resistant cell lines evaluated by clonogenic assay. Each point indicates the mean of at least three experiments. Standard deviation never exceeded 5%. **E.** Percentage (median value) of BrdU-positive cells after trastuzumab treatment (100 µg/ml). Values are the mean ± SD of three independent experiments. * significance at $p<0.05$ by t-test. **F.** Doubling times of parental and resistant cell lines. **G.** Cell cycle analysis of NCI N87 and NCI N87 HR by flow cytometry. Data are expressed as a percentage of distribution in each cell-cycle phase.

DISCUSSION

In gastric cancer patients, HER2/neu gene expression is an independent prognostic factor, and overexpression of the HER2 protein is correlated with poor prognosis and short-term survival [38, 39]. The effectiveness of trastuzumab and its subsequent approval as first-line treatment for HER2-overexpressing metastatic gastric cancer confirmed the importance of this receptor in gastric cancer. However, as already observed in other tumors, the majority of patients who initially show sensitivity to trastuzumab develop resistance within one year [17]. Aberrant HER2 activity and the activation of the HER2 receptor in human gastric tumors leads to receptor heterodimerization, mainly with HER3 and HER4 receptors [40], triggering a complex signal transduction cascade that modulates cancer cell survival, proliferation, mobility and invasiveness [41].

The main aim of our work was to investigate resistance mechanisms to trastuzumab in preclinical models of human gastric cancer. For this purpose we created trastuzumab resistant subclones starting from 3 HER2-overexpressing gastric cancer cell lines (AKG, KKP and NCI-N87 cells) with a high sensitivity to trastuzumab. These lines also expressed other HER family receptor members and showed genetic variants of HER2, HER3 and HER4.

After 8-12 months' exposure to increasing concentrations of trastuzumab, we successfully obtained trastuzumab-resistant AKG HR, KKP HR and NCI N87 HR subclones that grew in culture medium supplemented with high concentrations of the drug (up to 400 μg/

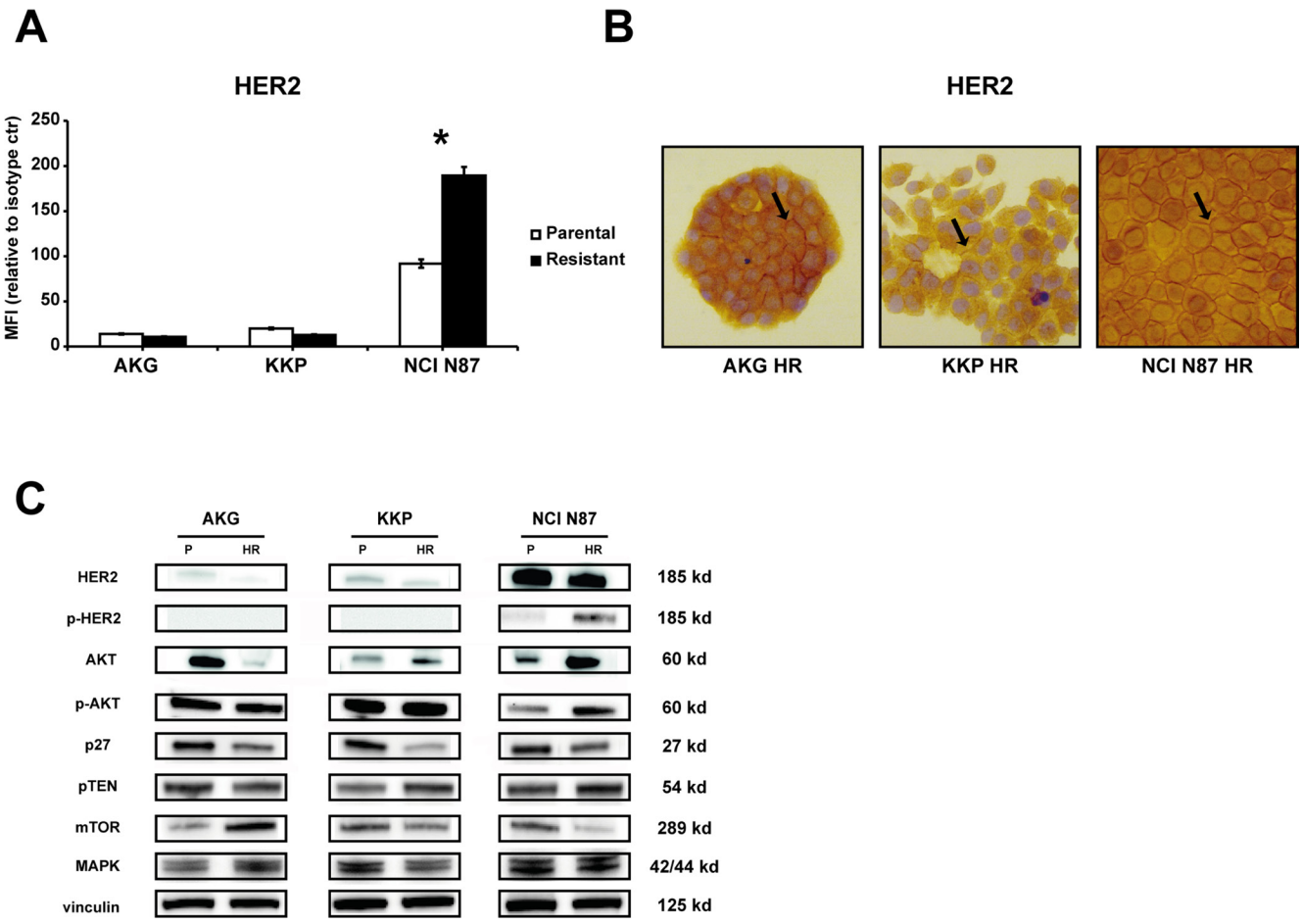


Figure 3: Characterization of trastuzumab targeting in resistant subclones: AKG HR, KKP HR and NCI N87 HR cells. A. HER2 protein levels on the cell surface were quantified by flow cytometry and expressed as mean fluorescence intensity (MFI) relative to isotype control. Statistical significance was denoted as * $p<0.05$. **B.** HER2 staining by IHC in gastric cancer cell lines. Sample reactivity was evaluated by light microscopy (× 200 magnification) by two independent observers. Marker positivity was evaluated in a semiquantitative manner, as described in the *Materials and Methods* section. **C.** Western blotting showed HER2, p-HER2, AKT, p-AKT, p27, PTEN, mTOR and MAPK protein expression in parental (P) and resistant (HR) cell lines. Vinculin expression indicated equal loading. All gels were run under the same experimental conditions and the experiments were repeated 3 times. The representative images were cropped and shown.

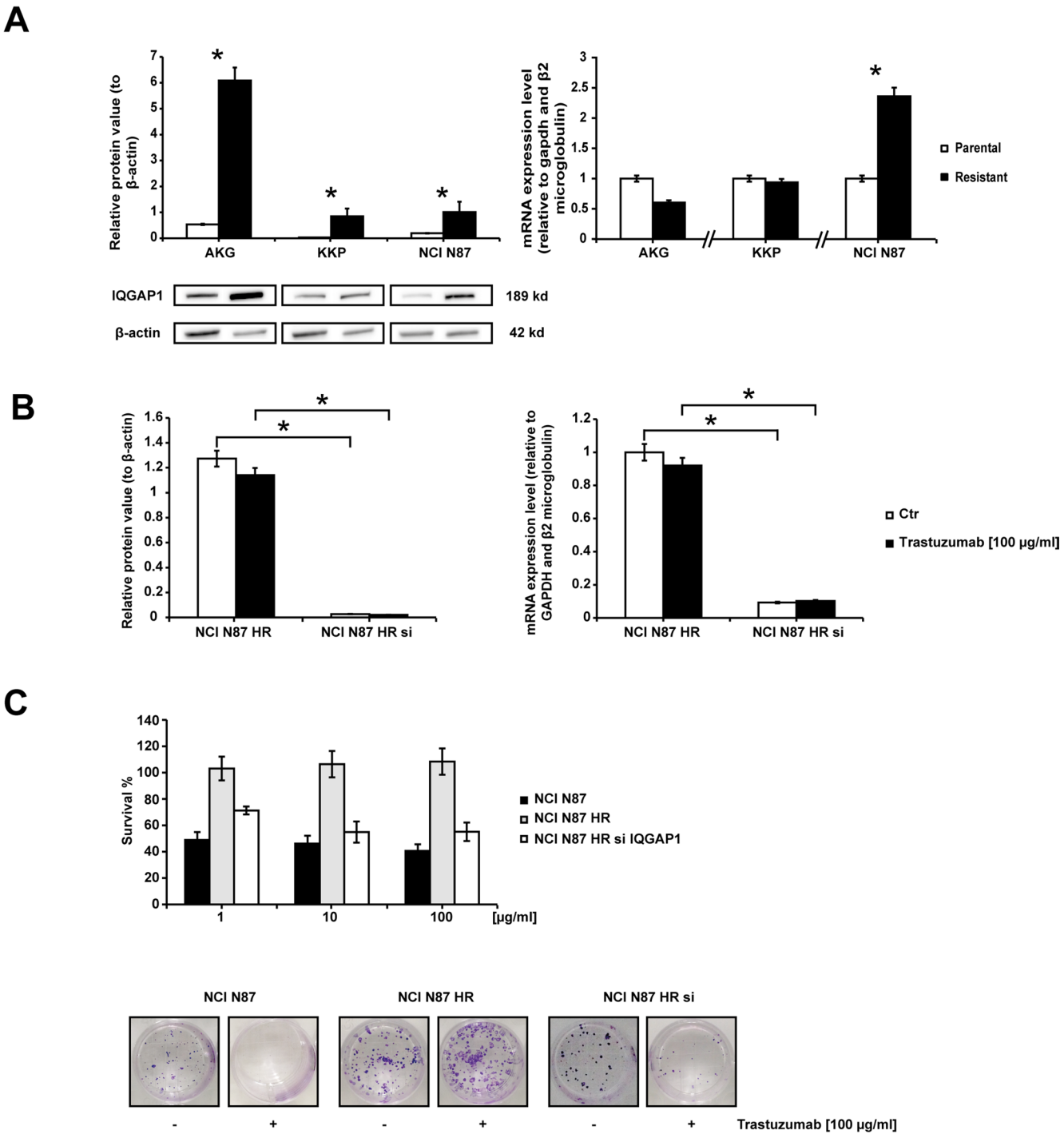


Figure 4: Trastuzumab sensitivity in subclone NCI N87 HR restored by IQGAP1 knockdown. A. IQGAP1 expression. The left panel shows the western blot of IQGAP1 in gastric cancer cell lines. β-actin expression indicated equal loading. Densitometric quantification of total IQGAP1 was calculated using Quantity One Software. All gels were run under the same experimental conditions and the experiments were repeated 3 times. The representative images were cropped and shown. The right graph shows IQGAP1 mRNA expression levels in resistant cells quantified with respect to parental cells and normalized to GAPDH and β2 microglobulin. Data are presented as mean +- SD. * $p<0.05$. **B.** IQGAP1 silencing. IQGAP1 protein expression levels (left graph) and IQGAP1 mRNA levels (right graph) in NCI N87 HR and NCI N87 HR IQGAP1-silenced before and after a 72 h-exposure to 100 µg/ml of trastuzumab. Western blot analysis of IQGAP1 was normalized to β-actin. IQGAP1 mRNA levels were measured by Real Time PCR and normalized to GAPDH and β2 microglobulin. Values are the mean ± SD of three independent experiments. **C.** The effect of IQGAP1 knockdown in NCI N87 HR cells was investigated in colony formation experiments carried out 72 h after transfection. The upper panel illustrates relative growth curves (means +- SD) and the bottom panel shows representative colony photos.

ml). Cell growth curves, BrdU incorporation and cell cycle analyses revealed that the biological features of trastuzumab-resistant cells differed from those of parental cells. In particular, the NCI N87 HR subclone grew more slowly, had a lower proliferative activity and showed a higher percentage of cells in G_0/G_1 phase and a lower percentage in S phase than NCI N87 cells. Similar results were reported by Zou et al. [42] who were the first group to obtain a trastuzumab-resistant subclone of NCI-N87 (N87 NCI/TR). In addition, our flow cytometry analyses revealed an increase in membrane HER2 levels only in the NCI N87 HR subclone with respect to its parental cells. We also detected a marked increase in p-HER2, AKT, p-AKT and MAPK protein levels and a reduction in p27 protein expression in NCI N87 HR cells. These results are in agreement with those from previous studies in which trastuzumab was reported to inhibit HER2+ tumor growth by stimulating endocytosis and degradation of the receptor, with subsequent impairment of downstream signaling through PI3K/AKT and MAPK cascades [43]. It was also recently reported that increased PI3K/AKT and MAPK cascade signaling inhibits p27 expression [43–46].

White et al. revealed that IQGAP1 governs trastuzumab function in HER2-overexpressing breast cancer. In particular, they reported that, in IQGAP1-silenced breast cancer cells, trastuzumab increased its capacity to decrease HER2 expression and HER2-stimulated activation of the PI3K/AKT cascade [37].

In the present work we showed that IQGAP1 knockdown in gastric cancer leads to the abrogation of trastuzumab resistance and to restored drug sensitivity. In particular, IQGAP1 protein levels in all trastuzumab resistant subclones were higher than those of parental cells, although no genetic variants were detected in the different cell lines used. However, the restoral of trastuzumab sensitivity through IQGAP1 silencing was only observed in NCI N87 HR cells which showed a strong activation of PI3K/AKT and MAPK signaling cascades, both features of IQGAP1-mediated trastuzumab resistance [37].

Multiple factors influence the resistance to molecular-targeting drugs and several studies have hypothesized that acquired resistance to trastuzumab might also be due to the alteration of the signaling cascade induced by HER3 and HER4 receptors [20, 23, 24]. Our models showed high levels of both receptors. In particular, AKG HR cell line expressed higher protein levels of HER3 than the parental line, mainly in the cell membrane. This finding is in agreement with data reported by Ma et al. [47] who considered HER3 overexpression to be a mechanism of resistance to trastuzumab. Increased HER3 expression also appears to promote both PI-3 K7Akt signaling and Scr kinase activity [48]. In our study, KKP HR cells expressed high levels of nuclear HER4, confirming recent findings about the involvement of HER4, especially in terms of its activation, cleavage and nuclear translocation, in resistance to trastuzumab in breast cancer cell lines [31, 49].

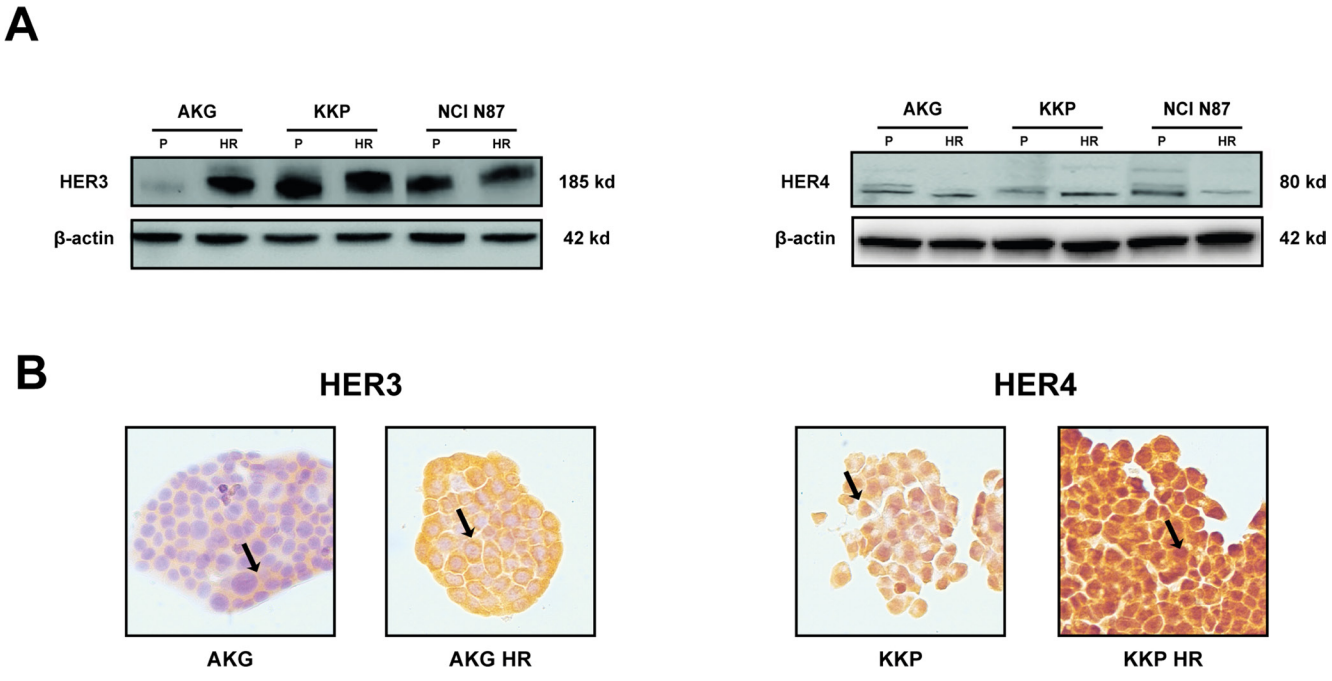


Figure 5: Characterization of resistant subclones: AKG HR, KKP HR and NCI N87 HR cells. A. HER3 and HER4 protein levels in parental cell lines (P) and their derivative subclones (HR) evaluated by western blot. The representative images were cropped and shown. Statistical significance was denoted as * $p<0.05$. **B.** HER3 and HER4 staining by IHC in AKG and KKP cells, respectively and in their derivative resistant subclones. Sample reactivity was evaluated by light microscopy (× 200 magnification) by two independent observers. Marker positivity was evaluated in a semiquantitative manner, as described in the *Materials and Methods* section.

We also investigated the presence of genetic variants potentially involved in acquired trastuzumab resistance. No genetic variants of the IQGAP1 gene were found in any of the studied cell lines. Furthermore, NGS analysis revealed that the resistant clone NCI N87 HR did not acquire additional gene variants with respect to the parental line. Conversely, KKP HR and AKG HR acquired one and two genetic variants, respectively, compared to their parental cell lines, and all were located in the C-terminal lobe of HER2, in a portion of the molecule called αF-helix. αF-helix, a highly hydrophobic component located in the middle of the C-lobe, plays a central role in anchoring key hydrophobic motifs. In particular, it forms the base of C- and R-spines, two motifs previously described by Kornev et al., [50, 51] which coordinate the N- and C-lobe movements of the kinase domain in the active conformation of the protein [52]. Given that both motifs are highly conserved through different types of active protein kinases, the assembly and anchorage of the spines to αF helix could be an important regulatory element. Furthermore, the activation loop, another important portion of the kinase domain, is firmly anchored to the hydrophobic αF helix. This is the most flexible part of the activation segment and requires phosphorylation to activate and increase the enzymatic activity of protein kinases, including ErbB family members [53, 54]. The genetic variants detected by our NGS analysis have never been reported before and may serve to maintain the active conformation of the HER2 receptor.

In conclusion, our study provides evidence of the existence of different mechanisms of resistance to trastuzumab in human gastric cancer. We also discovered that IQGAP1 is involved in trastuzumab resistance in gastric cancer cell lines and identified 2 new mutations of the HER2 gene that may be correlated with acquired resistance to the drug. Further studies are needed to explore these issues.

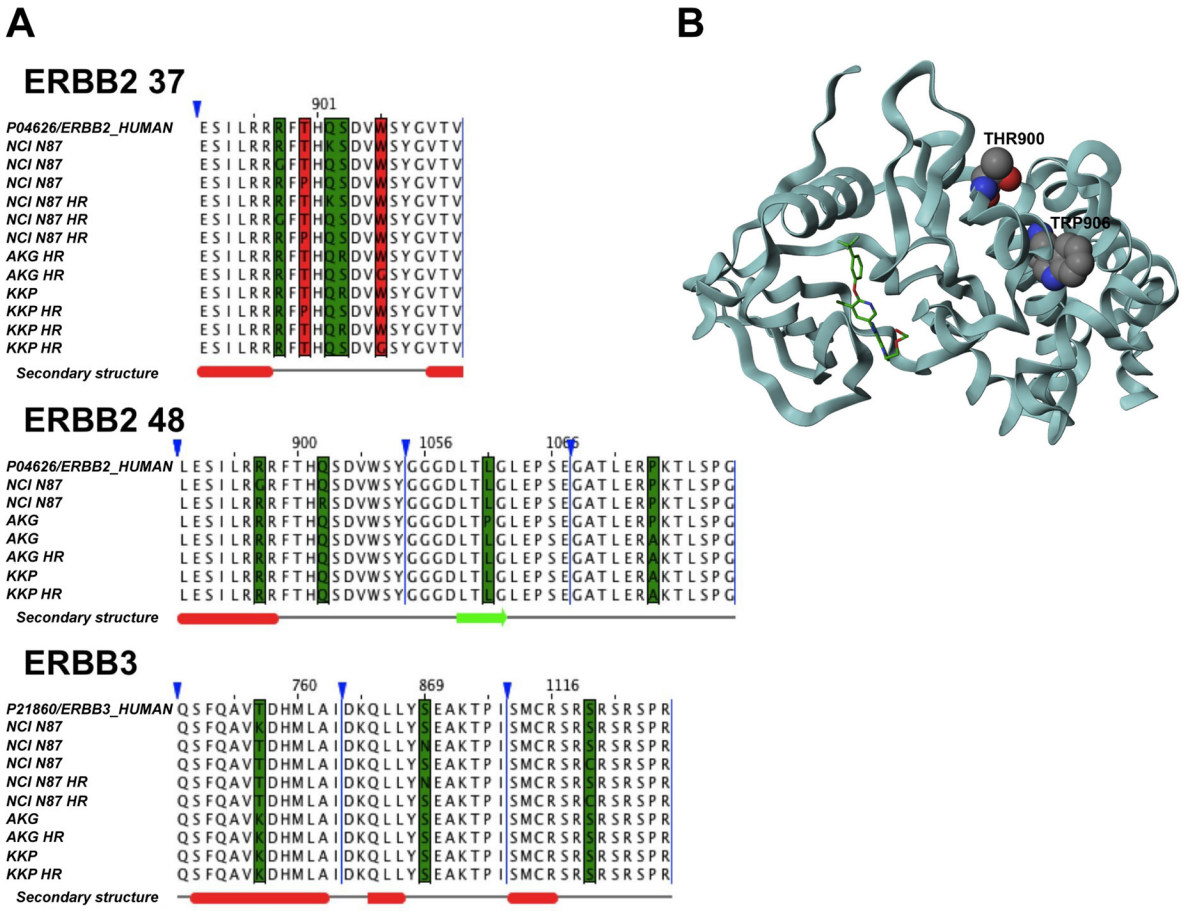


Figure 6: Comparison of sequence alignment between parental and trastuzumab resistant subclones. A. Sequence alignments of HER2 37, HER2 48 isoforms and HER3 highlights mutations in the cell lines under investigation. The red columns represent mutations associated with resistant cells, while green columns represent other kinds of mutations. Triangles denote hidden columns in the sequence. Secondary structure prediction denotes α-helix (red) and β-strand (green). **B.** The crystal structure of the kinase domain of HER2 (HER2-KD) in complex with SYR127063 (PDB code 3PP0). The ligand (green) binds to the HER2 ATP binding site. The mutations that confer resistance are located in residues 900 and 906 (in CPK notation) in a region located at the C-terminal lobe of the HER2-KD, which is predominantly α-helical.

MATERIALS AND METHODS

Cell lines

The study was performed on two cell lines (AKG, KKP) derived from human gastric adenocarcinoma (intestinal type), established and characterized in our laboratory [55, 56], and one commercial cell line obtained from a liver metastasis of a well differentiated gastric carcinoma (NCI-N87) and purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cell lines were maintained as a monolayer at 37°C and subcultured weekly. The culture medium was composed of DMEM/Ham's F12 (1:1) supplemented with fetal calf serum (10%), glutamine (2 mM), non-essential amino acids (1%) (Mascia Brunelli S.p.A., Milan, Italy), and insulin (10 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA). Cells were used in the exponential growth phase in all experiments.

Doubling time

For growth analysis, cells were plated in 12-well plates in triplicate at a concentration of 2×10^4 cells/well. Cells were collected and counted for the first 7 days after plating. Trastuzumab (*Herceptin*®) was purchased by the Oncology Pharmacy of our institute (IRST IRCCS). Proliferation doubling time was determined by the following formula: log2 (Cv/Cs), where Cv is the number of viable cells at harvest and Cs is the number of cells seeded. The sum of all previous population doublings determined the cumulative population doubling level at each passage. The Trypan blue exclusion test was used to evaluate the percentage of viable cells, which always exceeded 98% for the duration of the experiments.

Generation of trastuzumab-resistant subclones

We induce trastuzumab resistance by culturing trastuzumab-sensitive gastric cancer cell lines in the presence of progressively increasing doses of trastuzumab over a period of 12 months. The final concentration of trastuzumab used was 250 μg/ml for the trastuzumab-resistant subclone NCI-N87 HR and 400 μg/ml for the subclones AKG HR and KKP HR.

Immunohistochemistry

Cells were seeded in sterile culture slides (BD, Falcon, New Jersey, USA) and cultured in a humidified CO2 incubator for 72 h. They were then fixed in 4% (v/v) paraformaldehyde for 20 min and blocked for endogenous peroxidase activity with a 3% hydrogen peroxide solution. Antigen unmasking was performed using citrate buffer pH 6 for 40 min at 98.5°C. Rabbit monoclonal anti-human antibodies for HER3 (Cell Signaling Technology, Inc., Danvers, MA, USA) and HER4 (Santa Cruz Biotechnology, Dallas, Texas, USA) were used at a dilution of 1:250. Mouse monoclonal anti-human antibody for HER2 (Dako Corporation, Carpenteria, CA, USA) was used at a dilution of 1:100. Antibodies were incubated for 60 min at room temperature. Slides were washed with phosphate buffered saline (PBS), incubated with a universal biotinylated secondary antibody for 15 min and rinsed in PBS. They were then incubated with streptavidin-peroxidase conjugate (LSAB + Kit; Dako Corporation) for 15 min. Slides were rinsed again in PBS and antibody binding was detected by staining with diaminobenzidine/hydrogen peroxidase chromogen solution (DAB + liquid substrate–chromogen solution; Dako Corporation). Finally, the sections were rinsed in deionized water, counterstained by Mayer's hematoxylin, and mounted by Eukitt (Bio-Optica, Milan, Italy). Sample reactivity was evaluated by light microscopy (× 200) by two independent observers. Marker positivity was evaluated semi-quantitatively. Staining was evaluated in terms of the localization (nuclear, cytoplasmatic and membrane) of the selected proteins and the percentage of positive cells.

Clonogenic assay

Following a 72-h exposure to trastuzumab, 500 cells were seeded in 10-cm^2 dishes in 500 ml of medium. After 14 days, the resulting colonies were fixed and stained using 0.5% crystal violet in 25% methanol; colonies with more than 50 cells were quantified under inverted microscope (Olympus IX51 microscope, Olympus Corporation, Tokyo, Japan) by two independent observers. Five series of samples were prepared for each treatment dose [57].

Cytofluorimetric analysis

Flow cytometric analysis was performed using a FACS Canto flow cytometer (Becton Dickinson, San Diego, CA, USA) equipped with 488 nm (blue) and 633 (red) lasers. Data acquisition and analysis were performed using FACSDiva (Becton Dickinson) and ModFit 2.0 (DNA Modelling System, Verity Software House, Inc., Topsham, ME, USA). Samples were run in triplicate and 10,000 events were collected for each replica. Data were the average of three experiments, with errors under 5%.

Cell cycle distribution

After exposure to trastuzumab, cells were fixed in 70% ethanol, stained with propidium iodide (10 mg/ml, MP Biomedicals, Verona, Italy), RNAse (10 kunits/ml, Sigma-Aldrich) and NP40 (0.01%, Sigma Aldrich) overnight at 37C° in the dark, and analyzed by flow

cytometry. Data were expressed as fractions of cells in the different cycle phases.

Bromodeoxyuridine (BrdU) assay

After treatment with trastuzumab100 μg/ml, the cell culture medium was supplemented with 60 μM of BrdU and incubated for an additional 5 h. At the end of the incubation time, cells were fixed, incubated for 25 min with 2M of HCL and then washed with borax 0.1 M. Samples were incubated with anti-BrdU antibody 1:1000 (Sigma-Aldrich) for 60 min. After incubation with FITC-conjugated antibody (goat anti-mouse 1:250, Dako Corporation), cells were stained with 5 mg/ml of propidium iodide for 2 h at 4°C before flow cytometry acquisition.

Immunophenotypic analysis

Cells were fixed and immunophenotyping was performed using anti-HER2 (1:100) (Invitrogen, Life Technologies, Monza, Italy) antibody for 30 min at 4°C. After three washes, cells were incubated with RPE-conjugated goat anti-rabbit antibody 1:250 (Invitrogen) for 60 min in the dark. Appropriate isotype control was included for each sample.

Western blot

Cells were treated according to the previously described western blot procedure [58]. The following primary antibodies were used: anti-IQGAP1 (1:400), anti-HER2 (1:800) (Invitrogen, Thermo Fisher Scientific); anti-actin (1:1000) (Sigma-Aldrich); anti-HER4 (1:1000) (Abcam, Cambridge, UK); anti-vinculin (1:1000) (Thermo Fisher Scientific); anti-p27 (1:1000) (BD Biosciences, Milan, Italy); anti-HER3 (1:1000), anti-PTEN (1:1000), anti-mTOR (1:600), anti-MAPK (1:1000), anti-AKT (1:1000), anti-phospho-AKT (Ser^{473}) (1:1000) and anti-phospho-HER2 ($Tyr^{1221/1222}$) (1:1000) (Cell Signaling Technology). Precision Plus Protein™ WesternC™ Standards were used as molecular weight standards (Bio-Rad #161-0376). Quantity One Software (Bio-Rad) was used for analysis.

Small interfering RNA transfection

Silencer® Select Validated siRNA (Ambion, Carlsbad, CA, USA) was utilized for IQGAP1 silencing. A validated Universal Negative ControlTM (Invitrogen) was used as a control for transfection. The siRNA oligonucleotide showing the highest knockdown efficiency of IQGAP1 mRNA in the NCIN87cell line was used for the experiments. Cells were treated according to the previously described procedure [59]**.** Cells were treated after 72 h.

RNA extraction and real-time RT-PCR

Total RNA was extracted from cell lines using TRIzol® reagent according to the manufacturer's instructions (Invitrogen). Reverse transcription (RT) reactions were performed using an iScript TM cDNA Synthesis kit (Bio-Rad Laboratories). mRNA expression was analyzed by quantitative Real-Time PCR using the 7500 Real Time PCR system (Applied Biosystems, Thermo Fisher Scientific). The following TaqMan assays (Applied Biosystems, Thermo Fisher Scientific) were used: IQGAP1 (Hs00896595_m1) and its relative gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH Hs03929097_g1) and β2-microglobulin (Hs00984230_m1). Data showed the average of triplicates ± standard deviation (SD) and were representative of three independent experiments.

DNA extraction and next-generation sequencing (NGS)

Genomic DNA was extracted using QIAamp DNA MiniKit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. DNA quality was evaluated with High Sensitivity DNA Analysis Kit on Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and quantified using Qubit dsDNA BR Assay Kit (Invitrogen). DNA from these cell lines was subjected to target sequencing using a custom panel purchased from Agilent Technologies.

Sequences were achieved by designing primers to capture the entire coding region, exon-intron boundaries (±10 bp) and the promoter region of 4 genes using the Agilent HaloPlex Target Enrichment System (Table 3). Quantified libraries were sequenced on the Illumina MiSeq platform (San Diego, CA, USA) using 2 X 151 bp in pair-end mode and run on an Illumina V2 sequencing flow cell.

Raw demultiplexed reads from MiSeq sequencer were aligned against the human reference genome hg19 with BWA MEM [60]. GATK version 3.2.2 was used to recalibrate base qualities and realign aligned reads around indels [61]. Regions with coverage of less than or equal to 200x were discarded for downstream analyses. Somatic variant analysis was used to detect mutations: single nucleotide variants (SNVs) were identified using MuTect version 1.1.7 with standard parameters, and GATK IndelGenotyperV2 (with minFraction = 0.01 and minCnt = 5) was used to detect Indels. Genomic and functional annotation of detected variants was made by Annovar [62, 63]. Coverage statistics were performed by Depth of Coverage utility of GATK. BASH and R custom scripts were used to obtain the list of low coverage (200X) regions per sample.

Sequencing runs produced a total of 36,646,538 reads, of which 94.6% mapped on the hg19 human

Table 3: Genes selected for next generation sequencing (NGS) analysis

Gene	entrez ID	cytoband	genomic coordinates	number of exons
HER2	2064	17q12	chr17:37844316-37884317	All(31)+5'UTR
HER3	2065	12q13.2	chr12:56473788-56495859	All(28)+5'UTR
HER4	2066	2q34	chr2:212248319-213403372	All(28)+5'UTR
IQGAP1	8826	15q26.1	chr15:90931452-91043360	All(38)

reference genome, with a median coverage depth of 2779, 73X per sample. Only candidate somatic alterations with a read depth of at least 200 and a mutant allele fraction >1% were considered. Sequence results from parental and resistant subclones were compared to identify putative somatic mutations at the basis of the development of resistance.

Molecular modeling

The UniProt sequences P04626, P21860 and P46940 were taken as a reference for HER2, HER3 ad IQGAP1, respectively. Jalview V. 2.8.2 was used to obtain sequence alignments. The web service Clustal Omega was used with its default settings. Percentage identity coloring was used to generate figures. Three-dimensional figures were generated using an academic version of Maestro software V. 10.1.013.

Statistical analysis

All experiments were performed at least three times. Quantifiable data were derived from three independent experiments. Statistical analysis was carried out using GRAPH PAD PRISM 5.0 software by applying the Student t test for 2-group comparisons. Differences were considered significant at $p<0.05$.

ACKNOWLEDGMENTS

The authors thank Prof. Romano Danesi for his invaluable scientific contribution and Ursula Elbling for editing the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA: a cancer journal for clinicians. 2015.
2. LAUREN P. The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. an Attempt at a Histo-Clinical Classification. Acta Pathologica et Microbiologica Scandinavica. 1965; 64: 31-49.
3. Cunningham D, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, et al. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. The New England journal of medicine. 2006; 355: 11-20.
4. Smyth EC, Cunningham D. Gastric cancer in 2012: Defining treatment standards and novel insights into disease biology. Nature reviews. Clinical oncology. 2013; 10: 73-74.
5. Shah MA, Schwartz GK. Treatment of metastatic esophagus and gastric cancer. Seminars in oncology. 2004; 31: 574-587.
6. Ohtsu A, Shimada Y, Shirao K, Boku N, Hyodo I, Saito H, Yamamichi N, Miyata Y, Ikeda N, Yamamoto S, Fukuda H, Yoshida S, Japan Clinical Oncology Group Study (JCOG9205). Randomized phase III trial of fluorouracil alone versus fluorouracil plus cisplatin versus uracil and tegafur plus mitomycin in patients with unresectable, advanced gastric cancer: The Japan Clinical Oncology Group Study (JCOG9205). Journal of clinical oncology. 2003; 21: 54-59.
7. Dickson JL, Cunningham D. Systemic treatment of gastric cancer. European journal of gastroenterology & hepatology. 2004; 16: 255-263.
8. Yano T, Doi T, Ohtsu A, Boku N, Hashizume K, Nakanishi M, Ochiai A. Comparison of HER2 gene amplification assessed by fluorescence in situ hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer. Oncology reports. 2006; 15: 65-71.
9. Kim MA, Lee HJ, Yang HK, Bang YJ, Kim WH. Heterogeneous amplification of ERBB2 in primary lesions is responsible for the discordant ERBB2 status of primary and metastatic lesions in gastric carcinoma. Histopathology. 2011; 59: 822-831.
10. Marx AH, Tharun L, Muth J, Dancau AM, Simon R, Yekebas E, Kaifi JT, Mirlacher M, Brummendorf TH,

Bokemeyer C, Izbicki JR, Sauter G. HER-2 amplification is highly homogenous in gastric cancer. Human pathology. 2009; 40: 769-777.

11. Fassan M, Mastracci L, Grillo F, Zagonel V, Bruno S, Battaglia G, Pitto F, Nitti D, Celiento T, Zaninotto G, Fiocca R, Rugge M. Early HER2 dysregulation in gastric and oesophageal carcinogenesis. Histopathology. 2012; 61: 769-776.
12. Fassan M, Pizzi M, Realdon S, Balistreri M, Guzzardo V, Zagonel V, Castoro C, Mastracci L, Farinati F, Nitti D, Zaninotto G, Rugge M. The HER2-miR125a5p/miR125b loop in gastric and esophageal carcinogenesis. Human pathology. 2013; 44: 1804-1810.
13. Jorgensen JT, Hersom M. HER2 as a Prognostic Marker in Gastric Cancer - A Systematic Analysis of Data from the Literature. Journal of Cancer. 2012; 3: 137-144.
14. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet. 2010; 376: 687-697.
15. Satoh T, Xu RH, Chung HC, Sun GP, Doi T, Xu JM, Tsuji A, Omuro Y, Li J, Wang JW, Miwa H, Qin SK, Chung IJ, et al. Lapatinib plus paclitaxel versus paclitaxel alone in the second-line treatment of HER2-amplified advanced gastric cancer in Asian populations: TyTAN--a randomized, phase III study. Journal of clinical oncology. 2014; 32: 2039-2049.
16. Shimoyama S. Unraveling trastuzumab and lapatinib inefficiency in gastric cancer: Future steps (Review). Molecular and clinical oncology. 2014; 2: 175-181.
17. Okines AF, Cunningham D. Trastuzumab: a novel standard option for patients with HER-2-positive advanced gastric or gastro-oesophageal junction cancer. Therapeutic advances in gastroenterology. 2012; 5: 301-318.
18. Razis E, Bobos M, Kotoula V, Eleftheraki AG, Kalofonos HP, Pavlakis K, Papakostas P, Aravantinos G, Rigakos G, Efstratiou I, Petraki K, Bafaloukos D, Kostopoulos I, et al. Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic breast cancer. Breast cancer research and treatment. 2011; 128: 447-456.
19. Esteva FJ, Guo H, Zhang S, Santa-Maria C, Stone S, Lanchbury JS, Sahin AA, Hortobagyi GN, Yu D. PTEN, PIK3CA, p-AKT, and p-p70S6K status: association with trastuzumab response and survival in patients with HER2-positive metastatic breast cancer. The American journal of pathology. 2010; 177: 1647-1656.
20. Zhang S, Huang WC, Li P, Guo H, Poh SB, Brady SW, Xiong Y, Tseng LM, Li SH, Ding Z, Sahin AA, Esteva FJ, Hortobagyi GN, et al. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. Nature medicine. 2011; 17: 461-469.
21. Gallardo A, Lerma E, Escuin D, Tibau A, Munoz J, Ojeda B, Barnadas A, Adrover E, Sanchez-Tejada L, Giner D, Ortiz-Martinez F, Peiro G. Increased signalling of EGFR and IGF1R, and deregulation of PTEN/PI3K/Akt pathway are related with trastuzumab resistance in HER2 breast carcinomas. British journal of cancer. 2012; 106: 1367-1373.
22. Pastuskovas CV, Mundo EE, Williams SP, Nayak TK, Ho J, Ulufatu S, Clark S, Ross S, Cheng E, Parsons-Reponte K, Cain G, Van Hoy M, Majidy N, et al. Effects of anti-VEGF on pharmacokinetics, biodistribution, and tumor penetration of trastuzumab in a preclinical breast cancer model. Molecular cancer therapeutics. 2012; 11: 752-762.
23. Garrett JT, Olivares MG, Rinehart C, Granja-Ingram ND, Sanchez V, Chakrabarty A, Dave B, Cook RS, Pao W, McKinely E, Manning HC, Chang J, Arteaga CL. Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108: 5021-5026.
24. Hanker AB, Pfefferle AD, Balko JM, Kuba MG, Young CD, Sanchez V, Sutton CR, Cheng H, Perou CM, Zhao JJ, Cook RS, Arteaga CL. Mutant PIK3CA accelerates HER2-driven transgenic mammary tumors and induces resistance to combinations of anti-HER2 therapies. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110: 14372-14377.
25. Xia W, Petricoin EF, 3rd, Zhao S, Liu L, Osada T, Cheng Q, Wulfkuhle JD, Gwin WR, Yang X, Gallagher RI, Bacus S, Lyerly HK, Spector NL. An heregulin-EGFR-HER3 autocrine signaling axis can mediate acquired lapatinib resistance in HER2+ breast cancer models. Breast cancer research: BCR. 2013; 15: R85.
26. Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nature clinical practice.Oncology. 2006; 3: 269-280.
27. Mohd Sharial MS, Crown J, Hennessy BT. Overcoming resistance and restoring sensitivity to HER2-targeted therapies in breast cancer. Ann Ocol. 2012; 23: 3007-3016.
28. Kruser TJ, Wheeler DL. Mechanisms of resistance to HER family targeting antibodies. Experimental cell research. 2010; 316: 1083-1100.
29. Vlacich G, Coffey RJ. Resistance to EGFR-targeted therapy: a family affair. Cancer cell. 2011; 20: 423-425.
30. Li X, Duan Y, Qiao C, Zhou T, Yu M, Geng J, Feng J, Shen B, Lv M, Li Y. Anti-HER3 Monoclonal Antibody Inhibits Acquired Trastuzumab-Resistant Gynecologic Cancers. Technology in cancer research & treatment. 2015.

31. Mohd Nafi SN, Generali D, Kramer-Marek G, Gijsen M, Strina C, Cappelletti M, Andreis D, Haider S, Li JL, Bridges E, Capala J, Ioannis R, Harris AL, et al. Nuclear HER4 mediates acquired resistance to trastuzumab and is associated with poor outcome in HER2 positive breast cancer. Oncotarget. 2014; 5: 5934-5949. doi: 10.18632/oncotarget.1904.

32. White CD, Brown MD, Sacks DB. IQGAPs in cancer: a family of scaffold proteins underlying tumorigenesis. FEBS letters. 2009; 583: 1817-1824.

33. Patel V, Hood BL, Molinolo AA, Lee NH, Conrads TP, Braisted JC, Krizman DB, Veenstra TD, Gutkind JS. Proteomic analysis of laser-captured paraffin-embedded tissues: a molecular portrait of head and neck cancer progression. Clinical cancer research. 2008; 14: 1002-1014.

34. White CD, Khurana H, Gnatenko DV, Li Z, Odze RD, Sacks DB, Schmidt VA. IQGAP1 and IQGAP2 are reciprocally altered in hepatocellular carcinoma. BMC gastroenterology. 2010; 10: 125-230X-10-125.

35. Zhou R, Skalli O. Identification of cadherin-11 down-regulation as a common response of astrocytoma cells to transforming growth factor-alpha. Differentiation; research in biological diversity. 2000; 66: 165-172.

36. Walch A, Seidl S, Hermannstadter C, Rauser S, Deplazes J, Langer R, von Weyhern CH, Sarbia M, Busch R, Feith M, Gillen S, Hofler H, Luber B. Combined analysis of Rac1, IQGAP1, Tiam1 and E-cadherin expression in gastric cancer. Modern pathology. 2008; 21: 544-552.

37. White CD, Li Z, Dillon DA, Sacks DB. IQGAP1 protein binds human epidermal growth factor receptor 2 (HER2) and modulates trastuzumab resistance. The Journal of biological chemistry. 2011; 286: 29734-29747.

38. Kim JW, Im SA, Kim M, Cha Y, Lee KH, Keam B, Kim MA, Han SW, Oh DY, Kim TY, Kim WH, Bang YJ. The prognostic significance of HER2 positivity for advanced gastric cancer patients undergoing first-line modified FOLFOX-6 regimen. Anticancer Research. 2012; 32: 1547-1553.

39. Bayrak M, Olmez OF, Kurt E, Cubukcu E, Evrensel T, Kanat O, Manavoglu O. Prognostic significance of c-erbB2 overexpression in patients with metastatic gastric cancer. Clinical & translational oncology. 2013; 15: 307-312.

40. He XX, Ding L, Lin Y, Shu M, Wen JM, Xue L. Protein expression of HER2, 3, 4 in gastric cancer: correlation with clinical features and survival. Journal of clinical pathology. 2015.

41. Normanno N, Bianco C, Strizzi L, Mancino M, Maiello MR, De Luca A, Caponigro F, Salomon DS. The ErbB receptors and their ligands in cancer: an overview. Current Drug Targets. 2005; 6: 243-257.

42. Zuo Q, Liu J, Zhang J, Wu M, Guo L, Liao W. Development of trastuzumab-resistant human gastric carcinoma cell lines and mechanisms of drug resistance. Scientific reports. 2015; 5: 11634.

43. Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Seminars in oncology. 1999; 26: 60-70.

44. Lane HA, Beuvink I, Motoyama AB, Daly JM, Neve RM, Hynes NE. ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency. Molecular and cellular biology. 2000; 20: 3210-3223.

45. Neve RM, Sutterluty H, Pullen N, Lane HA, Daly JM, Krek W, Hynes NE. Effects of oncogenic ErbB2 on G1 cell cycle regulators in breast tumour cells. Oncogene. 2000; 19: 1647-1656.

46. Baselga J, Albanell J, Molina MA, Arribas J. Mechanism of action of trastuzumab and scientific update. Seminars in oncology. 2001; 28: 4-11.

47. Ma J, Lyu H, Huang J, Liu B. Targeting of erbB3 receptor to overcome resistance in cancer treatment. Molecular cancer. 2014; 13: 105-4598-13-105.

48. Kruser TJ, Wheeler DL. Mechanisms of resistance to HER family targeting antibodies. Experimental cell research. 2010; 316: 1083-1100.

49. Canfield K, Li J, Wilkins OM, Morrison MM, Ung M, Wells W, Williams CR, Liby KT, Vullhorst D, Buonanno A, Hu H, Schiff R, Cook RS, et al. Receptor tyrosine kinase ERBB4 mediates acquired resistance to ERBB2 inhibitors in breast cancer cells. Cell cycle (Georgetown, Tex.). 2015; 14: 648-655.

50. Kornev AP, Haste NM, Taylor SS, Eyck LF. Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. Proceedings of the National Academy of Sciences of the United States of America. 2006; 103: 17783-17788.

51. Kornev AP, Taylor SS, Ten Eyck LF. A helix scaffold for the assembly of active protein kinases. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105: 14377-14382.

52. Qiu C, Tarrant MK, Choi SH, Sathyamurthy A, Bose R, Banjade S, Pal A, Bornmann WG, Lemmon MA, Cole PA, Leahy DJ. Mechanism of activation and inhibition of the HER4/ErbB4 kinase. Structure (London, England: 1993). 2008; 16: 460-467.

53. Adams JA. Activation loop phosphorylation and catalysis in protein kinases: is there functional evidence for the autoinhibitor model?. Biochemistry. 2003; 42: 601-607.

54. Lew J. MAP kinases and CDKs: kinetic basis for catalytic activation. Biochemistry. 2003; 42: 849-856.

55. Bertoni L, Zoli W, Mucciolo E, Ricotti L, Nergadze S, Amadori D, Giulotto E. Different genome organization in

two new cell lines established from human gastric carcinoma. Cancer genetics and cytogenetics. 1998; 105: 152-159.

56. Zoli W, Ricotti L, Lenzi L, Roncuzzi L, Zini N, Amadori D, Gruppioni R, Sensi A, Gasperi-Campani A. Molecular genetics and in vitro sensitivity of a new human cell line, KKP, from a gastric adenocarcinoma. Cancer genetics and cytogenetics. 1998; 105: 43-49.
57. Leonard CE, Chan DC, Chou TC, Kumar R, Bunn PA. Paclitaxel enhances in vitro radiosensitivity of squamous carcinoma cell lines of the head and neck. Cancer research. 1996; 56: 5198-5204.
58. Pignatta S, Arienti C, Zoli W, Di Donato M, Castoria G, Gabucci E, Casadio V, Falconi M, De Giorgi U, Silvestrini R, Tesei A. Prolonged exposure to (R)-bicalutamide generates a LNCaP subclone with alteration of mitochondrial genome. Molecular and cellular endocrinology. 2014; 382: 314-324.
59. Arienti C, Tesei A, Carloni S, Ulivi P, Romeo A, Ghigi G, Menghi E, Sarnelli A, Parisi E, Silvestrini R, Zoli W. SLUG silencing increases radiosensitivity of melanoma cells in vitro. Cellular oncology (Dordrecht). 2013; 36: 131-139.
60. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics (Oxford, England). 2009; 25: 1754-1760.
61. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome research. 2010; 20: 1297-1303.
62. Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, Gabriel S, Meyerson M, Lander ES, Getz G. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nature biotechnology. 2013; 31: 213-219.
63. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic acids research. 2010; 38: e164.

Lung Cancer 96 (2016) 52–55

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Morphological and genetic heterogeneity in multifocal lung adenocarcinoma: The case of a never-smoker woman

Laura Bonanno[a,*], Fiorella Calabrese[b], Giorgia Nardo[c], Daniele Calistri[d], Michela Tebaldi[d], Gianluca Tedaldi[d], Valentina Polo[a,e], Stefania Vuljan[b], Adolfo Favaretto[a], PierFranco Conte[a,e], Alberto Amadori[a,e], Federico Rea[f], Stefano Indraccolo[c]

[a] Medical Oncology 2, Istituto Oncologico Veneto IRCCS, via Gattamelata 64, 35128 Padova, Italy
[b] Department of Cardiothoracic and Vascular Sciences, University of Padova, via Giustiniani 2, 35128 Padova, Italy
[c] Immunology and Molecular Oncology Unit, Istituto Oncologico Veneto IRCCS, via Gattamelata 64, 35128 Padova, Italy
[d] Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, via Maroncelli 40, 47014 Meldola, FC, Italy
[e] Department of Surgery, Oncology and Gastroenterology, University of Padova, via Giustiniani 2, 35124 Padova, Italy
[f] Thoracic Surgery Unit, Department of Cardiothoracic and Vascular Sciences, University of Padova, via Giustiniani 2, 35128 Padova, Italy

ARTICLE INFO

Article history:
Received 12 January 2016
Received in revised form 23 March 2016
Accepted 24 March 2016

Keywords:
Non-small cell lung cancer
Pathology
Next generation sequencing
EGFR
Multifocal lung tumors
Mutate allele frequency

ABSTRACT

Discrimination of multifocal primary lung cancers from lung metastases is crucial to allow for an appropriate clinical management. We report here a case of multifocal lung adenocarcinomas with different morphological and molecular patterns. Radical surgery of one lung nodule was performed at the time of diagnosis, and subsequently on two other lung nodules. At the time of distant relapse, biopsy was repeated for molecular characterization. The patient was treated with EGFR tyrosine kinase inhibitor according to the detection of EGFR exon 21 mutation in metastatic sample and in one of the three lung tumors, characterized by lower mutated allele frequency. The progression free survival was three months according to radiological criteria and the treatment was provided for six months, until clinical progression. Following the assessment of EGFR mutations by pyrosequencing, tumor samples were analyzed by a 30-gene next generation sequencing (NGS) panel, allowing to study intra- and inter-tumor heterogeneity and to confirm the three lung tumors as independent. Different molecular profiles of synchronous tumors and identical EGFR, PIK3CA and TP53 mutations in one of three primary lung tumors and the metachronous metastasis were identified.

In conclusion, morphological and molecular characterization of multiple lung nodules by NGS may help to define synchronous and metachronous adenocarcinomas, thus affecting surgical indication and systemic treatment. Intratumor heterogeneity may be associated with differential sensitivity to targeted treatment.

1. Background

The differentiation of multiple primary lung tumors from metastases is a key issue to allow for proper clinical management, since it affects the possibility of radical-intent local treatment. Up to 8% of non-small cell lung cancers (NSCLCs) present as multifocal [1,2].

In addition to more standardized clinical and pathological criteria [2–4], molecular profiling may help in characterizing multifocal tumors [5–7] and it allows to identify sub-clonal genetic alterations. A previous report showed higher frequency of molecular clonality among multiple lung tumors supposed to be synchronous lesions [8]. Overall, previous experiences indicated that more than 30% of multifocal NSCLCs showed different genetic profiles, consistently with biological independence of the tumors [9,10]. Next

Abbreviations: NSCLCs, non-small cell lung cancers; NGS, next generation sequencing; CT, computed tomography; ^{18}FDG-PET, 18-Fluoro-D-glucose positron emission tomography; PS, performance status; ECOG, eastern cooperative oncology group.

* Corresponding author.
E-mail addresses: laura.bonanno@ioveneto.it, laura.bonanno@hotmail.it (L. Bonanno), fiorella.calabrese@unipd.it (F. Calabrese), giorgia.nardo@gmail.com (G. Nardo), daniele.calistri@irst.emr.it (D. Calistri), michela.tebaldi@irst.emr.it (M. Tebaldi), gianluca.tedaldi@irst.emr.it (G. Tedaldi), valentina.polo@ioveneto.it (V. Polo), stefvu@hotmail.it (S. Vuljan), adolfo.favaretto@ioveneto.it (A. Favaretto), pierfranco.conte@unipd.it (P. Conte), albido@unipd.it (A. Amadori), federico.rea@unipd.it (F. Rea), stefano.indraccolo@unipd.it (S. Indraccolo).

http://dx.doi.org/10.1016/j.lungcan.2016.03.009
0169-5002/

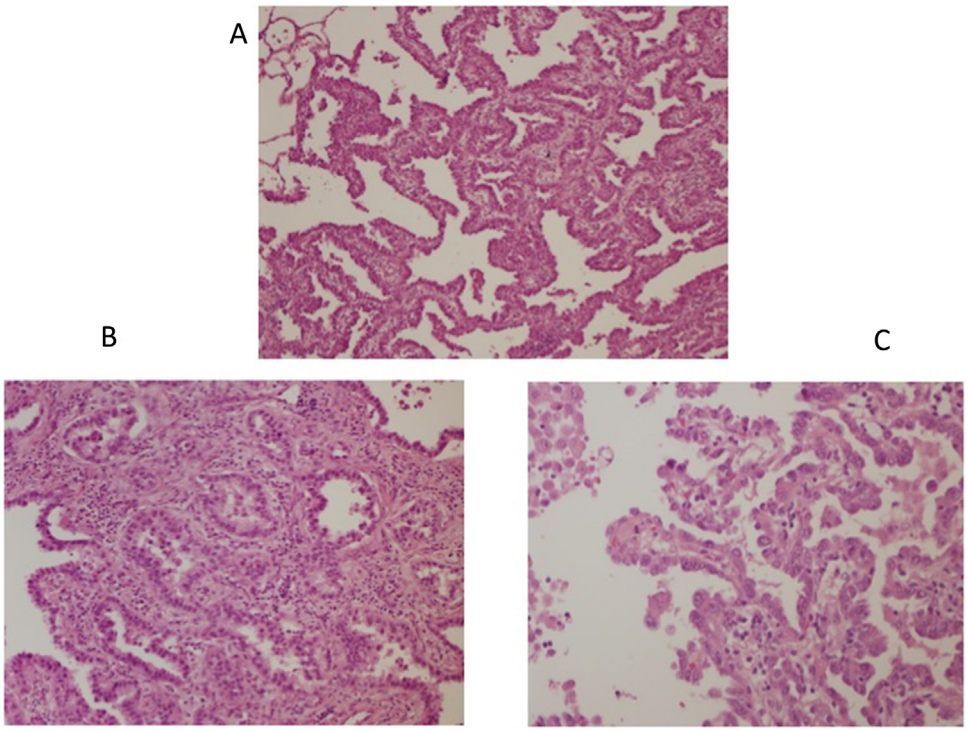


Fig. 1. Hematoxylin-eosin staining of lung nodules.
Hematoxylin-eosin staining of middle lobe nodule showing prevalent lepidic growth (A) and of the two left upper lobe nodules showing prevalent acinar (B) and papillary (C) morphology.

generation sequencing (NGS) is a method to perform multiple-gene assessment and may provide further information in molecular characterization of multifocal lung tumors [11,12].

After radical resection, the probability of developing a second lung cancer is approximately 1% per year [13] and the presence of multiple lung tumors is associated with chronic exposure to smoke-related carcinogens, consistently with the "field cancerization" theory, whereas it is rare in never-smokers [14]. Anyway, in this group of patients, the identification of different molecular pattern may have increased role also in metastatic disease, since it may affect systemic treatment decision-making and potentially change the outcome and the quality of life of metastatic patients [15].

2. Case presentation

In January 2011, a 68 year-old never-smoker female was diagnosed with three lung nodules at computed tomography (CT)-scan. 18-Fluoro-D-glucose positron emission tomography (^{18}FDG-PET) demonstrated pathological uptake of the right lesion (Supplementary Fig. 1). The other two contralater lesions were ground-glass opacities of about 1 cm (Supplementary Fig. 1). Middle-lobe lobectomy with homolateral mediastinal-hilar lymphadenectomy was performed and stage IA lung adenocarcinoma with prevalent lepidic growth was diagnosed [16] (Fig. 1). For the two contralateral lesions, radiological follow-up was planned. One year later, one of the two upper left lobe nodules slightly changed radiological features. For this reason, ^{18}FDG-PET was repeated and demonstrated pathological uptake of the lesion (Supplementary Fig. 2). After multidisciplinary evaluation, left upper lobectomy with lymphadenectomy was performed, since the two nodules were anatomically distinct (in a different lung and physically distinct) and either distant metastasis or node involvement was ruled out [3]. Histology confirmed the presence of two lung adenocarcinomas without node involvement. One lesion (left nodule #1) showed prevalent acinar morphology (50%) whereas papillary differentiation predominated in the other (80%) (Fig. 1) [16]. In both samples there was a component (20%) with lepidic growth. The three lesions were tested for the presence of EGFR mutations. Pyrosequencing disclosed an EGFR exon 19 (E746A750) deletion in the right lesion, whereas left nodule #1 had EGFR exon 21 (L858R) mutation and left nodule #2 resulted EGFR wild-type (Table 1). As the patient was apparently disease-free while pathological and molecular characterization indicated potential independence of the three tumors, systemic treatment was not recommended and radiological follow-up was planned. Thirty-three months later, multiple liver and spleen metastases were detected. The patient was symptomatic for fatigue, fever and weight loss. The performance status (PS) was 2 according to eastern cooperative oncology group (ECOG) scale. Ultrasound-guided liver biopsy was performed with the pathological diagnosis of lung adenocarcinoma localization. Morphological features were described as similar to the left nodule #1. Pyrosequencing was performed on metastatic site and exon 21 mutation was detected. Based on this finding, treatment with gefitinib was started. The patient experienced rapid clinical improvement. Three months later, CT-scan showed marked shrinkage of the main lesions, but a new one-centimeter liver nodule was detected. Because of persistent clinical benefit, the patient continued gefitinib and a liver biopsy with radiofrequency ablation of the progressing site was performed [17]. The biopsy confirmed the presence of L858R mutation, without resistance mutations. Table 1 shows different mutated allele frequency in the DNA extracted by the different lesions. In particular, the metastatic site has increased mutated allele frequency, when compared to the primary tumor (left nodule #1), taking into account the percentage of tumor cell DNA in the samples (Table 1). EGFR pyrosequencing thus disclosed sub-clonal exon 21 mutation in one lung nodule and potential selection of mutant clone in metastatic process may be hypothesized (Table 1). This finding challenges the current view that EGFR mutations develop early in the phylogenetic tree of lung adenocarcinomas [18,19].

Table 1
Pattern of EGFR mutations in multiple adenocarcinoma lesions.
from the same patient by pyrosequencing

Site	Prevalent morphology	EGFR-Pyrosequencing	Tumor cells percentage	Mutated allele frequency
Middle lobe	Lepidic	Exon 19 DelE746A750	70%	NA
Left upper lobe #1	Acinar	Exon 21 L858R	70%	8%
Left upper lobe #2	Papillary	Wild-type	70%	NA
Liver metastasis	Acinar	Exon 21 L858R	30%	18%
Liver progression	Acinar	Exon 21 L858R	45%	15%

NA: not available. The percentage of mutated allele may not be evaluated for exon 19 deletions with pyrosequencing.

Table 2
Results of NGS analysis.

Right Middle Lobe Nodule		Left Upper Lobe Nodule #1		Left Upper Lobe Nodule #2		Liver Metastasis	
Variant	Mutant allele Fraction	Variant	Mutant allele Fraction	Variant	Mutant allele Fraction	Variant	Mutant allele Fraction
EPHA3 (p.P422S)	0.11						
		PIK3CA (p.E542 K)	0.10			PIK3CA (p.E542 K)	0.32
EGFR (p. delE745-A750)	0.84						
		EGFR (p.L858R)	0.33			EGFR (p.L858R)	0.40
MET (p.G198D)	0.14						
		TP53 (p.R280T)	0.17			TP53 (p.R280T)	0.38
				PTEN (p.L182 V)	0.12		

NGS libraries were run on MiSeq (Illumina). Only variants found in > 10% of the reads (mutant allele fraction) and coverage > 600 reads are reported. The colors highlight identical mutations found in different tumor samples.

After three months, CT-scan showed further liver progression and chemotherapy was planned. In our case, limited benefit from gefitinib may be related to intra-tumor heterogeneity and low frequency of mutated allele, as previously described in Ref. [20,21].

In order to shed light on tumor genetic heterogeneity, a custom 30-gene panel using next generation sequencing (Illumina, San Diego, CA, Supplementary material) was performed on primary and metastatic tumor samples. NGS analysis confirmed the EGFR mutation pattern found by pyrosequencing and identified additional genetic differences among the three lung lesions (Table 2). By using cut-off values of > 600 reads and > 10% variant allele fraction, we identified mutations in several lung cancer-associated genes. In five out of ten cases, these variants were at low abundance (< 30% variant allele), suggesting the existence of sub-clones bearing different genetic alterations. The genetic profile of left lung nodule #1 and liver metastasis were identical, sharing the same EGFR, PIK3CA and TP53 mutations. This result further indicates that liver metastasis likely stems from lung nodule #1, whereas non-overlapping genetic fingerprints in the three lung lesions supported their independent origin (Table 2). The post-progression sample could not be analyzed by NGS due to the limited DNA amount available.

In the interpretation of these NGS results, it should be considered that as no exome sequencing was performed, the phylogenetic tree of the cancers could not be assessed in this case. Thus, these cancers could still be related to each other and the driver mutations found could have developed during the later stage and therefore only represent the branches of the tree.

3. Discussion and conclusions

In conclusion, we report a case of three primary lung adenocarcinomas in a never-smoker woman, with different morphological and genetic profiling. The data and clinical outcome highlight the importance of analyzing and matching morphological and molecular pattern of multifocal lung tumors.

In the reported case, morphological and molecular characteristics are concordant, as previously reported in a series of 33 adenocarcinomas and eight squamous cell carcinoma samples through extended NGS analyses [11]. Anyway, morphology-guided microdissection analysis is necessary to document this association [22–24], due to the potential effect of intratumor heterogeneity.

In the reported case, in addition to help in the definition of independent primary tumors, molecular characterization led to the choice of systemic treatment. Indeed, the use of ultrasensitive methods of EGFR mutations detection let us identify subclonal exon 21 mutation in one of the three primary lung adenocarcinomas, thus understanding the origin of liver metastasis and suggesting a clonal selection during the process of metastatic potential gain. In our patient, partial benefit from gefitinib with early oligoprogression may be related to intratumor heterogeneity and low frequency of mutated allele, as previous findings suggest [20,21]. This point opens new perspectives for studying new combination therapy approaches in oncogene addicted NSCLC.

These data also suggest a potential role for the detection of potentially druggable genetic alterations through liquid biopsies in multifocal NSCLCs. Indeed, since genetic material in plasma derives from all the tumor disease, the analysis of plasma from cancer patients could account for molecular heterogeneity, highlight which subclone of the neoplasm is leading metastatic process and provide spatial and temporal dynamic monitoring of molecular changes [25].

Consent

Written informed consent was obtained from the patient for publication of this Case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Authors' contribution

LB and SI conceived the manuscript, analyzed and interpreted the results; LB wrote the manuscript; FC and SV made pathological diagnosis and morphological characterization; GN, DC, MT, GT performed molecular analyses; LB and VP treated the patient; AF, PC and FR coordinated clinical management; FR performed surgery; AA coordinated molecular analyses. All the authors read and approved the final version of the manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgement

The work was partly supported by "IOV intramural Research Grant 2014-Translational Oncology"

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2016.03.009.

References

[1] A.F. Gazdar, J.D. Minna, Multifocal lung cancers—clonality vs field cancerization and does it matter, J. Natl. Cancer Inst. 101 (2009) 541–543.
[2] N. Martini, M.R. Melamed, Multiple primary lung cancers, J. Thorac. Cardiovasc. Surg. 70 (1975) 606–612.
[3] A.A. Loukeri, C.F. Kampolis, A. Ntokou, G. Tsoukalas, K. Syrigos, Metachronous and synchronous primary lung cancers: diagnostic aspects, surgical treatment, and prognosis, Clin. Lung Cancer 16 (2015) 15–23.
[4] N. Girard, C. Deshpande, C. Lau, D. Finley, V. Rusch, W. Pao, W.D. Travis, Comprehensive histologic assessment helps to differentiate multiple lung primary nonsmall cell carcinomas from metastases, Am. J. Surg. Pathol. 33 (2009) 1752–1764.
[5] K.R. Shen, B.F. Meyers, J.M. Larner, D.R. Jones, American College of Chest Physicians special treatment issues in lung cancer: ACCP evidence-based clinical practice guidelines (2nd edition), Chest 132 (2007) 290S–305S.
[6] C.T. Wu, M.W. Lin, M.S. Hsieh, S.W. Kuo, Y.L. Chang, New aspects of the clinicopathology and genetic profile of metachronous multiple lung cancers, Ann. Surg. 259 (2014) 1018–1024.
[7] N. Girard, I. Ostrovnaya, C. Lau, B. Park, M. Ladanyi, D. Finley, C. Deshpande, V. Rusch, I. Orlow, W.D. Travis, W. Pao, C.B. Begg, Genomic and mutational profiling to assess clonal relationships between multiple non-small cell lung cancers, Clin. Cancer Res. 15 (2009) 5184–5190.
[8] S. Shimizu, Y. Yatabe, T. Koshikawa, N. Haruki, S. Hatooka, M. Shinoda, M. Suyama, M. Ogawa, N. Hamajima, R. Ueda, T. Takahashi, T. Mitsudomi, High frequency of clonally related tumors in cases of multiple synchronous lung cancers as revealed by molecular diagnosis, Clin. Cancer Res. 6 (2000) 3994–3999.
[9] A. Warth, S. Macher-Goeppinger, T. Muley, M. Thomas, H. Hoffmann, P.A. Schnabel, R. Penzel, P. Schirmacher, S. Aulmann, Clonality of multifocal nonsmall cell lung cancer: implications for staging and therapy, Eur. Respir. J. 39 (2012) 1437–1442.
[10] Y.L. Chang, C.T. Wu, S.C. Lin, C.F. Hsiao, Y.S. Jou, Y.C. Lee, Clonality and prognostic implications of p53 and epidermal growth factor receptor somatic aberrations in multiple primary lung cancers, Clin. Cancer Res. 13 (2007) 52–58.
[11] S.J. Murphy, M.C. Aubry, F.R. Harris, G.C. Halling, S.H. Johnson, S. Terra, T.M. Drucker, M.K. Asiedu, B.R. Kipp, E.S. Yi, T. Peikert, P. Yang, G. Vasmatzis, D.A. Wigle, Identification of independent primary tumors and intrapulmonary metastases using DNA rearrangements in non-small-cell lung cancer, J. Clin. Oncol. 32 (2014) 4050–4058.
[12] W.R. Geurts-Giele, A.W. Dirkx-van der Velden, N.M. Bartalits, L.C. Verhoog, W.E. Hanselaar, W.N. Dinjens, Molecular diagnostics of a single multifocal non-small cell lung cancer case using targeted next generation sequencing, Virchows Arch. 462 (2013) 249–254.
[13] B.E. Johnson, Second lung cancers in patients after treatment for an initial lung cancer, J. Natl. Cancer Inst. 90 (1998) 1335–1345.
[14] E.J. Jung, J.H. Lee, K. Jeon, W.J. Koh, G.Y. Suh, M.P. Chung, H. Kim, O.J. Kwon, Y.M. Shim, S.W. Um, Treatment outcomes for patients with synchronous multiple primary non-small cell lung cancer, Lung Cancer 73 (2011) 237–242.
[15] M. Reck, D.F. Heigener, T. Mok, J.C. Soria, K.F. Rabe, Management of non-small-cell lung cancer: recent developments, Lancet 382 (2013) 709–719.
[16] W.D. Travis, E. Brambilla, M. Noguchi, A.G. Nicholson, K.R. Geisinger, Y. Yatabe, D.G. Beer, C.A. Powell, G.J. Riely, P.E. Van Schil, K. Garg, J.H. Austin, H. Asamura, V.W. Rusch, F.R. Hirsch, G. Scagliotti, T. Mitsudomi, R.M. Huber, Y. Ishikawa, J. Jett, M. Sanchez-Cespedes, J.P. Sculier, T. Takahashi, M. Tsuboi, J. Vansteenkiste, I. Wistuba, P.C. Yang, D. Aberle, C. Brambilla, D. Flieder, W. Franklin, A. Gazdar, M. Gould, P. Hasleton, D. Henderson, B. Johnson, D. Johnson, K. Kerr, K. Kuriyama, J.S. Lee, V.A. Miller, I. Petersen, V. Roggli, R. Rosell, N. Saijo, E. Thunnissen, M. Tsao, D. Yankelewitz, International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma, J. Thorac. Oncol. 6 (2011) 244–285.
[17] A.J. Weickhardt, B. Scheier, J.M. Burke, G. Gan, X. Lu, P.A. Bunn Jr., D.L. Aisner, L.E. Gaspar, B.D. Kavanagh, R.C. Doebele, D.R. Camidge, Local ablative therapy of oligoprogressive disease prolongs disease control by tyrosine kinase inhibitors in oncogene-addicted non-small-cell lung cancer, J. Thorac. Oncol. 7 (2012) 1807–1814.
[18] J. Zhang, J. Fujimoto, J. Zhang, D.C. Wedge, X. Song, J. Zhang, S. Seth, C.W. Chow, Y. Cao, C. Gumbs, K.A. Gold, N. Kalhor, L. Little, H. Mahadeshwar, C. Moran, A. Protopopov, H. Sun, J. Tang, X. Wu, Y. Ye, W.N. William, J.J. Lee, J.V. Heymach, W.K. Hong, S. Swisher, I.I. Wistuba, P.A. Futreal, Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing, Science 346 (2014) 256–259.
[19] C. Swanton, Intratumor heterogeneity: evolution through space and time, Cancer Res. 72 (2012) 4875–4882.
[20] A. Ono, H. Kenmotsu, M. Watanabe, M. Serizawa, K. Mori, H. Imai, T. Taira, T. Naito, H. Murakami, T. Nakajima, Y. Ohde, M. Endo, N. Yamamoto, Y. Koh, T. Takahashi, Mutant allele frequency predicts the efficacy of EGFR-TKIs in lung adenocarcinoma harboring the L858R mutation, Ann. Oncol. 25 (2014) 1948–1953.
[21] Q. Zhou, X.C. Zhang, Z.H. Chen, X.L. Yin, J.J. Yang, C.R. Xu, H.H. Yan, H.J. Chen, J. Su, W.Z. Zhong, X.N. Yang, S.J. An, B.C. Wang, Y.S. Huang, Z. Wang, Y.L. Wu, Relative abundance of EGFR mutations predicts benefit from gefitinib treatment for advanced non-small-cell lung cancer, J. Clin. Oncol. 29 (2011) 3316–3321.
[22] Z. Chen, X. Liu, J. Zhao, H. Yang, X. Teng, Correlation of EGFR mutation and histological subtype according to the IASLC/ATS/ERS classification of lung adenocarcinoma, Int. J. Clin. Exp. Pathol. 7 (2014) 8039–8045.
[23] H. Hu, Y. Pan, Y. Li, L. Wang, R. Wang, Y. Zhang, H. Li, T. Ye, Y. Zhang, X. Luo, L. Shao, Z. Sun, D. Cai, J. Xu, Q. Lu, Y. Deng, L. Shen, H. Ji, Y. Sun, H. Chen, Oncogenic mutations are associated with histological subtypes but do not have an independent prognostic value in lung adenocarcinoma, Onco Targets Ther. 7 (2014) 1423–1437.
[24] N. Cancer Genome Atlas Research, Comprehensive molecular profiling of lung adenocarcinoma, Nature 511 (2014) 543–550.
[25] L.A. Diaz Jr., A. Bardelli, Liquid biopsies: genotyping circulating tumor DNA, J. Clin. Oncol. 32 (2014) 579–586.

OncoTargets and Therapy

Dovepress
open access to scientific and medical research

Open Access Full Text Article

REVIEW

Cell-free DNA as a diagnostic marker for cancer: current insights

This article was published in the following Dove Press journal:
OncoTargets and Therapy
25 October 2016
Number of times this article has been viewed

Samanta Salvi[1]
Giorgia Gurioli[1]
Ugo De Giorgi[2]
Vincenza Conteduca[2]
Gianluca Tedaldi[1]
Daniele Calistri[1]
Valentina Casadio[1]

[1]Biosciences Laboratory, [2]Department of Medical Oncology, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

Abstract: The increasing knowledge of the molecular pathogenesis of cancer and the rapid development of new molecular techniques are promoting the study of early molecular alterations involved in cancer development in body fluids. Specific genetic and epigenetic alterations could be found in plasma, serum, and urine cell-free DNA (cfDNA) and could potentially be used as diagnostic biomarkers for several types of cancers. This review focuses on the role of cfDNA in diagnosis: a PubMed search was performed by selecting papers according to journal impact factor and robustness of statistical analysis. A comprehensive evaluation of "liquid biopsy", including cfDNA analysis, will be one of the critical challenges to better understand the early mechanisms of cancer development.

Keywords: cell-free DNA, diagnosis, cancer, liquid biopsy

Introduction

With a total of 1,658,370 cases each year in the US, cancer represents a prominent worldwide public health problem.[1] Screening programs and early diagnosis have an important impact in improving disease-free survival and reducing mortality in cancer patients. As noninvasive approaches for early diagnosis foster patient compliance, they can be included in screening programs.

Currently, noninvasive serum-based biomarkers widely used in clinical practice include carcinoma antigen 125 (CA 125), carcinoembryonic antigen, carbohydrate antigen 19-9 (CA19-9), and prostate-specific antigen (PSA) for the detection of ovarian, colon, and prostate[2,3] cancers, respectively.

These biomarkers generally have low specificity (high number of false-positive results): new noninvasive biomarkers have been relentlessly developed by researchers in the last years.

The increasing knowledge of the molecular pathogenesis of cancer and the rapid development of new molecular techniques are promoting the study of early molecular alterations in body fluids. Cell-free DNA (cfDNA) can be found in serum, plasma, urine, and other body fluids,[4] representing a "liquid biopsy", which is a circulating picture of a specific disease.[5]

The existence of cfDNA was firstly demonstrated about 70 years ago by Mandel and Metais;[6] cfDNA originates from necrotic or apoptotic cells, and it is generally released by all types of cells. About 40 years after the discovery of cfDNA, Stroun et al showed that specific cancer alterations could be found in the cfDNA of patients.[7] A number of following papers confirmed that cfDNA contains specific tumor-related alterations, such as mutations, methylation, and copy number variations (CNVs), thus confirming the existence of circulating tumor DNA (ctDNA).[8,9]

Correspondence: Valentina Casadio
Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, via Piero Maroncelli 40, 47014 Meldola, Italy
Email valentina.casadio@irst.emr.it

submit your manuscript | www.dovepress.com
Dovepress
http://dx.doi.org/10.2147/OTT.S100901

cfDNA in plasma or serum is the best characterized, while urine cfDNA (ucfDNA) is less known. However, some recent studies demonstrated that ucfDNA could also be a promising source of biomarkers.[10]

In blood, apoptosis seems to be the most frequent event that determines the amount of cfDNA. In cancer patients, however, the amount of cfDNA seems to be also influenced by necrosis.[11,12] Since apoptosis seems to be the main release mechanism, circulating cfDNA has a size distribution which reveals an enrichment in short fragments of about 167 bp,[13,14] corresponding to nucleosomes generated by apoptotic cells.

The present review focuses on the role of cfDNA in the diagnosis of tumors; a PubMed search was performed using the following phrases: cell-free DNA, liquid biopsy, diagnosis, early detection, ucfDNA, and circulating cfDNA. We selected the most recent and important studies on this topic according to journal impact factor and accuracy of statistical analysis.

Plasma/serum cfDNA

The evaluation of circulating cfDNA provides information regarding intratumor heterogeneity, reasons for primary resistance, detection of minimal residual disease, and disease evolution. In particular, the evaluation of ctDNA could allow for the reconstruction of the rearrangements and the epigenetic status of the tumor genome, and the identification of potentially metastatic clonal and subclonal cells.[15] The noninvasive sample collection and the easy-to-perform circulating cfDNA analysis could make a good diagnostic tool. ctDNA analysis could replace tissue biopsies as it can be easily repeated over time, allowing tumor burden and treatment response monitoring, and early identification of relapse.

Noteworthy, the circulating cfDNA has a specific profile. Indeed, a study on prenatal cfDNA showed a specific length of about 167 bp due to nuclease-cleaved nucleosomes,[14] suggesting that the major fragments were caused by apoptotic events both in healthy individuals and cancer patients. Interestingly, a recent study analyzing plasma DNA from 32 patients with stage IV colorectal cancer (CRC) showed a subset of patients (34.4%) with a biphasic size distribution (166 bp and 332 bp) of plasma DNA fragments associated with increased circulating tumor cell (CTC) numbers and elevated concentration of mutated plasma DNA component.[16] Distribution of different fragments could associate with tumor content in plasma samples, with a potential diagnostic significance. However, a more sensitive test is needed for early tumor stage detection.

We reviewed studies aiming to characterize cfDNA for concentration, cancer-related genetic alterations (such as mutations, CNV, and microsatellite instability [MSI]), and epigenetic alterations. Table 1 summarizes the sensitivity and specificity of the markers reported.

Circulating cfDNA concentration as a diagnostic marker

The amount of circulating cfDNA in serum and plasma seems to be significantly higher in patients with tumors than in healthy controls, especially in those with advanced-stage tumors than in early-stage tumors.[17–19] The variability of the amount of circulating cfDNA is higher in cancer patients than in healthy individuals,[16] and the amount of circulating cfDNA is influenced by several physiological and pathological conditions, including proinflammatory diseases.[20,21] However, in a study conducted on 50 patients with resectable non-small-cell lung cancer (NSCLC), 101 patients with chronic respiratory inflammation, and 40 healthy volunteers, the authors found a significantly higher amount of circulating cfDNA in plasma of NSCLC patients than in subjects with chronic respiratory inflammation and healthy individuals, with 90% sensitivity and 80.5% specificity in discriminating NSCLC patients from healthy individuals (area under the curve [AUC] =0.90).[22]

Noteworthy, circulating cfDNA amount was influenced by various issues. Firstly, cfDNA amount was significantly higher in serum than in plasma,[23,24] due to clotting of white blood cells in serum,[23] suggesting that serum is a worse source for tumor-specific DNA analysis because of the possible presence of wild-type DNA.

Besides, circulating cfDNA is less stable, with a variable half-life in the circulation ranging from 15 minutes to several hours.[25] For these reasons, diagnostic studies based on the amount of circulating cfDNA provide insufficiently robust and consistent results.

Cancer-related genetic alterations

Deep-sequencing data

Several studies aimed at correlating rearrangements in matched tissue and plasma samples were conducted to confirm that circulating cfDNA analysis can be used as a diagnostic tool.

A next-generation sequencing (NGS) evaluation on 50 cancer genes covering 2,800 COSMIC mutations in 60 tumor tissues and 31 plasma samples from 17 metastatic breast cancer patients showed a 76% concordance between tissue and plasma. The authors concluded that plasma can be prospectively tested as an alternative to metastatic biopsies.[26]

Table 1 Sensitivity and specificity of serum/plasma cfDNA markers

Marker	Method	Cancer patients	Controls	Cancer type	Sensitivity	Specificity	Reference
ctDNA	CAPP-Seq	13	5	NSCLC	0.85	0.96	Newman et al[30]
Cyclin E CNV and length index	qPCR	88	70	Ovarian serous carcinoma	0.73	0.97	Salani et al[34]
MSI (BAT-26)	Real-time PCR	44	44	CRC	0.70	0.67	Mokarram et al[35]
DNA concentration	DNA DipStick™ Kit	84	43	NSCLC	Cutoff: 6–25 ng/mL =0.75 Cutoff: 26–125 ng/mL =0.54	Cutoff: 6–25 ng/mL =0.86 Cutoff: 26–125 ng/mL =1.00	Sozzi et al[37]
cfDNA concentration (cutoff: 60 ng/mL)	Picogreen	54	31	Ovarian cancer	Stage I/II =0.47 Stage III/IV =0.56	1.00	Chang et al[38]
Allelic imbalance of 8 SNPs	Digital SNP analysis	54	31	Ovarian cancer	Stage I/II =0.87 Stage III/IV =0.95	1.00	Chang et al[38]
DNA integrity (fragments of 200 bp, 1,300 bp, 1,800 bp, and 2,400 bp)	Real-time PCR	123	67	Prostate cancer	0.70	0.81	Hanley et al[40]
KRAS exon 2 mutations or *BRAF* V600E mutation	qPCR	124	71	CRC	0.38	1.00	Mouliere et al[44]
KRAS mutations at codon 12	qPCR	58	21	Pancreatic cancer	0.70	1.00	Dianxu et al[42]
GSTP1 methylation	MS-PCR	31	44	Prostate cancer	0.95	0.87	Dumache et al[51]
RASSF1 methylation	qMS-PCR	93	76	Breast cancer	0.62	0.87	Hoque et al[47]
APC methylation	MS-PCR	60	40 adenomatous colorectal polyp +60 healthy control	CRC	0.57	0.89	Pack et al[66]
SHOX2 methylation	Methylation-specific HeavyMethyl assay	188	155	Lung cancer	0.60	0.90	Kneip et al[69]
SEPT9 methylation	Real-time PCR	53	1,457 non-CRC subjects	CRC	0.48	0.92	Church et al[53]
SEPT9 methylation	Real-time PCR	70	100	Lung cancer	0.44	0.96	Powrózek et al[54]
THBD methylation	Digital MethyLight assay	107	98	CRC	0.71	0.80	Lange et al[70]
RASSF1A and *BRCA* methylation	Sensitive MS-PCR	50	40	Ovarian cancer	0.82	1.00	Ibanez de Caceres et al[58]
RASSF1A, CACLA, and EP300 methylation	Microarray-based assay	30	30 benign disease +30 healthy controls	Ovarian cancer	0.90	0.87	Liggett et al[61]
CDKN2A, *DLEC1*, *DAPK1*, and *UCHL1* methylation	MS-PCR	40	41	Nasopharyngeal carcinoma	0.85	0.95	Tian et al[64]
APC, *GSTP1*, and *TIG1* methylation	Real-time PCR after methylation-sensitive restriction endonuclease treatment	45	45	Bladder cancer	0.80	0.93	Ellinger et al[67]

Abbreviations: ctDNA, circulating tumor DNA; CAPP-Seq, cancer-personalized profiling by deep sequencing; NSCLC, non-small-cell lung cancer; CNV, copy number variation; qPCR, quantitative PCR; MSI, microsatellite instability; PCR, polymerase chain reaction; CRC, colorectal cancer; cfDNA, cell-free DNA; SNPs, single-nucleotide polymorphisms; qMS-PCR, quantitative MS-PCR; MS-PCR, methylation-specific PCR.

These results were confirmed in an independent cohort of 34 patients with 18 different tumor types: 46 genes covering more than 6,800 COSMIC mutations in tissue and plasma samples were analyzed. Twenty-seven out of 34 patients showed a 97% concordance between mutations found in tissue and in ctDNA.[27] ctDNA-based NGS analyses could revolutionize the management of patients with potentially curable or metastatic disease.[28]

In a recent extensive study, Bettegowda et al used a digital polymerase chain reaction (PCR)-based method to

identify ctDNA for early detection. They evaluated 640 plasma samples from patients with various cancer types, showing differences in the ctDNA samples: more than 75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head-and-neck cancers presented evaluable ctDNA. However, ctDNA was found only in 48%–73% of localized tumors, such as colorectal, gastroesophageal, pancreatic, and breast adenocarcinoma.[29] Newman et al performed an ultrasensitive and economical method called cancer-personalized profiling by deep sequencing (CAPP-Seq) for quantifying ctDNA.[30] CAPP-Seq was implemented by the capture of recurrent mutations or gene fusions in NSCLC by complementary hybridization in solution. After evaluating 1 plasma sample from 5 controls, and 35 plasma samples from 13 patients with NSCLC, the authors identified ctDNA in 85% of patients with stage II–IV disease (sensitivity), while 96% of controls had no ctDNA (specificity). Stage I patients had a ctDNA/cfDNA ratio tenfold lower than stage II–IV patients, suggesting that DNA is less released into circulation at an early stage of disease, due to less apoptotic events or less vascularization. In conclusion, they found a significant correlation between ctDNA levels and the tumor volume.

Another study on *EGFR* deep sequencing in 288 plasma and matched biopsies from NSCLC patients showed a good concordance between the 2 sample types. However, in about 50% of patients, the alterations were found in tissues but were not confirmed in plasma samples.[31] Sensitivity in finding plasma alterations was even lower in early stages.

Copy number variation

A study conducted on 90 primary breast cancer patients, 30 metastatic patients, and 98 female controls compared CNV of *HER2* in tissue and plasma samples. *HER2* copy number was found in 95% of plasma-matched tissue. No amplification was found in circulating cfDNA of the controls, but only 8 out of 68 patients with primary tumor and 5 out of 30 patients with metastatic tumor showed *HER2* amplification in circulating cfDNA, suggesting a poor role in diagnosis for low-positive cases.[32]

Clonal and subclonal CNV was recently evaluated in ctDNA using massively multiplexed PCR and NGS. The authors matched tissue and plasma samples from 11 patients with stage II breast cancer for CNV detection. They showed that this technique was able to detect subclonal mutations in plasma, which were otherwise missed in tumor tissue biopsies.[33] The authors found CNV with very low allelic values in ctDNA in 8 out of 11 (72.7%) patients with stage II breast cancer, concluding that this technique is suitable for early diagnosis.

Salani et al evaluated cyclin E CNV in the plasma of 88 ovarian serous carcinoma patients and 70 controls. The authors also studied the integrity index of cyclin E. Combination of cyclin E copy number (400 bp) and DNA length index had an AUC value of 0.936.[34] Since the analysis of cyclin E had a higher sensitivity (95.6%) than cytology (74%), it may be suitable for false-negative cytology cases.

Microsatellite instability

BAT-25 and BAT-26 mononucleotide microsatellites were tested in tissue and serum from 44 healthy individuals and 44 CRC patients by real-time PCR and high-performance liquid chromatography (HPLC). BAT-26 resulted more sensitive than BAT-25 in identifying MSI tumors, and real-time PCR methods showed higher specificity than HPLC.[35] Sensitivity and specificity values are reported in Table 1.

A study on serum from 34 patients with primary (n=8) and metastatic (n=24) breast cancers evaluated 4 microsatellite loci of chromosomes 10q22-23, 16q22-23, 17q11-12, and 17q21. The authors showed loss of heterozygosity at different loci in 16 patients and MSI in only 1 patient suggesting that genomic aberrations on chromosomes 10, 16, and 17 are detectable in circulating cfDNA and could represent diagnostic information.[36] However, further evaluations on healthy controls are needed to confirm the diagnostic role of genetic aberrations.

A study on 84 patients with NSCLC and 43 healthy controls evaluated DNA concentration and MSI at loci located at 3p14.2 (D3S1300, FHIT locus), 3p21 (D3S1289), 3p23 (D3S1266), 3p24.2 (D3S2338), and 3p25–26 (D3S1304), which are hotspots of deletions in lung cancer. DNA concentration was higher even in stage I patients than in controls with an AUC of 0.844. Moreover, all patients except 3 with MSI in the plasma had a stage I tumor, suggesting a potential diagnostic use in lung cancer screening.[37] None of these alterations were found in healthy controls.

Circulating cfDNA concentration and allelic status were evaluated as diagnostic markers by 8 single-nucleotide polymorphisms in combination with CA 125 levels in plasma DNA from 54 ovarian cancer patients and 31 controls. For circulating cfDNA concentration and allelic imbalances, the authors showed an area under the receiver operating characteristic (ROC) curve of 0.90 and 0.95 for patients and controls, respectively. Moreover, combination of the serum CA 125 level and the circulating cfDNA concentration increased the area under the ROC curve from 0.78 (CA 125 alone) to 0.84.[38]

Circulating cfDNA integrity

Circulating cfDNA was also evaluated in terms of integrity. Madhavan et al evaluated circulating cfDNA from breast cancer patients (n=383) and from healthy controls (n=100) by measuring ALU and LINE1 repetitive DNA elements using quantitative PCR. They observed a hierarchical decrease in circulating cfDNA integrity and an increase in circulating cfDNA concentration from healthy controls to primary and to metastatic breast cancer patients.[39] They reported an AUC of 0.75.

Hanley et al evaluated circulating cfDNA integrity in plasma from 123 prostate cancer (PCa) patients and 67 controls. The authors analyzed 4 different fragment sizes (200 bp, 1,300 bp, 1,800 bp, and 2,300 bp), providing a score of integrity status. Circulating cfDNA integrity was positive in 86 out of 123 patients and in 13 out of 67 controls. Moreover, circulating cfDNA integrity identified 63% of the PCa patients negative for PSA levels.[40]

Mutations

KRAS mutations are the most studied alterations in different types of cancers, in tissue and body fluids.

Analysis of *KRAS* mutations in plasma of 44 pancreatic patients vs 37 controls showed a 27% sensitivity and a 100% specificity. Moreover, patients with mutation showed a significantly shorter survival than patients with wild-type *KRAS*.[41]

A following study on *KRAS* mutations at codon 12 in plasma samples from 58 pancreatic cancer patients and 21 healthy controls showed good test accuracy. In particular, the authors found *KRAS* mutations in 71% of the patients and none in healthy controls. Moreover, when the analysis was combined with CA19-9 evaluation, sensitivity rose from 73% (CA19-9 alone) to 90% (both tests), suggesting a better early detection tool.[42]

Spindler et al evaluated plasma cfDNA levels and tumor-specific *KRAS* mutations in a cohort of 229 metastatic CRC patients and 100 healthy individuals. They found higher levels of cfDNA in CRC patients than healthy individuals with an AUC of 0.9486 and a 85% overall concordance of *KRAS* mutations in plasma and tissue.[43]

An extensive study was conducted by Mouliere et al on the plasma of 124 CRC patients and 71 healthy individuals aiming at evaluating multi-markers, such as the total circulating cfDNA concentration, the presence of point mutations, the proportion of mutated allele, and the circulating cfDNA integrity index. They showed that the point mutations of *KRAS* (exon 2 mutations: G12V, G12A, G12D, G12S, G12C, G12R, and G13D) and *BRAF* (V600E) were found in 42 out of the 124 plasma samples analyzed. Moreover, they found that more than 80% of circulating cfDNA was shorter than 145 bp, with higher – but more fragmented – DNA concentration in plasma from patients than controls.[44] *BRAF* mutation (V600E) was also informative for monitoring melanoma in serum of patients.[45]

Though the *KRAS* mutations analysis reported low sensitivity in circulating cfDNA, the analysis comparing tissue and circulating cfDNA plasma samples revealed higher sensitivity. Bettegowda et al analyzed matched tissue and plasma samples from 206 patients with metastatic CRCs, showing a sensitivity of 87.2% and a specificity of 99.2% of ctDNA detection.[29]

Epigenetics

Epigenetic events, such as DNA methylation in CpG islands, occur early in cancer development suggesting a potential role of DNA methylation as a biomarker for early diagnosis.[46] Blood-based test could improve sensitivity and specificity of current screening; to this aim, a number of potential methylation biomarkers from plasma or serum were tested.[47,48] DNA methylation is the most investigated event in cfDNA, and several markers have been proposed.

Global genomic hypomethylation is a hallmark of cancer in humans. Chen et al investigated the role of hypomethylation of Alu elements in tumor tissue and matched serum of glioma patients and healthy controls by bisulfite sequencing. They showed a correlation of Alu hypomethylation between tumor and serum samples; ROC curve analysis showed an AUC for diagnosis of 0.861, suggesting that the detection of Alu hypomethylation in serum may be used in clinical practice for the diagnosis of glioma.[49]

GSTP1 methylation in PCa is an early event in carcinogenesis, representing one of the most studied epigenetic markers with a diagnostic role. The meta-analysis conducted by Wu et al on GSTP1 methylation in urine, plasma, and serum showed 0.50%–0.75% sensitivity and 0.80%–0.95% specificity.[50] Another important recent study showed 95% sensitivity and 87% specificity.[51] Although it is not approved by the US Food and Drug Administration (FDA), it could nevertheless be associated with PSA in serum.[52]

Methylation of *SEPT9* promoter region in circulating cfDNA of CRC gave promising results at first assessment (72%–90% sensitivity and 88%–90% specificity), but it presented low sensitivity in the subsequent PRESEPT study.[53] Even though the test still requires some improvements, it is under review for FDA approval for clinical use. *SEPT9* test for CRC detection has been used for lung cancer with 44% sensitivity and 96% specificity.[54]

Hypermethylation of the promoter regions of *RASSF1A* has a role in different cancer types. Hoque et al showed that *RASSF1A* methylation in plasma of breast cancer patients reached 62% sensitivity and 87% specificity.[47] Analysis of *RASSF1A* and *RARbeta2* methylation provided 95% of diagnostic coverage in breast cancer patients and 60% in patients with benign lesions and did not present false-positive results in healthy women.[55]

Cassinotti et al highlighted that methylation of *RASSF1A* combined with *CYCD2*, *HIC1*, *PAX*5, *RB1*, and *SRBC* distinguished CRC patients and controls with 84% sensitivity and 68% specificity, whereas *RASSF1A* combined with *HIC1* and *MDG1* differentiated patients with adenomatous polyps and controls with 55% sensitivity and 65% specificity.[56] *RASSF1* combined with *VHL* methylation analyzed in cell-free serum DNA in patients with renal cell carcinoma reached high specificity but low sensitivity for renal cell carcinoma diagnosis.[57]

Ibanez de Caceres et al identified *RASSF1A* and *BRCA* hypermethylation in serum DNA of ovarian cancer patients with 82% sensitivity and 100% specificity with sensitive methylation-specific PCR (MS-PCR).[58]

MethDet56 is a novel method for identifying new biomarkers, consisting in a microarray panel of frequently methylated genes for measuring the quantity of methylated target sequence following digestion with endonuclease Hin6I and PCR amplification of undigested fragments.[59] At first, it was applied to pancreatic cancer, and then to ovarian cancer, selecting several genes able to identify ovarian cancer.[60,61] The methylation panel of *RASSF1A*, *CALCA*, and *EP300* distinguished between patients with ovarian cancer and healthy controls with 90% sensitivity and 87% specificity, whereas methylation of *RASSF1A* and *PGR* differentiated between ovarian cancer samples and benign ovarian disease with 80% sensitivity and 73.3% specificity.[61] The methylation panel of *RASSF1A*, *UCHL1*, *NPTX2*, *SARP2*, *ppENK*, and *p16 (CDKN2A)* showed that they are able to distinguish between plasma DNA derived from pancreatic cancer samples and control samples by MS-PCR and confirm by direct sequencing after bisulfite treatment. Moreover, *CDKN2A* was differentially methylated between pancreatic cancer and chronic pancreatitis, which is a recognized risk factor for pancreatic cancer.[62] Methylated *CDKN2A* in plasma is useful for detecting lung cancer, but it seems to work better when it is a part of a biomarker panel rather than as a single gene.[63]

Promoter hypermethylation of *CDKN2A* combined with *DLEC1*, *DAPK1*, and *UCHL1* analyzed with MS-PCR in serum DNA of patients with nasopharyngeal carcinoma achieved 85% sensitivity and 95.1% specificity, which is higher than that achieved by the individual gene.[64]

Cell-free plasma DNA methylation levels of *GSTP1*, *p16*, and *APC* genes were found significantly higher in PCa patients than in cancer-free controls with pyrosequencing approach by Delgado-Cruzata et al.[65]

APC is a biomarker investigated in different diseases. Pack et al showed that *APC* methylation in plasma has 57% sensitivity and 84% specificity for CRC detection. It is also significantly increased in stage I CRC, and the most sensitive (57%) and specific (89%) marker when compared to the promoter methylation of other 4 genes.[66] Hypermethylation of *APC*, *GSTP1*, and *TIG1* in serum cfDNA is able to distinguish bladder cancer and controls with 80% sensitivity and 93% specificity.[67] Radpour et al identified a methylation panel of 8 tumor suppressor genes including *APC* in circulating cfDNA which is higher in patients with breast cancer than in controls. This panel could achieve >90% sensitivity and specificity, for the development of a blood-based test for breast cancer diagnosis.[68]

APC, *RARb*, and *CDH13* were found to be differentially methylated in cfDNA between patients with lung cancer and healthy controls,[63] although with low sensitivity. Also, *SHOX2* gene promoter methylation was found to be a potential biomarker for lung cancer detection reaching 60% sensitivity and 90% specificity.[69]

Other methylated genes could have a role of cancer type-specific biomarkers. *CST6* shows a differentially methylated pattern between breast cancer and control plasma samples using bisulfite conversion and MS-PCR in circulating cfDNA. *CST6* is also included in an 8-gene biomarker panel which reaches 90% sensitivity and specificity in patients with early-stage breast cancer vs 30 healthy controls as shown using PCR after bisulfite treatment.[68]

The promoter region methylation of *THBD* differentiated CRC and control plasma samples with 71% sensitivity and 80% specificity.[70]

Urine cfDNA

Urine sample is very advantageous for noninvasive detection of cancer. Given its complexity, it could be a source for a variety of biomarkers, from proteins to nucleic acids.[71] Some urinary biomarkers for early diagnosis of prostate and bladder cancers are already FDA approved (eg, NMP22, FISH Urovysion, PCA3).[72,73] Most of the published data regarding urine focus on biomarkers from exfoliated cells, and very little is known about the role of ucfDNA.

ucfDNA originates from cells coming in direct contact with urine (necrotic or apoptotic cells) or from cfDNA in blood. Glomerular filtration acts as a "dimensional selection": only small DNA fragments from circulation (about 100 bp) can penetrate through the pores of the glomerular barrier, appearing in urine.[74,75] As a consequence, ucfDNA could provide important information on specific alterations of circulating cfDNA and genomic DNA coming from cells shedding into urine, thus being useful for identifying both cancers of the urological tract and other solid tumors.[76]

All papers published on ucfDNA for diagnostic purposes are preliminary studies conducted on small series of patients and still far from any clinical application. The development of new molecular technologies (eg, NGS or digital PCR), alongside a broader case series analysis, will offer a deeper insight into the practical clinical translation of these promising findings.

Table 2 summarizes the sensitivity and specificity of the markers analyzed in ucfDNA.

Cancer-related genetic alterations

ucfDNA can be used for studying genetic alterations from tumors distal to the urological tract. Fifteen years ago, 2 studies demonstrated that specific cancer-associated mutations detectable in plasma and serum were also present in urine,[73,75] suggesting that ucfDNA may be studied for detecting specific alterations.

ucfDNA genetic alterations are mostly evaluated for urological cancers, even though some studies demonstrated that urine could effectively produce a picture of DNA alterations coming from circulation.[76]

Su et al demonstrated that k-*RAS* gene mutations were detectable in urine of patients with adenomatous polyps or CRC and that mutations in urine better correlate with tissue than plasma samples. In a following study on 20 patients, they further confirmed their previous hypothesis[77] that urine could be even more representative of k-*RAS*-mutated DNA than serum or plasma. They used a restriction-enriched PCR to analyze k-*RAS* mutations and found 95%, 35%, and 40% mutation incidence in urine, serum, and plasma samples, respectively. Although remarkable, their findings are unusable for diagnostic purposes (low number of analyzed cases), even though 95% mutation incidence in urine samples suggests a good test sensitivity.

A number of studies have been published on ucfDNA characteristics in urological cancers, especially bladder.

Firstly, ucfDNA quantity was evaluated by Chang et al using picogreen,[78] though it resulted inaccurate for proper distinction between bladder cancer patients and healthy individuals, as confirmed by Zancan et al.[79] On the other hand, Chang et al demonstrated that the detection of a long (400 bp) DNA fragment could be a potential diagnostic marker, with 86% sensitivity and 72% specificity.[78] Casadio et al confirmed the ucfDNA integrity, with a real-time PCR approach, detecting 3 long amplicons belonging to 3 oncogenes frequently amplified in bladder cancer (*c-MYC*, *BCAS1*, *HER2*).[10] They obtained 73% sensitivity, with 84% and 83% specificity in healthy individuals and in patients with urological symptoms, respectively. Interestingly, Szarvas et al[80] analyzed 12 microsatellite markers mapped on 6 different chromosomes. They obtained 80% sensitivity and 81% specificity. After comparing the results obtained in urine supernatant vs urine sediments, they reported higher sensitivity in the cell-free fraction.

Table 2 Sensitivity and specificity of urine cfDNA markers

Marker	Method	Cancer patients	Controls	Cancer type	Sensitivity	Specificity	Reference
Microsatellite analysis	PCR and fluorescent DNA sequencer	44	36	Bladder	0.80	0.81	Szarvas et al[80]
DNA integrity β-Actin (400 bp)	Real-time PCR	46	98	Bladder	0.86	0.72	Chang et al[78]
DNA quantity	GeneQuant Pro Quant-iT DNA high sensitivity assay kit Real-time PCR NanoDrop 1000	45	87	Bladder	0.57	NA	Zancan et al[79]
DNA integrity	Real-time PCR	52	46 symptomatic individuals 32 healthy individuals	Bladder	0.73	0.84	Casadio et al[10]
DNA integrity	Real-time PCR	29	25	Prostate	0.79	0.84	Casadio et al[81]
DNA integrity	Real-time PCR	67	64	Prostate	0.5	0.44	Salvi et al[82]
Vimentine hypermethylation	qMethyLight PCR	20	20	CRC	0.75	0.90	Song et al[89]

Abbreviations: cfDNA, cell-free DNA; NA, not available; PCR, polymerase chain reaction; qMethyLight PCR, quantitative MethyLight PCR.

ucfDNA integrity was also evaluated for early diagnosis of PCa: despite an initial study with promising results,[81] this marker failed to have a good early diagnostic role in the confirmatory study.[82]

Following the technological advancements, some recent papers[83–85] with no diagnostic purposes used NGS approaches, microarray, or digital PCR on ucfDNA. These studies are important because they demonstrated the feasibility of NGS approaches on cfDNA in urine,[85] even presenting a higher tumor genome burden in the cell-free fraction than in the cell sediment.[84] All these results indicate further applications of ucfDNA in tumors.

Epigenetics

Besides serum and plasma circulating cfDNA, *GSTP1* methylation also appears to be a promising diagnostic marker in ucfDNA as shown in a study,[86] although it was conducted on a small case series with no robust statistical results.

LINE-1 hypomethylation in bladder cancer is a promising DNA methylation biomarker for diagnostic purposes.[87] Ghanjati et al found that DNA methylation profiles of *LINE-1* promoter regions in ucfDNA of urothelial carcinoma patients can be detected by bisulfite genomic sequencing. *LINE-1* hypomethylation may be used for diagnostic purposes as unmethylated full-length *LINE-1* sequences prevail in urine of cancer patients.[88]

Bisulfite conversion is used for urological tumor detection as DNA fragments are longer than 300 bp. Bisulfite conversion, however, is unsuitable for other types of tumor detection due to further DNA fragmentation. For this reason, Song et al developed a quantitative MethyLight PCR-based assay to detect hypermethylated vimentin in the low-molecular weight (LMW) voided urine of CRC patients. The assay targeted a 39-nucleotide segment of the hypermethylated region of vimentin gene, detecting hypermethylated vimentin in 75% of LMW urine DNA from CRC patients and in 10% of urine samples of healthy controls. As a consequence, a urine test using epigenetic markers may be evaluated for CRC screening.[89]

Feng et al demonstrated the feasibility of urine hypermethylation of *DAPK1*, *RARB*, *TWIST1*, and *CDH13* genes for cervical cancer screening with sensitivity similar to that of an exfoliated cervical cytology.[90]

Discussion

cfDNA is an undeniable source of biomarkers for assisting clinicians in early cancer detection, monitoring patients under treatment, and predicting drug response or disease progression.[91,92] It is noteworthy that the studies on the role of circulating cfDNA in earlier stages of cancer are not as many as those on advanced cancer. This is probably due to the fact that ctDNA in circulation is more representative in patients with advanced and metastatic than local diseases, and that the likelihood of finding alterations increases alongside the aggressiveness of the disease.[29] However, we strongly believe that the technological advancements (eg, NGS approaches and digital PCR-based methods) will improve sensitivity in early detection and provide more robust data.

The ideal early diagnostic marker should be noninvasive and highly accurate, with a good cost/benefit ratio and allow simple interpretation of the results. Despite that cfDNA seems to be a good source of markers with such characteristics, and the high number of publications, none of the cfDNA-related markers have yet entered the clinical practice. Marker performance varies widely depending on the population, the sample storage, the test performance, and the result analysis. For these reasons, comparison between cfDNA-based biomarkers is inappropriate, unless within a single study, and translation to clinical practice is still difficult. In addition, pilot studies on the same marker are mostly retrospective, and the sample size and the statistical power are often inadequate for proving the robustness of a cfDNA biomarker. Lastly, studies reporting diagnostic accuracy of early diagnostic markers, including cfDNA, should follow the Standards for Reporting of Diagnostic Accuracy guidelines for publication.[93]

Epigenetic alterations seem to be the most promising biomarkers based on circulating cfDNA studies. Epigenetic events, especially methylation of specific tumor suppressor genes promoter, are often early phenomena in the process of carcinogenesis. For this reason, they are more suitable for early detection than mutations or other genetic alterations. One example of a highly promising approach is the analysis of *GSTP1* in PCa,[50] which is detectable in circulation and urine in a high number of cancer patients, without being present in healthy individuals. However, this promising marker also did not enter the clinical practice due to the lack of inter-studies reproducibility; several methylation studies used different approaches such as bisulfite conversion and immunoprecipitation enzymatic digestion, leading to noncomparable results.

ucfDNA has been mostly evaluated for urological cancers. Despite the limited number of studies and that the restricted sample size does not allow for valid conclusions, ucfDNA appears to be a promising source of early diagnostic markers. NGS approach seems to be feasible in

urine samples, showing that many efforts have been recently made to improve the technology for the study of ucfDNA. This will lead to further advancements in the employment of ucfDNA.

Clinical applications and future perspectives

Using ctDNA characterization for the early diagnosis of tumors has a great potential for clinical application; however, some limitations have to be considered. First of all, even if ctDNA could be distinguished from total cfDNA using somatic mutations analysis, the very low presence of ctDNA (often only <0.1%) needs more sensitive and reproducible methods. Secondly, cfDNA characteristics could be different among patients, forcing a qualitative analysis and specific optimization procedure for each patient. Despite these limitations and the low number of large studies on diagnostics, there are a number of potential clinical applications encouraging the search for new, sensitive, and robust methods. A very promising application in early diagnosis is adding ctDNA detection to conventional markers used for screening programs; in this context, the detection of somatic mutations might suggest an early development of disease. To reach these clinical applications, several sensitive methods have been already proposed: first of all, massively parallel sequencing or NGS,[94,95] and then digital PCR. Nowadays, ctDNA could be analyzed for gene-specific panel or whole exome/genome using NGS with the advantages of multiple mutations data from only 1 analysis. In parallel, digital PCR analysis offers a high level of sensitivity (up to about 0.01%) and specificity for only few molecular targets[94] with real quantification of mutated sample percentage compared to total samples. Improvement methods will combine the multiplicity data from NGS results and the more sensitive and precise digital PCR, for moving to real clinical and routine applications.

Moreover, a more detailed picture of disease status is also given by the knowledge about all liquid biopsy aspects such as CTCs, small extracellular vesicles, and cell-free RNA. However, for an early diagnosis, ctDNA seems to be the most promising marker for the real clinical application, thanks to the most easy detection and stability compared to CTCs and cell-free RNA.

The next achievement in cfDNA evaluation will be robustness: larger prospective studies, with more sensitive and reproducible methods, are needed. Also, other laboratories must confirm cfDNA alterations as early diagnostic markers before being translated into clinical practice.

A deeper knowledge of cancer development and evolution will lead to more sensitive and robust analysis methods useful for the characterization of all aspects of liquid biopsy, thus providing answers for each clinical query.

Acknowledgment

The authors wish to thank Veronica Zanoni for editing the manuscript.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin*. 2015;65(1):5–29.
2. Terry KL, Schock H, Fortner RT, et al. A prospective evaluation of early detection biomarkers for ovarian cancer in the European EPIC cohort. *Clin Cancer Res*. Epub 2016 Apr 8.
3. Zhang Y, Yang J, Li H, Wu Y, Zhang H, Chen W. Tumor markers CA19-9, CA242 and CEA in the diagnosis of pancreatic cancer: a meta-analysis. *Int J Clin Exp Med*. 2015;8(7):11683–11691.
4. Chan AK, Chiu RW, Lo YM; Clinical Sciences Reviews Committee of the Association of Clinical Biochemists. Cell-free nucleic acids in plasma, serum and urine: a new tool in molecular diagnosis. *Ann Clin Biochem*. 2003;40(Pt 2):122–130.
5. De Mattos-Arruda L, Caldas C. Cell-free circulating tumour DNA as a liquid biopsy in breast cancer. *Mol Oncol*. 2016;10(3):464–474.
6. Mandel P, Metais P. Les acides nucleiques du plasma sanguin chez l' homme [The nucleic acids in blood plasma in humans]. *C R Seances Soc Biol Fil*. 1948;142(3–4):241–243.
7. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology*. 1989;46(5):318–322.
8. Goessl C, Krause H, Müller M, et al. Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. *Cancer Res*. 2000;60(21):5941–5945.
9. Frenel JS, Carreira S, Goodall J, et al. Serial next-generation sequencing of circulating cell-free DNA evaluating tumor clone response to molecularly targeted drug administration. *Clin Cancer Res*. 2015;21(20):4586–4596.
10. Casadio V, Calistri D, Tebaldi M, et al. Urine cell-free DNA integrity as a marker for early bladder cancer diagnosis: preliminary data. *Urol Oncol*. 2013;31(8):1744–1750.
11. Hao TB, Shi W, Shen XJ, et al. Circulating cell-free DNA in serum as a biomarker for diagnosis and prognostic prediction of colorectal cancer. *Br J Cancer*. 2014;111(8):1482–1489.
12. Zonta E, Nizard P, Taly V. Assessment of DNA integrity, applications for cancer research. *Adv Clin Chem*. 2015;70:197–246.
13. Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. *Clin Chem*. 2015;61(1):112–123.
14. Lo YM, Chan KC, Sun H, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci Transl Med*. 2010;2(61):61ra91.
15. Kaiser J. Medicine. Keeping tabs on tumor DNA. *Science*. 2010;327(5969):1074.
16. Heitzer E, Auer M, Hoffmann EM, et al. Establishment of tumor-specific copy number alterations from plasma DNA of patients with cancer. *Int J Cancer*. 2013;133(2):346–356.
17. Sozzi G, Conte D, Leon M, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. *J Clin Oncol*. 2003;21(21):3902–3908.

18. Kim K, Shin DG, Park MK, et al. Circulating cell-free DNA as a promising biomarker in patients with gastric cancer: diagnostic validity and significant reduction of cfDNA after surgical resection. *Ann Surg Treat Res*. 2014;86(3):136–142.
19. Shao X, He Y, Ji M, et al. Quantitative analysis of cell-free DNA in ovarian cancer. *Oncol Lett*. 2015;10(6):3478–3482.
20. Raptis L, Menard HA. Quantitation and characterization of plasma DNA in normals and patients with systemic lupus erythematosus. *J Clin Invest*. 1980;66(6):1391–1399.
21. Shapiro B, Chakrabarty M, Cohn EM, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer*. 1983;51(11):2116–2120.
22. Szpechcinski A, Chorostowska-Wynimko J, Struniawski R, et al. Cell-free DNA levels in plasma of patients with non-small-cell lung cancer and inflammatory lung disease. *Br J Cancer*. 2015;113(3):476–483.
23. Lee TH, Montalvo L, Chrebtow V, Busch MP. Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. *Transfusion*. 2001;41(2):276–282.
24. Chan KCA, Yeung S-W, Lui W-B, Rainer TH, Lo YMD. Effects of preanalytical factors on the molecular size of cell-free DNA in blood. *Clin Chem*. 2005;51(4):781–784.
25. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer – a survey. *Biochim Biophys Acta*. 2007;1775(1):181–232.
26. Rothé F, Laes J-F, Lambrechts D, et al. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. *Ann Oncol*. 2014;25(10):1959–1965.
27. Lebofsky R, Decraene C, Bernard V, et al. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. *Mol Oncol*. 2015;9(4):783–790.
28. Lianos GD, Mangano A, Cho WC, Dionigi G, Roukos DH. Circulating tumor DNA: new horizons for improving cancer treatment. *Future Oncol*. 2015;11(4):545–548.
29. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6(224):224ra24.
30. Newman AM, Bratman SV, To J, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med*. 2014;20(5):548–554.
31. Uchida J, Kato K, Kukita Y, et al. Diagnostic accuracy of noninvasive genotyping of EGFR in lung cancer patients by deep sequencing of plasma cell-free DNA. *Clin Chem*. 2015;61(9):1191–1196.
32. Page K, Hava N, Ward B, et al. Detection of HER2 amplification in circulating free DNA in patients with breast cancer. *Br J Cancer*. 2011;104(8):1342–1348.
33. Kirkizlar E, Zimmermann B, Constantin T, et al. Detection of clonal and subclonal copy-number variants in cell-free DNA from patients with breast cancer using a massively multiplexed PCR methodology. *Transl Oncol*. 2015;8(5):407–416.
34. Salani R, Davidson B, Fiegl M, et al. Measurement of cyclin E genomic copy number and strand length in cell-free DNA distinguish malignant versus benign effusions. *Clin Cancer Res*. 2007;13(19):5805–5809.
35. Mokarram P, Rismanchi M, Alizadeh Naeeni M, et al. Microsatellite instability typing in serum and tissue of patients with colorectal cancer: comparing real time PCR with hybridization probe and high-performance liquid chromatography. *Mol Biol Rep*. 2014;41(5):2835–2844.
36. Schwarzenbach H, Müller V, Stahmann N, Pantel K. Detection and characterization of circulating microsatellite-DNA in blood of patients with breast cancer. *Ann N Y Acad Sci*. 2004;1022:25–32.
37. Sozzi G, Conte D, Mariani L, et al. Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. *Cancer Res*. 2001;61(12):4675–4678.
38. Chang HW, Lee SM, Goodman SN, et al. Assessment of plasma DNA levels, allelic imbalance, and CA 125 as diagnostic tests for cancer. *J Natl Cancer Inst*. 2002;94(22):1697–1703.
39. Madhavan D, Wallwiener M, Bents K, et al. Plasma DNA integrity as a biomarker for primary and metastatic breast cancer and potential marker for early diagnosis. *Breast Cancer Res Treat*. 2014;146(1):163–174.
40. Hanley R, Rieger-Christ KM, Canes D, et al. DNA integrity assay: a plasma-based screening tool for the detection of prostate cancer. *Clin Cancer Res*. 2006;12(15):4569–4574.
41. Castells A, Puig P, Móra J, et al. K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. *J Clin Oncol*. 1999;17(2):578–584.
42. Dianxu F, Shengdao Z, Tianquan H, et al. A prospective study of detection of pancreatic carcinoma by combined plasma K-ras mutations and serum CA19-9 analysis. *Pancreas*. 2002;25(4):336–341.
43. Spindler KLG, Pallisgaard N, Andersen RF, Brandslund I, Jakobsen A. Circulating free DNA as biomarker and source for mutation detection in metastatic colorectal cancer. *PLoS One*. 2015;10(4):1–14.
44. Mouliere F, El Messaoudi S, Pang D, Dritschilo A, Thierry AR. Multimarker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer. *Mol Oncol*. 2014;8(5):927–941.
45. Shinozaki M, O'Day SJ, Kitago M, et al. Utility of circulating B-RAF DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy. *Clin Cancer Res*. 2007;13(7):2068–2074.
46. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature*. 2004;429(6990):457–463.
47. Hoque MO, Feng Q, Toure P, et al. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. *J Clin Oncol*. 2006;24(26):4262–4269.
48. Guerrero-Preston R, Hadar T, Ostrow KL, et al. Differential promoter methylation of kinesin family member 1a in plasma is associated with breast cancer and DNA repair capacity. *Oncol Rep*. 2014;32(2):505–512.
49. Chen J, Gong M, Lu S, et al. Detection of serum Alu element hypomethylation for the diagnosis and prognosis of glioma. *J Mol Neurosci*. 2013;50(2):368–375.
50. Wu T, Giovannucci E, Welge J, Mallick P, Tang WY, Ho SM. Measurement of GSTP1 promoter methylation in body fluids may complement PSA screening: a meta-analysis. *Br J Cancer*. 2011;105(1):65–73.
51. Dumache R, Puiu M, Motoc M, Vernic C, Dumitrascu V. Prostate cancer molecular detection in plasma samples by glutathione S-transferase P1 (GSTP1) methylation analysis. *Clin Lab*. 2014;60(5):847–852.
52. Van Neste L, Herman JG, Otto G, Bigley JW, Epstein JI, Van Criekinge W. The epigenetic promise for prostate cancer diagnosis. *Prostate*. 2012;72(11):1248–1261.
53. Church TR, Wandell M, Lofton-Day C, et al. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. *Gut*. 2014;63(2):317–325.
54. Powrózek T, Krawczyk P, Kucharczyk T, Milanowski J. Septin 9 promoter region methylation in free circulating DNA-potential role in noninvasive diagnosis of lung cancer: preliminary report. *Med Oncol*. 2014;31(4):917.
55. Skvortsova TE, Rykova EY, Tamkovich SN, et al. Cell-free and cell-bound circulating DNA in breast tumours: DNA quantification and analysis of tumour-related gene methylation. *Br J Cancer*. 2006;94(10):1492–1495.
56. Cassinotti E, Melson J, Liggett T, et al. DNA methylation patterns in blood of patients with colorectal cancer and adenomatous colorectal polyps. *Int J Cancer*. 2012;131(5):1153–1157.
57. de Martino M, Klatte T, Haitel A, Marberger M. Serum cell-free DNA in renal cell carcinoma: a diagnostic and prognostic marker. *Cancer*. 2012;118(1):82–90.
58. Ibanez de Caceres I, Battagli C, Esteller M, et al. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. *Cancer Res*. 2004;64(18):6476–6481.
59. Melnikov AA, Scholtens D, Talamonti MS, Bentrem DJ, Levenson VV. Methylation profile of circulating plasma DNA in patients with pancreatic cancer. *J Surg Oncol*. 2009;99(2):119–122.

60. Melnikov A, Scholtens D, Godwin A, Levenson V. Differential methylation profile of ovarian cancer in tissues and plasma. *J Mol Diagn*. 2009; 11(1):60–65.
61. Liggett TE, Melnikov A, Yi Q, et al. Distinctive DNA methylation patterns of cell-free plasma DNA in women with malignant ovarian tumors. *Gynecol Oncol*. 2011;120(1):113–120.
62. Park JW, Baek IH, Kim YT. Preliminary study analyzing the methylated genes in the plasma of patients with pancreatic cancer. *Scand J Surg*. 2012;101(1):38–44.
63. Warton K, Samimi G. Methylation of cell-free circulating DNA in the diagnosis of cancer. *Front Mol Biosci*. 2015;2:13.
64. Tian F, Yip SP, Kwong DL, Lin Z, Yang Z, Wu V. Promoter hypermethylation of tumor suppressor genes in serum as potential biomarker for the diagnosis of nasopharyngeal carcinoma. *Cancer Epidemiol*. 2013; 37(5):708–713.
65. Delgado-Cruzata L, Hruby GW, Gonzalez K, et al. DNA methylation changes correlate with Gleason score and tumor stage in prostate cancer. *DNA Cell Biol*. 2012;31(2):187–192.
66. Pack SC, Kim HR, Lim SW, et al. Usefulness of plasma epigenetic changes of five major genes involved in the pathogenesis of colorectal cancer. *Int J Colorectal Dis*. 2013;28(1):139–147.
67. Ellinger J, El Kassem N, Heukamp LC, et al. Hypermethylation of cell-free serum DNA indicates worse outcome in patients with bladder cancer. *J Urol*. 2008;179(1):346–352.
68. Radpour R, Barekati Z, Kohler C, et al. Hypermethylation of tumor suppressor genes involved in critical regulatory pathways for developing a blood-based test in breast cancer. *PLoS One*. 2011;6(1):e16080.
69. Kneip C, Schmidt B, Seegebarth A, et al. SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer in plasma. *J Thorac Oncol*. 2011;6(10):1632–1638.
70. Lange CP, Campan M, Hinoue T, et al. Genome-scale discovery of DNA-methylation biomarkers for blood-based detection of colorectal cancer. *PLoS One*. 2012;7(11):e50266.
71. Calistri D, Casadio V, Bravaccini S, Zoli W, Amadori D. Urinary biomarkers of non-muscle-invasive bladder cancer: current status and future potential. *Expert Rev Anticancer Ther*. 2012;12(6):743–752.
72. Grossman HB, Messing E, Soloway M, et al. Detection of bladder cancer using a point-of-care proteomic assay. *JAMA*. 2005;293(7):810–816.
73. Wei JT, Feng Z, Partin AW, et al. Can urinary PCA3 supplement PSA in the early detection of prostate cancer? *J Clin Oncol*. 2014;32(36): 4066–4072.
74. Botezatu I, Serdyuk O, Potapova G, et al. Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. *Clin Chem*. 2000;46(8 Pt 1):1078–1084.
75. Su YH, Wang M, Brenner DE, et al. Human urine contains small, 150 to 250 nucleotide-sized, soluble DNA derived from the circulation and may be useful in the detection of colorectal cancer. *J Mol Diagn*. 2004;6(2):101–107.
76. Su YH, Wang M, Aiamkitsumrit B, Brenner DE, Block TM. Detection of a K-ras mutation in urine of patients with colorectal cancer. *Cancer Biomark*. 2005;1(2–3):177–182.
77. Su YH, Wang M, Brenner DE, Norton PA, Block TM. Detection of mutated K-ras DNA in urine, plasma, and serum of patients with colorectal carcinoma or adenomatous polyps. *Ann N Y Acad Sci*. 2008;1137:197–206.
78. Chang HW, Tsui KH, Shen LC, Huang HW, Wang SN, Chang PL. Urinary cell-free DNA as a potential tumor marker for bladder cancer. *Int J Biol Markers*. 2007;22(4):287–294.
79. Zancan M, Galdi F, Di Tonno F, et al. Evaluation of cell-free DNA in urine as a marker for bladder cancer diagnosis. *Int J Biol Markers*. 2009;24(3):147–155.
80. Szarvas T, Kovalszky I, Bedi K, et al. Deletion analysis of tumor and urinary DNA to detect bladder cancer: urine supernatant versus urine sediment. *Oncol Rep*. 2007;18(2):405–409.
81. Casadio V, Calistri D, Salvi S, et al. Urine cell-free DNA integrity as a marker for early prostate cancer diagnosis: a pilot study. *Biomed Res Int*. 2013;2013:270457.
82. Salvi S, Gurioli G, Martignano F, et al. Urine cell-free DNA integrity analysis for early detection of prostate cancer patients. *Dis Markers*. 2015;2015:574120.
83. Birkenkamp-Demtröder K, Nordentoft I, Christensen E, et al. Genomic alterations in liquid biopsies from patients with bladder cancer. *Eur Urol*. 2016;70(1):75–82.
84. Togneri FS, Ward DG, Foster JM, et al. Genomic complexity of urothelial bladder cancer revealed in urinary cfDNA. *Eur J Hum Genet*. 2016;24(8):1167–1174.
85. Xia Y, Huang CC, Dittmar R, et al. Copy number variations in urine cell free DNA as biomarkers in advanced prostate cancer. *Oncotarget*. Epub 2016 Apr 26.
86. Bryzgunova OE, Morozkin ES, Yarmoschuk SV, Vlassov VV, Laktionov PP. Methylation-specific sequencing of GSTP1 gene promoter in circulating/extracellular DNA from blood and urine of healthy donors and prostate cancer patients. *Ann N Y Acad Sci*. 2008;1137: 222–225.
87. Schulz WA, Steinhoff C, Florl AR. Methylation of endogenous human retroelements in health and disease. *Curr Top Microbiol Immunol*. 2006;310:211–250.
88. Ghanjati F, Beermann A, Hermanns T, et al. Unreserved application of epigenetic methods to define differences of DNA methylation between urinary cellular and cell-free DNA. *Cancer Biomark*. 2014;14(5): 295–302.
89. Song BP, Jain S, Lin SY, et al. Detection of hypermethylated vimentin in urine of patients with colorectal cancer. *J Mol Diagn*. 2012; 14(2):112–119.
90. Feng Q, Hawes SE, Stern JE, et al. Promoter hypermethylation of tumor suppressor genes in urine from patients with cervical neoplasia. *Cancer Epidemiol Biomarkers Prev*. 2007;16(6):1178–1184.
91. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011;11(6):426–437.
92. Schwarzenbach H, Alix-Panabières C, Müller I, et al. Cell-free tumor DNA in blood plasma as a marker for circulating tumor cells in prostate cancer. *Clin Cancer Res*. 2009;15(3):1032–1038.
93. Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *Clin Chem*. 2015;61(12):1446–1452.
94. Chaudhuri AA, Binkley MS, Osmundson EC, Alizadeh AA, Diehn M. Predicting radiotherapy responses and treatment outcomes through analysis of circulating tumor DNA. *Semin Radiat Oncol*. 2015;25(4): 305–312.
95. Ignatiadis M, Lee M, Jeffrey SS. Circulating tumor cells and circulating tumor DNA: challenges and opportunities on the path to clinical utility. *Clin Cancer Res*. 2015;21(21):4786–4800.