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"Biological significance of germline alterations in *BRCA1* and *BRCA2* genes and response to DNA damage agents in hereditary breast cancer"

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Chapter 1

Introduction

Breast cancer (BC) is still the most common neoplasm and the leading cause of death among women worldwide, accounting for a quarter of newly diagnosed malignancies in women [1]. In Italy, its incidence is about 40.000 new cases/year, with an overall prevalence of 416.000 cases [2]. The introduction of mammography screening has led to an increase of the incidence of breast cancer and an evident reduction of mortality in the group of women of 50-69 years in European countries [3]. In contrast, survival benefits for age group of 40-49 years are still object of debate. In Italy, screening campaigns have been recently extended to women aged 45-49 years in some regions [4]. In addition, the improvements in terms of treatment approach and outcomes have contributed to reduce mortality, especially in younger age groups [5]. However, the complexity of biological heterogeneity of breast cancer subtypes has been reported to be responsible for diversified outcomes and response to therapy [6]. The traditional classification based on immunohistochemistry markers (ER, PR, HER2) has been overcome by molecular stratification of tumors into main 6 different intrinsic subtypes according to gene expression profiles [7-9]: luminal A (ER+), luminal B (ER+/HER2-enriched), HER2+ (HER2-enriched), basal-like, claudin-low, and normal-like (Fig. 1.1). In spite of the good reproducibility derived from this classification method, unclassifiable breast tumors are not rare.

Among numerous risk factors (lifestyle and environmental factors), genetic predisposition remains the most determinant element affecting breast cancer development after gender and aging [10]. A positive history of breast cancer is associated with a doubled risk for a proband and the presence of more than one affected relatives increases this risk [11]. In this context, pathogenic mutations in *BRCA1* and *BRCA2* genes have been reported to be responsible for the large majority of hereditary breast cancers [12]. With the advent of more sensitive technologies, the identification of high-risk individuals with unknown genetic variants in breast cancer susceptibility genes and candidates genes has been increased [13]. These findings have led to an emerging need to better characterize these alterations in terms of biological and clinical affects in order to design efficient prophylactic and therapeutic programs for specific carriers. In this scenario, this

project has been proposed to contribute to the detection and to the interpretation of new potential high-risk variants in hereditary breast cancer patients.

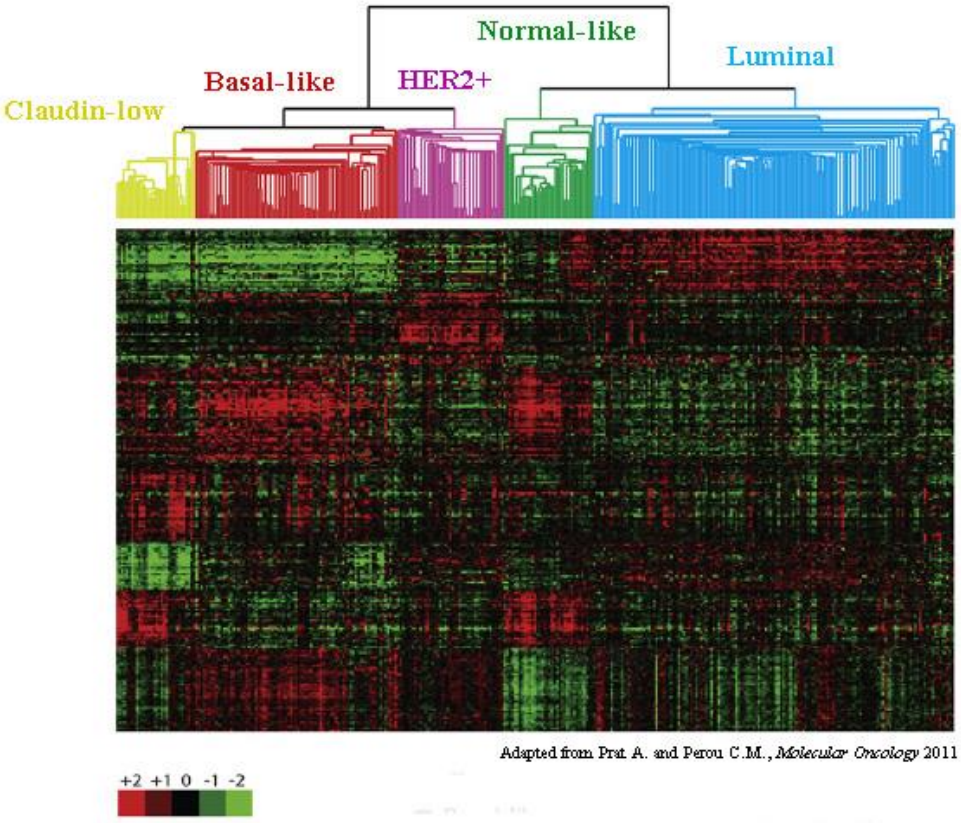

Figure 1.1 Breast cancer is a heterogeneous disease and includes at least five molecular subtypes according to hierarchical clustering based on gene expression

1.1 Hereditary breast cancer

A considerable proportion (15-20%) of all breast cancer cases have familial or hereditary background (Fig. 1.2) [14]. Specifically, approximately 5-10% have a hereditary origin, that are due to a single inherited mutation in known breast cancer susceptibility genes. Up to 30% of these cases are related to the presence of germline pathogenic mutations in the tumour suppressor high-risk genes *BRCA1* and *BRCA2* (~25%), and in other risk genes (~5%) [12]. Individuals with pathogenic *BRCA* mutations are therefore classified at an increased risk of developing breast/ovarian cancers and, less frequently, other malignancies [12]. The remaining 70% fraction accounts for breast cancer cases related to unknown predisposing genes or loci, which probably consist of rare high-risk variants and polygenic mechanisms acting together to confer high breast-cancer risk [15]. Moreover, other hereditary cancer syndromes have been reported to be associated with an increased lifetime risk of breast cancer development caused by genetic abnormalities in different genes, such as Li-Fraumeni (*p53*), Cowden (*PTEN*), Peutz-Jeghers (*STK11*) and Hereditary Diffuse Gastric Cancer (*CDH1*), which are characterized by more frequent cancer cases in family members than *BRCA* carrier family [16].

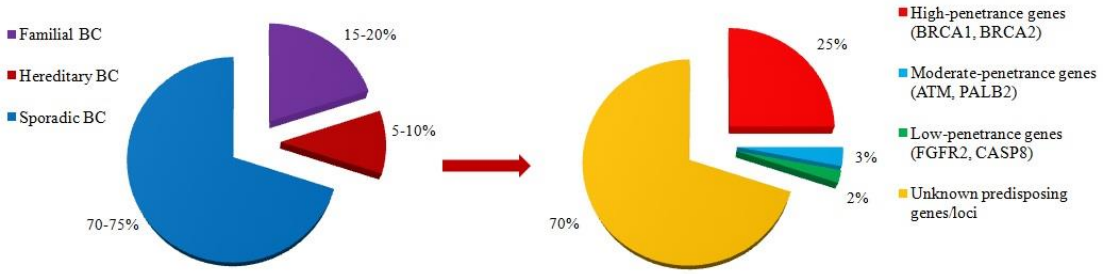

Figure 1.2 Global prevalence of sporadic, familial and hereditary breast cancer. About 30% of all inherited forms of breast cancers can be explained by mutations in single genes.

1.1.1 Genetic counseling and testing for hereditary breast cancer

The National Society of Genetic Counselors (NSGC) defined the first guidelines concerning cancer risk assessment, also known as genetic counseling, for breast cancer associated with the presence of pathogenic mutations in *BRCA1* and *BRCA2* genes [17]. The main purpose is the collection of genetic, biological and environmental information related to the individual's risk of cancer in order to estimate the likelihood of a hereditary cancer syndrome in a family and an individual patient's lifetime risk for cancer. This process allows the identification of patients who may undergo genetic testing for known hereditary breast cancer syndromes. During the past few years, genetic testing for breast/ovarian cancer susceptibility genes has been incorporated into clinical practice. The main criteria of inclusion for high-risk patients are essentially based on the presence on known mutations within family, early onset of disease and/or a positive family history of breast cancer. Table 1.1 reports the complete list of approved guidelines for *BRCA1* and *BRCA2* testing according to the National Comprehensive Cancer Network (NCCN) [18]. Of note, diagnosis of breast cancer at ≤ 45 years and of triple negative breast cancer at ≤ 60 years, represent two important criteria. Generally, genetic testing results include four potential answers which are associated with different clinical risk:

a) Positive result:
- high-risk variants: presence of a mutation with evidence of disrupting normal protein functions, defined as pathogenic or deleterious mutation;
- uncertain-risk variants: presence of a mutation with no evidence of pathogenicity or neutrality, defined as a variant of unknown or uncertain clinical significance (VUS);
- no-risk variants: presence of a mutation with evidence of neutrality, defined as neutral or benign variants.

b) Negative result: absence of any genetic alteration.

In the case of pathogenic mutations, prevention and clinical indications are recommended and proposed to carrier patients (detailed information are given in paragraph "Management of *BRCA* mutation carrier patients"). However, they have been reported to

be detected in only about 20% of familial breast cancer [19]. Therefore, a negative result in either *BRCA*1 or *BRCA2* genes, doesn't imply absence of mutation in different breast cancer susceptibility genes and of cancer risk. Accordingly, individuals within family with a strong history of breast cancer have been shown to present a four-fold increase of cancer risk and additional genes conferring significant risk of breast cancer are emerging [20]. In this context, multi-gene panels are designed for genetic testing of those high-risk individuals tested negative for *BRCA1*/*2* mutations and of individuals with a family history suggesting the presence of multiple hereditary syndromes. Specific criteria for the access to these customized gene panels have been defined [18].

Table 1.1 National Comprehensive Cancer Network eligibility criteria for *BRCA1/2* genetic testing of breast cancer patients

➢ Individual from a family with a known pathogenic *BRCA1*/*BRCA2* gene mutation
➢ Personal history of breast cancer[a] + one or more of the following: o Breast cancer diagnosis at ≤ 45 ys o Breast cancer diagnosis at ≤ 50 ys with: ✓ An additional primary[b] breast cancer ✓ ≥ 1 close blood relative[c] with breast cancer at any age ✓ ≥ 1 close relative with pancreatic cancer ✓ ≥ 1 relative with pancreatic cancer (Gleason score ≥ 7) ✓ An unknown or limited family history o Breast cancer diagnosis at ≤ 60 ys with: ✓ Triple negative breast cancer o Breast cancer diagnosis at any age with: ✓ ≥ 1 close blood relative[c] with breast cancer at ≤ 50 ys ✓ ≥ 2 close blood relatives[c] with breast cancer at any age ✓ ≥ 1 close blood relative[c] with ovarian[d] carcinoma ✓ ≥ 2 close blood relatives[c] with pancreatic cancer (Gleason score ≥ 7) and/or prostate cancer at any age ✓ A close male blood relative[c] with breast cancer ✓ Ethnicity associated with higher mutation frequency (*e.g.* Ashkenazi Jewish)
➢ Personal history of ovarian[d] carcinoma
➢ Personal history of male breast cancer
➢ Personal history of prostate cancer (Gleason score ≥ 7) at any age with: o ≥ 1 close blood relative[c] with ovarian carcinoma at any age o ≥ 1 close blood relative[c] with breast cancer diagnosis at ≤ 50 ys o 2 relatives with breast, pancreatic or prostate cancer at any age
➢ Personal history of pancreatic cancer at any age with: o ≥ 1 close blood relative[c] with ovarian carcinoma at any age o ≥ 1 close blood relative[c] with breast cancer diagnosis at ≤ 50 ys ➢ 2 relatives with breast, pancreatic or prostate cancer at any age
➢ Personal history of pancreatic cancer and Ashkenazi Jewish ancestry
➢ Family history only: o First- or second-degree blood relative[c] meeting any of the above criteria o Third-degree blood relative[c] with breast cancer and/or ovarian[d] carcinoma ➢ ≥ 2 close blood relatives[c] with breast cancer and/or ovarian[d] carcinoma

[a] Invasive and ductal carcinoma in situ breast cancers should be included.

[b] Bilateral (contralateral) disease or two or more clearly separate ipsilateral tumors either synchronously or a synchronously.

[c] First-, second-, and third-degree relatives on same side of family.

[d] Fallopian tube and primary peritoneal cancers.

1.1.2 Breast cancer susceptibility genes

Cancer susceptibility genes are defined as genes whose genetic alterations predispose to hereditary cancer. Penetrance represents the entity of risk of cancer development related to a gene, and it is measured as the proportion of individuals carrying a specific mutation with clinical manifestation of disease [21]. According to a penetrance grade scale, low-penetrance, moderate-penetrance and high-penetrance genes present a relative risk of cancer of 1.5, 1.5-5 and > 5, respectively [21]. Advances in molecular sequencing technology have led to identify multiple breast cancer susceptibility genes different from *BRCA* genes (Fig. 1.3).

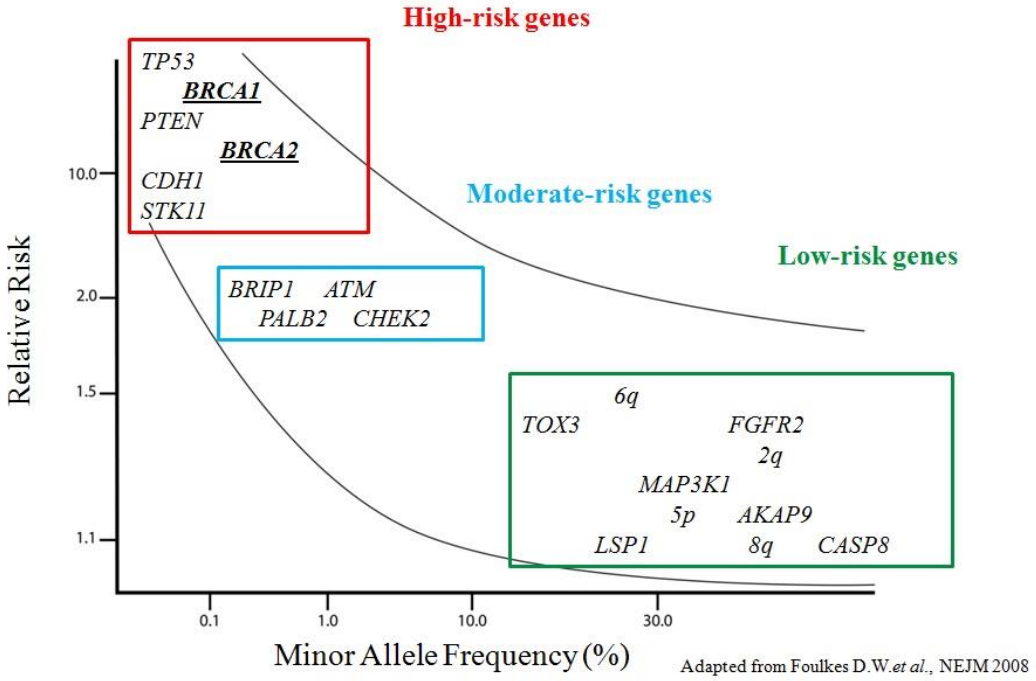

Figure 1.3 Contribution of hereditary susceptibility genes and loci to the development of hereditary breast cancer. *BRCA1* and *BRCA2* mutations are responsible for about 30% of all familiar cases.

1.1.2.1 High-penetrance genes

BRCA1 and *BRCA2* are the two major familial breast/ovarian cancer risk genes, which have identified by genome-wide linkage analysis and positional cloning [21]. Mutations in these genes are transmitted in autosomal dominant manner, in which the mutated gene is the dominant gene located in one of the autosomal chromosomes. They account for about 30% of the familial clustering of breast cancer. The estimated lifetime risk of breast cancer among *BRCA* mutation carriers varies across different studies, ranging from 41% to 90% [22-24]. Recent meta-analysis and prospective analysis reported 57% and 60% estimates for *BRCA1* mutation carriers, and 49% and 55% for *BRCA2* mutation carriers, respectively [23-24]. Similarly, variable prevalence of *BRCA1* and *BRCA2* mutations within different breast cancer patient cohorts have been published [25-28]. The ethnicity, geography localization and the composition of study patient population, and the technique used to test for mutations contribute to all this variability. Moreover, recurrent founder mutations have been identified in specific populations, including Ashkenazi Jews, in Iceland, The Netherlands, Sweden, Norway, Germany, France, Spain, Canada and countries of Eastern and Southern Europe [29]. The most well-described *BRCA1*/*BRCA2* founder effect occurs in the Ashkenazi Jewish population, which is commonly tested for three different recurrent mutations, c.68_69delAG and c.5266dupC in *BRCA1* and c.5946delT in *BRCA2* genes, accounting for > 90% of all detected mutations [29]. In Italy, few founder mutations have been reported in distinct regions, including four *BRCA1* mutations (c.3228_3229delAG, c.3285delA, c.1380dupA, c.5062_5064del3) in Tuscany, *BRCA2* c.8537_8538delAG in Sardinia and *BRCA1* c.4964_4982del19 in the southern part of Calabria [29]. Germline mutations in the other high-risk genes *TP53*, *PTEN* and *STK11* have been described in <1% of breast cancer families and usually associated with rare cancer syndromes, Li–Fraumeni, Cowden and Peutz–Jeghers syndromes, respectively [30-34]. In particular, though the small proportion of Li–Fraumeni syndrome (0.1%) among breast cancer patients, germline mutations in *p53* gene confer a significant increase of lifetime risk of early onset (< 45 years) breast cancer [35-38]. Individuals with mutation in the tumor suppressor genes *PTEN* present a lifetime risk up to 50% for breast cancer [32, 39]. Genetic alterations in *STK11* gene have been detected in 70-80% of patients with Peutz–Jeghers syndrome with an increased breast cancer risk up to 50% [40-42]. Therefore, specific guidelines concerning these breast cancer hereditary syndromes

have been also developed in order to propose genetic counseling and testing to affected individuals [18].

1.1.2.2 Moderate- and low-penetrance genes

Traditional linkage analysis in high risk breast cancer patients allowed the identification of high-penetrance genes [43-44]. On the contrary, moderate- and low-penetrance alleles are inherited without generating disease with high-penetrance profiles and are therefore difficult to track (Fig. 1.3). In this context, genome-wide associations studies (GWAS) have been helpful for the identification of these genes, consisting in the evaluation of association of genetic markers with breast cancer cases in comparison to matched population of unaffected controls. In particular, candidate gene association screening has contributed to the identification of mutations in genes functionally related to *BRCA1* and/or *BRCA2*. They are moderate-penetrance genes and include *CHEK2, ATM, BRIP1*, *PALB2* and *NBS1* [45]. It has been observed that breast cancer risk caused by alterations in these genes is significant higher in familial and/or early onset breast cancer cases [46]. However, only a small fraction, about 5%, of breast cancer predisposition is explained by mutations in these genes [47].

Furthermore, different common (up to 40%) polymorphisms have been identified and associated with 10% to 20% lifetime risk in breast cancer cases through GWAS analysis, including single-nucleotide polymorphisms (SNPs) in *MAP3K1*, *FGFR2*, *LSP1*, *TNRC19, H19* and *CASP*8 [48-54]. Interestingly, the combination of the effects derived from these low-penetrance variants is supposed to confer an increase to breast cancer risk. A large collaborative study has been recently conducted in order to calculate polygenic risk scores based on 30.000 breast cancer cases and controls [55]. The resulting stratification have shown a threefold increased risk of developing breast cancer for individuals in the highest 1% of the score, and a stronger effect of family history and early onset of disease (< 40 years) for individuals in the lowest 1% of the score [55]. Despite all these findings, more than 70% of the familial breast cancer cases still remains unexplained.

1.1.2.3 The advancement in sequencing molecular techniques

After the discovery of *BRCA1* and *BRCA2* as high-risk breast cancer genes, different mutation screening methods have been developed for the identification of mutations, such as direct Sanger sequencing [56], denaturing high-performance liquid chromatography [57], denaturing gradient-gel electrophoresis [58] and single-stranded conformational polymorphism high-resolution melting [59]. These techniques are based on the highly sensitive screening of PCR-amplified individual exons, derived from DNA usually extracted from blood samples. Before progress in molecular techniques, Sanger sequencing approach has been the ideal standard method for the identification of mutations. However, limited sensitivity and incapacity of performing parallel multiple target analysis represent evident limits which need to be overcome. In parallel with sequencing analysis detecting only modifications of small numbers of bases (single base changes, small insertions, small deletions), a minor fraction of all *BRCA1*/*2* mutations may account for large genomic rearrangements (LRs) of DNA sequence able to disrupt gene function. After preliminary studies reported that 6%-18% of individuals tested negative for *BRCA1*/*2* mutation were carriers of LRs in the same genes, in 2007 Multiplex Ligation-dependent Probe Amplification (MLPA) technology was introduced in genetic testing as secondary integrative analysis [20, 35, 60]. In particular, it is a PCR-based technique which allows the determination of the relative copy number of different DNA target sequences using a single primer pair [61-63]. Amplicons of different length are produced because of the presence of differential fluorescent stuffer sequences contained in each probe.

The advent of next-generation sequencing (NGS) technology has changed the scenario of medical genetic testing in terms of sensitivity, efficiency and costs [64-68]. Specifically, a wide spectrum of mutations can be detected, including single nucleotide substitutions, small insertions and deletions, large genomic duplications and deletions, and rare variations [69]. This high-throughput method consists in 1) the design of probes targeting genes of interest, 2) the capture of the targeted genomic regions and 3) the extensive parallel sequencing of the captured DNA. Targeted NGS allows the analysis of amplicons derived from multiple PCR reactions or from different enrichment approaches. Interestingly, the design of amplicon size of < 175 base pairs (bp) is useful for the application of NGS to formalin-fixed tissue samples, whose DNA is highly fragmented

[70]. Several NGS-based platforms are currently used, including the Genome Analyzer/HiSeq/MiSeq (Illumina Solexa), the SOLiD System (Thermo Fisher Scientific), the Ion PGM/Ion Proton (Thermo Fisher Scientific), and the HeliScope Sequencer (Helicos BioSciences) [71-72]. Therefore, the possibility of testing in parallel multiple genes has led to the introduction of multigene panels in clinical laboratories for the screening of mutations in different breast cancer susceptibility genes for high-risk patients [73]. Genetic counseling remains a crucial step for the selection of individuals who may benefit from these analyses.

Based on NGS system, whole-genome sequencing (WGS) and whole-exome sequencing (WES) can be performed with a less expensive sequencing cost per genome/exome. WES represents the most used technique for the identification of rare high-risk genetic variants because of its lower costs and littler amount of data to interpret [74]. In the last few years, more than 45 different WES studies have been conducted for hereditary cancers [75]. Specifically, the first 12 familial breast cancer studies have contributed to identify a moderate number of cancer susceptibility genes [76]. However, this approach presents two main limits regarding the incomplete covering of exons (10%-15% is not covered) and the omission of non coding regulatory regions of the genome [77-78]. Therefore, new efficient and collaborative sequencing efforts are necessary for the detection of new breast cancer susceptibility genes in order to improve risk assessment and genetic counseling.

1.2 *BRCA1* and *BRCA2* genes

The two major familial breast cancer susceptibility genes, *BRCA1* and *BRCA2*, are tumor suppressor genes which encode two proteins acting at different stages of the same pathway of genome protection, the homologous recombination (HR) DNA repair pathway (Fig. 1.4). This process allows the high-fidelity repair of DNA double-strand (ds) breaks (DSBs) occurring in mammalian cells and represents one of the most important pathway underlying the protection of genome integrity in proliferating cells [79]. Interestingly, correlation between HR deficiency and human disease was revealed when BRCA1 and BRCA2 proteins were described as crucial players of HR [80-81]. However, hereditary breast cancer could be also related to the presence of mutations in other HR proteins, as recent findings have reported for PALB2 and RAD51 [82-85].

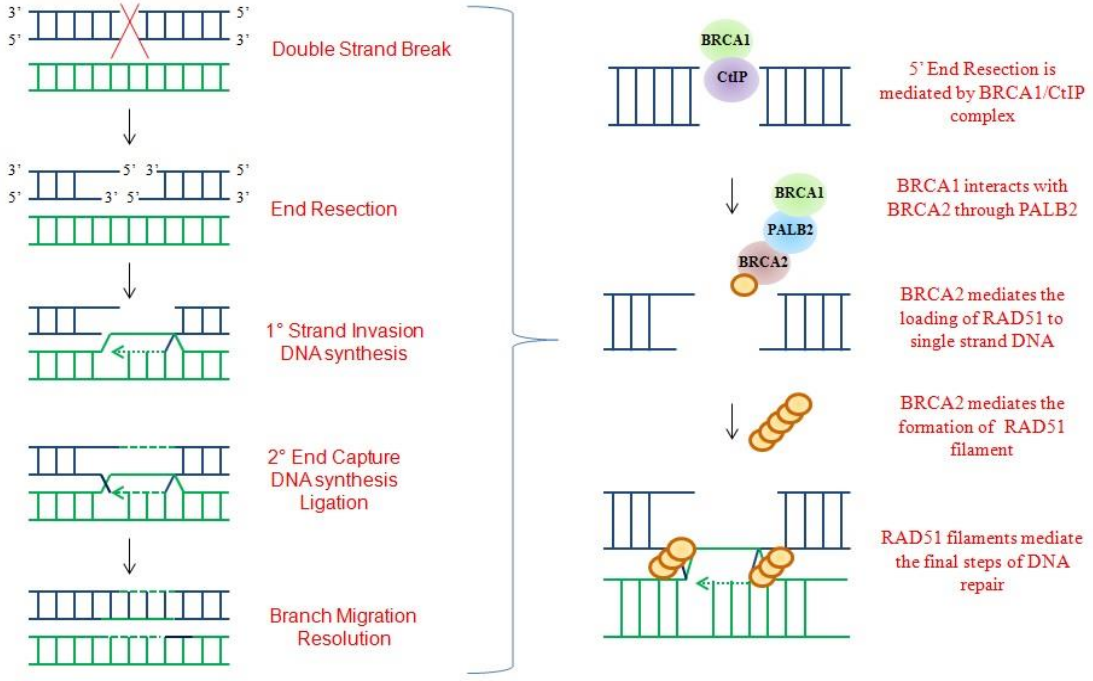

Figure 1.4 Overview of the roles of BRCA1 and BRCA2 proteins in homologous recombination DNA repair mechanism. BRCA1 and BRCA2 act at different steps of homologous recombination DNA repair process.

1.2.1 Protein structures and HR-related biological roles

Human *BRCA1* and *BRCA2* genes encode two proteins of 1863 and 3418 amino acids, respectively. Each one contains different functional domains through which BRCA1 and BRCA2 interact with several proteins of HR pathway and exert their functions (Fig. 1.5). First evidence of their involvement in HR process are based on the findings of their co-localization with the recombinase RAD51 in nuclear foci in mitotic cells [86-89].

BRCA1 protein is mainly composed of three regions, including the amino terminal Really Interesting New Gene (RING) domain followed by a nuclear localization sequence (NLS), a large central portion encoded by exon 11 followed by a coiled-coil domain, and tandem BRCA1 carboxy-terminal repeats (BRCTs) at the carboxy-terminus of the protein (Fig 1.5) [90]. All these structured domains concur to form macro-protein complexes which define BRCA1 involvement in multiple cellular functions, transcription regulation, cell cycle checkpoints activation and DNA repair [91]. Interestingly, numerous *BRCA1* mutation have been identified within the RING and BRCT domains, supporting their role

in the suppression of breast and ovarian cancer [92-94]. Actually, the biological activity of BRCA1 in HR pathway remains one of the most well-defined functions in relation to its tumor suppressor role [95]. BRCA1 is involved in two distinct steps of HR, 5' to 3'resection of DSBs and PALB2/BRCA2 mediated loading of RAD51 onto the generated single strand DNA (ssDNA) (Fig. 1.4) [96-100]. Specifically, BRCA1 co-localizes with the resection complex MRE11-RAD50-NBS1 (MRN) after DNA damage and directly interacts with the resection factor CtIP [101-104]. The proposed model consists in the interaction of BRCA1 with phosphorylated CtIP through its carboxy-terminal BRCT domain to cooperate with the MRN nuclease to mediate resection [101, 103-105]. In the same direction of promoting DNA resection, BRCA1 has been shown to act as antagonize of the resection suppressor 53BP1 [106-107]. In contrast, BRCA1 complexed with the Abraxas-RAP80 macro-complex has been reported to act as anti-resection factor through

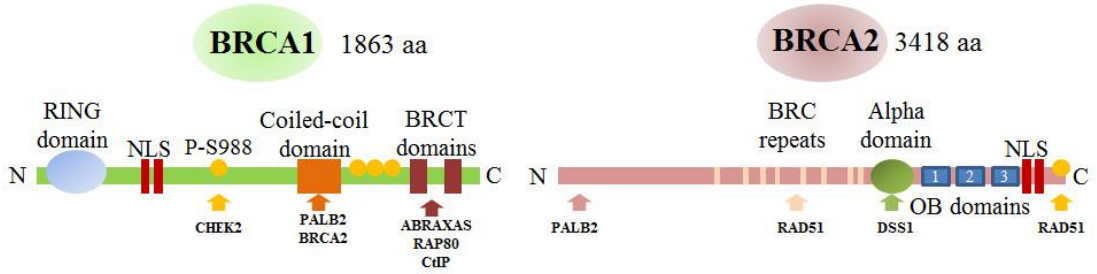

Figure 1.5 Structure of BRCA1 and BRCA2 proteins with their relevant functional domain and molecular interactors. BRCA1 and BRCA2 proteins contain different functional domains through which they interact with other relevant proteins of homologous recombination mechanism.

the association with ubiquitylated histones at DNA DSBs [108]. Overall, these two opposing BRCA1 roles may contribute to explain different grades of resection defects in BRCA1 deficient cells [105, 109-110].

Beside the pleiotropic aspect of BRCA1 (not discussed here), BRCA2 has its fundamental role in HR. The structure of BRCA2 protein is essentially composed by DNA-binding domain (DBD), that binds to ssDNA/ds DNA and to DSS1 protein, and eight BRC repeats which interact with RAD51 (Fig. 1.5) [111-112]. Specifically, DBD region is subdivided in five components consisting in a 190-amino-acid α-helical domain, three oligonucleotide binding (OB) folds that are ssDNA-binding modules, and a tower domain

(TD) that emerges from OB2 and binds dsDNA. In addition to BRC repeats, BRCA2 interacts with RAD51 also through a distinct domain in the carboxyl terminus of the protein. BRCA2 acts directly in the final step of HR, when RAD51 has to be recruited to DSBs in order to repair the damage [113] (Fig. 1.4). This interaction promotes the process of DNA repair via RAD51, contrasting the activation of single-strand annealing (SSA) repair mechanism which is RAD51 independent [114-115]. Unlike RAD51, BRCA2 contains an additional NLS domain which has been suggested to be required to mediates the transport of RAD51 into the nucleus and to DSB sites [116]. Accordingly, *BRCA2*-deficient cells have been shown not to form RAD51 foci after DNA damage [113]. The mechanism of recombination is based on the release of RAD51 from BRCA2 to generate a nucleoprotein filament on ssDNA, allowing its invasion and pairing with a homologous DNA duplex, and the final strand exchange between the matched DNA molecules [113; 115-116]. Interestingly, breast cancer patients have been found to harbor point mutations in BRC repeat region of *BRCA2*, suggesting a potential association between the functional effect of such variants and disease onset [117]. Additionally, a different mechanism of regulation of the recombinational repair has been described. It involves the phosphorylation of residue 3291 in the C-terminal region of BRCA2 and consequent modulation of interaction with RAD51 [118]. However, this binding has been supposed not to be essential for HR, but important in case of absence of functional BRC repeats [119-120]. However, it's not clear how the two RAD51-related regions cooperate to control RAD51 activity.

The role of BRCA2 as DNA-binding protein has been elucidated after the identification of functional domains in structural studies [112]. In particular, the helical domain interacts with DSS1 which has been found to maintain BRCA2 protein stability and to support HR in human cells [112, 121-123]. Interestingly, potential deleterious mutations within BRCA2 DBD region have been identified in breast cancer patients [112]. In addition, specific mutations, compromising the structural integrity of DBD region, have been predicted to affect the function of BRCA2 protein in HR [124-126]. Surprisingly, deletion of the entire DBD domain didn't abrogate BRCA2 function in HR supported by PALB2-mediated recruitment of BRCA2 to DNA [126].

The connection between the functional activity of BRCA1 and BRCA2 proteins has been also documented by the common physically interaction with PALB2. This 1186 amino

acid protein contains two different domains, a coiled-coil domain and WD40 β-propeller domain, which are able to bind to BRCA1 coiled-coil domain and to N-terminus of BRCA2, respectively [97-100, 127]. The formation of BRCA1-PALB2-BRCA2 macrocomplex has been reported to be essential for the BRCA1/PALB2-mediated recruitment of BRCA2 and RAD51 to DSB sites, and for the BRCA2-mediated loading of RAD51 onto ssDNA [97, 99-100]. Additionally, the phosphorylation of S988 amino acid in BRCA1 protein by the kinase CHK2 positively regulates the assembling of BRCA1-PALB2-BRCA2 complex [128].

Therefore, BRCA1 and BRCA2 contribute to regulate the finely tuned molecular cascade of DNA repair HR pathway.

1.2.2 Online databases of *BRCA* genetic alterations

The introduction of analysis of the entire coding sequence of the *BRCA* genes in clinical genetic practice has led to the identification of thousands of different mutations with different biological and clinical significance. Therefore, the collection of these genetic alterations in free public databases was necessary in order to make easier and more efficient the circulation of data regarding *BRCA* variants, in particular those newly identified and without a significance in relation to breast cancer onset. Importantly, HGVS nomenclature (http://www.hgvs.org/) should be assigned unequivocally to variants in order to avoid ambiguity in the comparison of data on the same alteration. The final goal was to translate molecular data into clinical benefit. The Breast Cancer Information Core Database (BIC database, www.research.nhgri.nih.gov/bic/) is the first public archive of *BRCA* mutations, which contains the whole series of reported *BRCA1* and *BRCA2* coding variants, resulting until now in about 4000 mutations in the two genes (1790 in *BRCA1* and 2000 in *BRCA2*), identified from various population studies. Of them, 1300 mutations are defined as pathogenic. It is made up of deleterious, neutral and VUS variants registered by users of different research and clinical laboratories. Specific genetic, molecular and biological information are reported for each variant: designation, number of records, position in gene, nucleotide change, type of mutation, predicted effect on protein, clinical importance, clinical classification and functional data when present (Table 1.2). In particular, an internal BIC-linked database, CIRCOS, has been created as a visualization web resource displaying functional information of all *BRCA1* missense

variants documented in BIC [129]. Overall, pathogenic mutations, covering about 70-80% of all *BRCA* alterations, include mainly frameshift, non sense and intervening sequence types affecting protein functions. Among reported missense mutations, only 6% of them are classified as deleterious. Moreover, LRs fraction represents 1% of all the coding variants. The remaining 30% of variants in BIC database is represented by VUS, whose significance at biological and clinical levels remains to be elucidated. The current number of VUS is estimated at around 1500 [130-132]. Besides the BIC database, ClinVAR at the National Center for Biotechnology Information (NCBI) website is another free accessible archive of germline (and sporadic) *BRCA* mutations (not only) presented with associated clinical significance supported by data of clinical testing laboratories, research laboratories, locus-specific databases, OMIM®, GeneReviews™, UniProt, expert panels and practice guidelines [172]. Each submitter may be update his recorded variant at any time.

Since VUS represents an important intriguing clinical issue, specific databases have been created to organize results of analysis from different laboratories [133-141]. In particular, IARC/LOVD (http://chromium.liacs.nl/LOVD2/cancer/home.php) database classifies *BRCA* variants according to the IARC classification scheme (see next Section "Characterization of variants of unknown significance"), which represents the most used system for the characterization of VUS [135-136]. Moreover, this database includes only those VUS with literature references and links to published source data. Submissions are updated by a named expert curator on a voluntary basis.

Recently, the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) (http://www.enigmaconsortium.org) consortium has been set up with the aim of collecting large datasets of data on VUS in cancer susceptibility gene from international studies and promote large-scale collaborations. Overall, ENIGMA has obtained 6000 submissions of unique VUS, identified in over 13000 families from more than 17 countries [142].

However, a non-constant ongoing update of deposited information, such as variant frequency, characterization or re-classification, and the use of a non-univocal standardized nomenclature, represents the main limit to be overcome for the creation of worldwide system of classification.

Table 1.2 Description of major information included in BIC database for each *BRCA1/2* mutation

Type of information	Description
# of times recorded	Number of family members carrying mutation
Accession number	Unique identifier generated at the time mutation is entered into database
Exon	*BRCA1* or *BRCA2* exon in which mutation has been identified
NT	Nucleotide number in the transcript (cDNA) at which mutation occurs
Codon	Triplet codon in which mutation occurs
Base Change	Nucleotide difference compared to reference sequences
AA change	Resulting change in the encoded amino acid sequence
BIC Designation	Designation of described mutation according to BIC nomenclature guidelines
HGVS cDNA	Designation of described mutation according to HGVS nomenclature guidelines for cDNA sequence
HGVS Protein	Designation of described mutation according to HGVS nomenclature guidelines for protein sequence
HGVS Genomic	Designation of described mutation according to HGVS nomenclature guidelines for genomic sequence
dbSNP	NCBI accession number of described mutation
Mutation Type	Type of described mutation: 3'UTR 3-prime Untranslated Region 5'UTR 5'UTR 5-prime Untranslated Region F Frameshift IFI In Frame Insertion IFD In Frame Deletion IVS Intervening Sequence M Missense N Nonsense P Polymorphism S Splice Syn Synonymous UV Unclassified Variant
Clinically Importance	Clinically significance based on available data: Yes Clinically Important No Not Clinically Important Unknown Undetermined Clinically Significance
Clinical Classification	Class of BIC Classification based on a combination of systems[a]
Functional Data	When available, data from functional studies and *in silico* analysis, collected in CIRCOS database

[a] Plon SE *et al.* 2008 Hum Mutat; Spurdle AB *et al.* 2008 Hum Mutat; Walker LC *et al.* 2013 Hum Mutat; InSiGHT 2014 Nat Genet

1.2.3 Molecular/clinical features and prophylaxis of *BRCA1/2*-related patients

About 3-5% of all breast cancers have been associated with the presence of pathogenic *BRCA1*/*BRCA2* germline mutations [143]. *BRCA*-related tumors have been shown to display different pathological characteristics compared to tumors derive from non carrier patients. In particular, invasive ductal carcinoma is the prominent (> 80%) histology type arising in *BRCA1*/*BRCA2* mutation carriers and *BRCA1*-related tumors present a higher frequency of medullary carcinomas (9%) compared to sporadic tumors (2%) [143-144]. On the contrary, the majority of *BRCA2* mutation carriers develop lobular and tubular carcinomas [143; 145]. In addition, *BRCA1*-related tumors have been reported to shown a more aggressive phenotype, including higher tumor grade, proliferation index, necrotic areas and higher frequency of somatic abnormalities in prognostically important genes such as p53 [143, 146-150]. Interestingly, these tumors were likely to be associated with a triple-negative histology (70%) and a basal/myoepithelial phenotype, which often correlates with poor prognosis, although mutations have also been reported in up to 20% of luminal breast tumors [146, 151-154]. Conversely, *BRCA2*-related tumors show patterns of expression more similar to sporadic tumor, with overexpression of ER, PR (luminal traits) and of the cytokeratins CK8 and CK18 [155]. In this scenario, data regarding contralateral risk of breast cancer have been clearly reported: when the first *BRCA1*-related breast cancer develops under 40 years of age, the contralateral risk is 60% and for *BRCA2* is 25% [156].

However, data regarding *BRCA* mutations and breast cancer prognosis are still inconsistent, probably due to the relative rarity of such mutations in the population, the large variation in sample sizes of several studies and additional environmental and concurrent genetic factors [156-159]. Recently, a systematic review of all published studies, reporting survival data related to *BRCA* mutation carriers, has explored how study design differences could determine the disparities in outcome [160]. The authors concluded that these patients did not present a worse breast cancer survival in the adjuvant setting [160].

Therefore, individuals tested positive for pathogenic germline mutations in *BRCA1*/*2* genes are candidates to surveillance and preventive strategies, including contralateral/bilateral mastectomy and premenopausal bilateral salpingo-oophorectomy,

which have been substantially shown to reduce the risk of developing the disease and to improve survival [161-162].

1.2.4 DNA damage agents in *BRCA* deficient-related tumors

Medically treatment of hereditary *BRCA*-related breast cancer is currently generally dictated by histology, stage and immunohistochemistry (ER, PR, HER2) rather than the identification of *BRCA* status. In the last years, elucidation of mechanisms underlying the biological roles of BRCA1 and BRCA2 proteins and therefore of the molecular effects of their genetic aberrations, have contributed to investigate new options of treatment for these patients, in particular novel targeted therapies. The notion that inactivation of *BRCA1* and *BRCA2* produces the impairment of HR DNA repair, has led to propose studies on the use of DNA cross-linking agents (platinum salts) and poly(ADP-ribose) polymerase (PARP) inhibitors [163]. Specifically, cisplatin or carboplatin are platinum-based drugs which induce the formation of intra- or inter-strand cross-links in DNA molecules. This damage type requires HR mechanism for its correct repair, showing that defects in this pathway confer drug sensitivity in cancer cells. Accordingly, positive encouraging results have been recently published regarding sensitivity to cisplatin, which has been correlated with high response rate in patients with *BRCA1* mutations [164-165]. However, the most promising data come from preclinical and clinical studies on the use of PARP inhibitors, which result less toxic than cisplatin. The mechanism of action of these drugs is based on the inhibition of PARP1 enzyme, which is involved in base excision repair (BER) process directed to protect from ssDNA lesions [163]. Accumulation of this type of DNA damage can be responsible for degeneration and formation of DSBs during DNA replication [163]. *BRCA*-mutated cells, which present defects in HR pathway, have been demonstrated to be highly sensitive to PARP inhibitors [166-168]. Accordingly, early clinical trials have reported significant efficiency of PARP inhibitors in *BRCA*-deficient breast and ovarian cancers [169-170]. In particular, phase I and II trial results confirmed the efficacy of PARP inhibitors in recurrent or advanced ovarian cancer as well as an another phase II trial specifically for Olaparib (AZD 2281) in maintenance therapy of patients with recurrent, platinum-sensitive, high-grade serous ovarian cancer [169;171-173]. Consequent phase III prospective controlled randomized trial is now recruiting *BRCA* carrier patients with stage III-IV high-grade serous tumors

after first-line treatment with platinum-based therapy (ID: GOG 304). Finally, Olaparib (AZD-2281) was the first oral drug approved by FDA for patients with deleterious germline *BRCA*-mutated advanced ovarian cancer treated with three or more lines of chemotherapy [174]. Similarly, Olaparib and Velaparib (ABT-888) have demonstrated to contribute to improve PFS and safety in triple negative and *BRCA*-related advanced breast cancers [175]. Researchers are now waiting for results from an ongoing phase III trial (OlympiA) consisting in analyzing the effects of Olaparib in adjuvant therapy in patients with HER2 negative *BRCA*-related breast tumors. On the contrast, a different PARP inhibitor drug, Iniparib, was evaluated as non-functional after negative results obtained from phase studies investigating Iniparib as adjuvant treatment for metastatic triple-negative breast cancer [176]. All these studies contribute to make evident the need of a selection of breast cancer patients who will respond to PARP inhibitors or different targeted therapies.

1.3. Characterization of variants of unknown significance

During the past few years, genetic testing for breast/ovarian cancer susceptibility genes has been incorporated into clinical practice, however up to 20% of families tested for *BRCA* mutations show at least one VUS [177-178]. The proportion may fall to 5% in a well-characterized population [179-181]. With the generation of more sensitive technologies, the detection of mutations has been increased, and the proportion of VUS cases is likely to increase. BIC database collects almost 1800 distinct *BRCA1* and *BRCA2* VUS, consisting in missense mutations, small in-frame deletions, splicing variants and variants in regulatory sequences. The majority of them are rare variants and case-control association studies are not able to determine their cancer risk. In the clinical practice, such results are very difficult to interpret and manage because of the relative lack of data in favor or against surveillance and prophylactic approaches. Regular update of the International genetic databases, co-segregation analysis, functional analysis and epidemiology may be helpful to better understand the potential impact of these variants on the lifetime risk of developing cancer. In fact, different studies have demonstrated that some VUS may have important biological effects resulting in the alteration of the normal activity of BRCA1 and -2 proteins [182]. Specifically, variants occurring within determinant functional domains have been reported to negatively affect BRCA nuclear

functions including DNA repair, transcriptional activity and cell cycle regulation, explaining their potential pathogenicity in terms of increased risk for the development of breast cancer [182-183]. Therefore, the correct characterization and interpretation of VUS is becoming increasingly important. Currently, several research groups are performing analysis of VUS in order to increase biological information and to improve clinical management of carriers of specific VUS. In this context, IARC scheme has been developed to provide a unique classification scale [134]. It includes five different classes defined by the probability for an allelic variant of being pathogenic: “non-pathogenic” or of “no clinical significance” (Class 1, < 0.001), “likely non pathogenic” or of “little clinical significance" (Class 2, 0.001–0.049), “uncertain” (Class 3, 0.05–0.949), “likely pathogenic” (Class 4, 0.95–0.99) and “definitely pathogenic” (Class 5, > 0.99). The IARC group had also propose clinical recommendation for each class (Table 1.3). The classification of VUS is based mainly on the development and application of multifactorial likelihood prediction models [181]. They essentially consist in integrated analysis of direct and indirect data obtained from different types of methods.

Table 1.3 Classification scheme of variant of unknown significance (VUS) according to the International Agency for Cancer Research (IARC)

Class	Probability of Pathogenicity	Genetic Testing	Surveillance
1	< 0.001	No testing of at-risk relatives	Not recommended
2	0.001 – 0.049	No testing of at-risk relatives	Not recommended
3	0.05 – 0.949	No testing of at-risk relatives	Based on family history
4	0.95 – 0.99	Testing of at-risk relatives	High-risk
5	> 0.99	Testing of at-risk relatives	High-risk

This table is adapted from Moghadasi S. *et al.* 2015 Hum Mutat

1.3.1. Direct methods

Direct methods provide information at genetic level of identified VUS. They include the frequency of the variant in cases and controls, its co-segregation with the disease in families, co-occurrence with a deleterious mutation in the same gene, and personal and family history of cancer of the carriers of VUS. Each approach is directed to provide a value of cancer risk associated to a VUS. A direct estimate can be obtained by case-control studies, which however require unavailable large numbers of individuals with the specific allelic variant [184]. Another powerful resource is represented by the study of personal and family history, and the observation of the segregation of VUS with the disease [185-186]. In particular, when a variant is present a family with multiple cases of breast cancer, it is supposed to be more likely associated with the disease [185]. In parallel, Mohammadi L. *et al.* developed an algorithm to calculate likelihood ratios (LRs) of a VUS being pathogenic, based on gender, genotype, present age and/or age of onset for breast and/or ovarian cancer [186]. In this case, the limit may be the impossibility of including additional affected family members, mainly for ethical reasons. Finally, the co-occurrence of a VUS *in trans* with a deleterious mutation can be indicative of its non pathogenicity. In the case of dominant condition where a pathogenic variant has already been identified, the presence of a second sequence variant seen *in trans* may help exclude pathogenicity, since a second pathogenic variant would be lethal [187-188]. However, careful evaluation of potential more aggressive phenotypic effect of variant should be taken into consideration in order not to exclude its pathogenicity.

1.3.2. Indirect methods

Indirect evidence includes histopathological tumor features, the occurrence of loss of heterozygosity (LOH) in tumor DNA and, limited to missense variants, the severity of the amino acid change and its conservation across species. Specific immunohistochemical and molecular characteristics can be considered distinct traits of tumors derived from carriers of pathogenic mutations. As described before, *e.g. BRCA1*-related tumors are usually characterized by a basal-like phenotype [147]. However, pathogenic mutations are preferentially protein-truncating variants and histopathology of tumors may not resemble profiles of those derived from carriers of potentially pathogenic missense VUS. Thus, correlation between a VUS and phenotype of related-tumor has to be significantly demonstrated in large datasets.

LOH analyses are integrated into multifactorial classification models based on the classical Knudson's two-hit hypothesis, which states that following the event of a germline mutation a second inactivating somatic mutation involves the loss of the wild-type allele, as occurs in the 80% of tumors [189-192]. The calculation of LRs implies the inclusion of LOH frequency of control groups (*BRCA* negative individuals). However, precise frequency of LOH of *BRCA1* and *BRCA2* loci in non *BRCA*-related familial tumors are unknown.

The lack of data sufficient to make available likelihood methods able to classify VUS, contributes to the establishment of complementary approaches, including *in vitro* functional assays and the use of *in silico* tools capable to distinguish VUS of different levels of risk [131; 193]. In particular, functional assays measure the effect of variant on protein activity whose functions are well understood. However, the link between these effects and cancer risk may be difficult to establish. Based on known function of BRCA1 and BRCA2 proteins, specific methods have been developed to analyze *BRCA* alterations, such as transcriptional and BARD1-correlated ubiquitin ligase activity for BRCA1, and homologous recombination assay for BRCA2 [194-196]. Transcriptional assay is based on the transcriptional activity of C-terminus region of BRCA1 and it consists on the DBD fusion experiments performed in yeast and mammalian cells [197]. Similarly, ubiquitin ligase activity of BRCA1 is linked to the interaction at RING domain with BARD1 and the enzyme UbcH5a. Pathogenic mutations have already been identified in this domain and have been correlated with the loss of this ligase activity [198]. In relation to BRCA2, the effective functional assay is represented by homologous recombination repair assay which allows the Green fluorescent protein (GFP)-dependent measurement of fidelity of DSB repair in mutated mammalian cells through the pDR-GFP-I-SceI approach [196; 199]. This assay can be applied to variants located within BRCT domain, RAD51 binding domain and PALB2 binding domain [179, 200-201].

Missense and splicing site mutations represent the majority of VUS. In these cases, a clear effect on the protein integrity can't be established. Additionally to previously described approaches, bioinformatics tools have been developed to predict the structure of protein encoded by a specific missense VUS or the nature of modifications on mRNA splicing induced by a splicing site mutation (Table 1.4). Currently, many *in silico* programs (as freely accessible programs or softwares with a license) are available and provide scores

based on the prior probability that a sequence change will disrupt the ‘‘normal’’ chemical structure of BRCA protein [202].

For missense mutations located within known functional domains of BRCA protein, Align-GVGD (http://agvgd.iarc.fr/alignments.php) is used for the assessment of these substitutions and is able to predict the neutrality of a mutation combining protein multiple sequence alignments with biochemical characteristics of amino acids [203]. In addition, PolyPhen (http://genetics.bwh.harvard.edu/pph/), SIFT (http://blocks.fhcrc.org/sift/SIFT.html) and PROVEAN (http://provean.jcvi.org/index.php) softwares predict the effects of UVs on structure and function of protein. In particular, PolyPhen is based on an algorithm which assigns a VUS to one of three classes defined as probably damaging or deleterious, possibly damaging, or benign [204]. Similarly, SIFT and PROVEAN results provide scores for which a protein variant is predicted to have a "deleterious" or a "neutral" effect [205-206]. Both of them can be applied to naturally occurring non synonymous polymorphisms or laboratory-induced missense mutations [205-206]. Recently, computational analyses of the effect of missense variants on protein structure have also been applied to the classification of VUS [207-208].

Potential splicing aberrations can be analyzed by different web-based programs (Table 1.4). The analysis can be performed at two levels in order to obtain different information: 1] modifications in consensus sequences of donor and acceptor splice sites within intronic or exonic regions (*e.g.* Human Splicing Finder, Gene Splicer); 2) creation of exonic or intronic splice enhancer (ESE/ISE), or/and of exonic or intronic splice silencer (ESS/ISS) (*e.g.* Human Splicing Finder, ESE finder) [209]. The programs are based on the recognition of multiple sequences recognized as potential binding sites of splicing factors [209]. However, the sensitivity and specificity of this these programs are not well defined [185]. In addition, a variant that is predicted to affect mRNA splicing, but not confirmed to alter a functional domain, should be considered of uncertain significance.

Overall, multivariate models may comprehend the integration of multiple type of data (genetic, epidemiological, *in vitro*, *in silico*, tumor histopathological features) in order to better characterize the role of a specific VUS in cancer risk and to improve clinical management of VUS carriers.

Table 1.4 The most used *in silico* tools for the prediction of effect of *BRCA* VUS on protein and mRNA splicing

Bioinformatic Tool	Score adopted and/or Results
Missense Mutations	
Align-GVGD[a]	7-scale scoring system: C0-C65. C0 = neutral C15-C25 = uncertain C35-C65 = likely deleterious
Polyphen2[b]	Calculation of the Naive Bayes posterior probability that a given mutation is damaging and reports estimates of false positive (the chance that the mutation is classified as damaging when it is in fact non damaging) and true positive (the chance that the mutation is classified as damaging when it is indeed damaging) rates. A mutation is also defined qualitatively, as benign, possibly damaging or probably damaging.
SIFT[c]	Score ranging from 0 to 1. The amino acid substitution is predicted damaging is the score is ≤ 0.05, and tolerated if the score is > 0.05.
PROVEAN[d]	If the PROVEAN score is equal to or below a predefined threshold (-2.5), the protein variant is predicted to have a "deleterious" effect, otherwise the variant is predicted to have a "neutral" effect.
Splicing Mutations	
Human Splicing Finder	Combination of 12 different algorithms to identify and predict mutations' effect on splicing motifs including the acceptor and donor splice sites, the branch point and auxiliary sequences known to either enhance or repress splicing: Exonic Splicing Enhancers (ESE) and Exonic Splicing Silencers (ESS).
Gene Splicer	Combination of three models of three models for splice-site prediction. The final score for a given sequence is obtained by a combined contribution of the three methods.
ESE finder	Rapid analysis of exon sequences to identify putative ESEs responsive to the human SR splicing factors F2/ASF, SC35, SRp40 and SRp55, and to predict whether exonic mutations disrupt such elements.

SR proteins, Ser/Arg-rich proteins

[a] Align Grantham Variation and Grantham Deviation.

[b] Polymorphism Phenotyping v2

[c] Sorting Intolerant From Tolerant

[d] Protein Variation Effect Analyzer

Chapter 2

Aims of the study

Although the large number of studies investigating *BRCA* alterations and their clinical role in different populations and ethnicities, there is a lack of a systematic analysis on these alterations in Italian cohorts, including the analysis of VUS, that may contribute to elucidate biological impact and differential treatment response to current chemotherapies and to new targeted therapies, particularly DNA damaging agents.

In this project, we proposed to investigate the role of *BRCA1* and *BRCA2* alterations in terms of clinical impact, biological effects and potential response to therapy in a consecutive cohort of Italian hereditary breast cancer patients, with particular attention to the significance of genotype-phenotype associations for VUS.

Three distinct phases have been defined:

1. Clinical impact: evaluation of the distribution of type of germline *BRCA1* and *BRCA2* alterations, and of their clinico-pathological associations in a cohort of *BRCA* breast cancer patients.

2. Biological impact: evaluation of the biological role of *BRCA* VUS in terms of molecular and *in silico* characterization in relation to their potential functional effect.

3. Response to therapy: evaluation of the potential effects of DNA damaging agents as single agents in relation to the presence of specific *BRCA* alterations in *in vitro* experiments.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1. Study population and data collection

All breast cancer patients who attended the Genetic Counseling Clinic (GCC) for breast/ovarian cancer at Humanitas Cancer Center between June 2008 and May 2014 were retrospectively evaluated. During the GCC personal and familial cancer history was collected and compared against the Institutional eligibility criteria for *BRCA1/2* testing (Table 3.1). If the data met at least one of the criteria, the opportunity to be tested was discussed with the patient and all the implications were explained. Upon the informed consent signature, genetic analyses were performed at Cogentech laboratories (Consortium for Genomic Technologies, Milan, Italy). All the genetic mutations detected were evaluated and interpreted for their significance according to BIC and LOVD databases (http://research.nhgri.nih.gov/bic/ and http://www.lovd.nl/3.0/home). All the patients tested positive for *BRCA1* and/or *BRCA2* mutational analysis were enrolled in the case arm of the study. Patients who tested negative for the same analysis were evaluated for the control arm, and enrolled if at least two first-degree relatives from the same family arm were affected with breast and/or ovarian cancer. A database was created in order to collect all the patients' demographic, lifestyle, clinical, histopathological and genetic information. This study was approved by the Ethics Committee at the Humanitas Clinical and Research Center.

Table 3.1 Institutional eligibility criteria applied for the selection of breast cancer patients for *BRCA1/2* genetic testing

Personal criteria	Familial criteria
Early age onset breast cancer (≤ 40 ys)	Breast cancer diagnosed ≤ 50 ys + one first degree relative diagnosed with breast cancer ≤ 50 ys
Ovarian cancer at age ≤ 50 ys	Breast cancer diagnosed ≤ 50 ys + one first degree relative diagnosed with ovarian cancer
Two primary breast cancer with the first occurring at age ≤ 50 ys	Breast cancer diagnosed ≤ 50 ys + one first degree relative diagnosed with bilateral breast cancer
Breast and ovarian cancer at any age	Breast cancer diagnosed at any age + two first degree relatives diagnosed with ovarian cancer
Male breast cancer at any age	Ovarian cancer at any age + one first degree relative diagnosed with ovarian cancer
Triple negative breast cancer at age ≤ 50 ys	Breast cancer diagnosed ≤ 50 ys + one first degree relative diagnosed with breast cancer ≤ 50 ys
Particular ethnicity (Ashkenazi Jewish)	Breast cancer diagnosed ≤ 50 ys + one first degree relative diagnosed with ovarian cancer
BRCA mutation carrier present in the family	

ys, years

3.1.2. Breast cancer cell lines

MCF7, MDAMB231 and HCC1937 were obtained by American Type Culture Collection (ATCC) and were grown according to manufacturers' instructions. Briefly, MCF7 and MDAMB231 were grown in Dulbecco's modified Eagle's medium (DMEM) medium, and HCC1937 in RPMI1640 medium. Medium was supplemented with heat-inactivated 10% Fetal Bovine Serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. All cell lines were grown in a humidified atmosphere at 37°C and 5% CO2.

3.2. Methods

3.2.1. *BRCA1* and *BRCA*2 mutation analysis and molecular characterization

BRCA1 and *BRCA2* mutation analysis was performed on peripheral blood specimens by Sanger sequencing and by Multiplex-Ligation-dependent Probe Amplification. *BRCA1/2* mutations were classified according to their potential functional effect as known mutations or VUS. ER, PR, Ki-67, and HER2 status of breast tumors were extracted from pathology reports with data obtained from IHC analysis of sections from formalin-fixed paraffin-embedded (FFPE) primary mammary tumor blocks. HER2 status was assessed by IHC and FISH analysis. Breast tumors classification and cut-off values for receptor markers and Ki-67 were set according to St. Gallen guidelines [210]. All mutations are described according to the HUGO-approved systematic nomenclature (http://www.hgvs.org/mutnomen/) or to the traditional mutation nomenclature of BIC database (http://research.nhgri.nih.gov/bic/).

3.2.2. *In silico* protein prediction model for *BRCA1* and *2* variants

In silico analysis of the *BRCA1* and *BRCA2* VUS was performed using different freely available tools including Align-GVGD [http://agvgd.iarc.fr/agvgd_input.php], PolyPhen-2 [http://genetics.bwh.harvard.edu/pph2/index.shtml] and PROVEAN [http://provean.jcvi.org/seq_submit.php] web-based platforms and Human Splicing Finder (HSF) for alternative splicing prediction [211]. The *BRCA1* CIRCOS web tool resource was used for extracting all the current information on *BRCA1* missense variants, including multidimensional genomic data [212].

3.2.3. Structural protein modeling for *BRCA* variants

Molecular modeling of protein variants has been based on the availability of three-dimensional data for one region of BRCA1. The PDB file 4IFI reports the X-ray solved structure of the 1646-1859 region, and it has been used to simulate the Trp1837Arg substitution variant. This variant has been modeled by the MODELLER software, with the help of the mutate_model script [213]. Solvent exposure of amino acids has been evaluated with the NACCESS software [http://wolf.bms.umist.ac.uk/naccess]. Models of wild type and variant proteins have been observed by visual inspection and graphically represented with the InsightII software (Accelrys, Inc., San Diego, USA).

3.2.4. Immunohistochemistry assay

Two-micron thick sections of FFPE tissues were cut and used for immunohistochemical staining of BRCA1, BRCA2 and γ-H2AX proteins. In particular, we used a previously validated BRCA1 antibody [214]. For all antibodies staining, antigen retrieval was carried out by heating slides in 0.1 M citrate buffer in a water-bath set to 98°C for 25 minutes. Blocking steps were performed for all antibodies. Slides were then autostained for 1 hour with the mouse monoclonal anti-BRCA1 (GTX70113, Genetex, 8F7 clone, 1:200) and anti-BRCA2 (NBP1-41189, Novus Biologicals, 1:100) antibodies, and with the rabbit polyclonal γ-H2AX antibody (ab2893, Abcam, 1:1000). Two-step visualization with the MACH4 for BRCA1, and MACH1 for BRCA2 and γ-H2AX, were performed using a Universal HRP-Polymer Kit (BioCare Medical). Diaminobenzidine tetrahydrochloride (Dako) was used as chromogen. Nuclei were counterstained with a hematoxylin solution (Medite, Bergamo, Italy). BRCA1, BRCA2 and γ-H2AX nuclear staining was evaluated in tumor cells. Different features were evaluated, including staining intensity, protein location and total percentage of positive cells in the tissue section. BRCA1 and BRCA2 nuclear expression was scored as < or > 90%. BRCA1 cytoplasmic staining was scored as present or absent. Normal mammary glands were used as internal positive control. γ-H2AX nuclear staining was scored as low or high according to 50% cut off. Tumors from *BRCA* mutation non carriers were used as control group.

3.2.5. RNA isolation and qRT-PCR analysis

Total RNA was extracted from two 10-micron thick sections of each FFPE tumor tissue using High Pure miRNA Isolation Kit (Roche) according to manufacturer's instructions. RNA purity and concentration were determined from readings of absorbance using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). RNA samples with A260/A280 ratio of 1.8-2.0 were used for qRT-PCR analysis of *BRCA1*, *BRCA2* and *γ-H2AX* expression. Briefly, cDNA was obtained from retrotranscription of RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) according to manufacturer's instructions. Quantitative RT-PCR was performed using the following PrimePCR™ assays (Bio-Rad) diluted in the SsoAdvanced™ Universal Probes Supermix (Bio-Rad): *BRCA1* (Custom design, Forward: GGCTATCCTCTCAGAGTGACATTTTA, Reverse: GCTTTATCAGGTTATGTTGCATGGT), *BRCA2* (Assay ID: qHsaCEP0052184). Expression level of *β-actin* (Assay ID: HsaCEP0036280) was used for normalization. RT-PCR was performed in duplicate for each case on ABI Prism 7900HT real-time RT-PCR detection system (Applied Biosystems™) as described by the manufacturer (Bio-Rad). Forty cycles of amplification were performed and fluorescent signals of probes were used to generate threshold cycle (C_t) to calculate gene expression levels. The $\Delta\Delta C_t$ method for relative quantification of gene expression was used to determine gene expression levels. The ΔC_t was calculated by subtracting the C_t of housekeeping gene from the C_t of each gene of interest. The $\Delta\Delta C_t$ was calculated by subtracting the ΔC_t of the reference sample from the ΔC_t of each sample. RNA extracted from normal breasts was used as reference sample for the calculation of $\Delta\Delta C_t$.

3.2.6. CRISPR/Cas9-mediated transfection for the generation of mutated cells in *BRCA1* gene

3.2.6.1. Design of plasmids and donor DNA as HDR template

Two different pCas9-Guide vectors expressing human codon-optimized Cas9 and guide RNA (gRNA), and one donor double-stranded(ds)DNA were designed in order to generate MDA-MB-231 breast cancer cells harboring the specific point mutation c.5509

T>C (BIC entry: W1837R) in *BRCA1* gene. A designing tool at the Blue Heron website (wwws.blueheronbio.com) was used to select the two gRNA sequences (gRNA1 and gRNA2) with the best sequence specificity. Functional cassette containing GFP reporter gene was introduced in pCas9-Guide vectors (pCas-Guide-EF1a-GFP) for the monitoring of transfection efficiency and the sorting out of transfected GFP positive cells. Donor dsDNA was obtained from the annealing of two designed oligos, according to manufacturer's protocol (www.OriGene.com), and was used as template for Homologous DNA Repair (HDR).

3.2.6.2. Optimization of *in vitro* transfection

Transfection efficiency was firstly evaluated with the visual monitoring of green GFP-related signal using a fluorescent microscope at 24, 48 and 72 hours post transfection (Olympus TH-200 microscope). Secondly, absolute numbers of transfected cells were determined via Fluorescence Activated Cell Sorting (FACS). Briefly, 18-24 hours before transfection MDA-MB-231 were plated at a density of 10^5 cells/well in 12-well culture plates in complete growth medium. After overnight incubation, mixtures containing different amounts of pCas-guide vectors gRNA1 or gRNA2, of donor dsDNA and of TransIT-X2 transfection reagent (Mirus) were diluted in 100 µl of Opti-MEM (Life Technologies, Inc.) and incubated at RT for 30 minutes. DNA mixtures were used to treat cells and culture medium was changed 6 hours post transfection. After three days, cells were washed with PBS, trypsinized, collected by centrifugation and resuspended in cell sorting medium for FACS analysis (FACSAria Cell Sorter, BD Biosciences). Transfection efficiency of G1 and G2 vectors were compared. Cell viability was also evaluated in order to ensure sufficient cells for FACS enrichment. Positive and negative controls were included in each experiment.

3.2.6.3. Validation of genome editing

3.2.6.3.1. Clonal expansion of transfected cells

FACS was used to sort out GFP positive cells obtained from transfection under optimized conditions. Fluorescent positive cells were plated into 96-well culture plates with ten cells per well in 100 µl of complete medium. For each experimental replicate, two plates of cells were seeded. Cells were grown and monitored for single colonies per well for three

weeks. Each colony was expanded into one well of a 24-well culture plate and one hundred thousand cells were collected for immediate genomic analysis. The remaining cells were frozen and stored in 5% DMSO.

3.2.6.3.2. Screening of mutated cell colonies

Genomic DNA from single colonies was extracted using Exgene^{TM} Cell SV kit (GeneAll Biotechnology, Korea) and analysed for the determination of quality and concentration with Nanodrop ND-1000 spectrophotometer (Thermo Scientific). PCR amplification was performed in a 50 µl reaction using 50 ng of DNA, 0.4 µl DNA Polymerase (1U/ µl; Biotools, Madrid, Spain), 10x Standard Reaction Buffer (Biotools, Madrid, Spain), 200 µM of dNTP mix (Applied Biosystems), 7.5 pMoli of forward and reverse primers (Forward 5'-AGGACCCTGGAGTCGATTGAT-3', Reverse 5'-AGCATCTTGCTCAATTGGTGG-3'). Two µl of PCR products were analysed on 1.5% agarose gel to verify the amplification of a single DNA fragment. Purified PCR products were obtained using spin column purification (Expin PCR SV, GeneAll Biotechnology, Korea) and total DNA was quantified. Sanger sequencing analysis was used to verify the insertion of desired point mutation among expanded clonal cell lines.

3.2.7. Drug sensitivity assays

3.2.7.1. Cell proliferation assay

Cell viability was assessed using the XTT Cell Proliferation Kit II (Roche Applied Science) according to manufacturer's instructions. Cells were plated at a density of 5×10^3 cells/well in 96-well flat-bottomed plates. Following overnight incubation, triplicate wells were treated with increasing concentrations of Cisplatin and Olaparib (Selleck Chemicals) for 72 hours. XTT solution was added to each well and plates were incubated for at least 4 hours. The absorbance was read at 450 nm and at 690 nm (as reference wavelength). For each drug concentration, the percentage of proliferation inhibition was calculated as ratio between the average values of non-treated and of treated cells. Non-treated cells and empty wells were used as positive and negative controls, respectively.

3.2.7.2. Cell apoptosis assay

Annexin-FITC and Propidium Iodide (PI) assays (Immunostep, Salamanca, Spain) were performed and analysed on FACSCalibur flow cytometry system (Becton-Dickinson, San Jose, CA, USA). Briefly, cells were seeded at a density of 10^5 cells/ml in 12-well culture plates and, following overnight incubation, treated in 10% FBS with Cisplatin or Olaparib alone for 72 hours. After treatment, cells were harvested, washed with PBS, stained and analyzed by flow cytometry according to manufacturer's instructions. Wild type and mutated breast cancer cells were used for the analysis.

3.2.8. Statistical analysis

Associations between demographic and clinical patients' characteristics, and *BRCA1/2* mutational status were explored. Characteristics were described as frequencies and proportions. Differences between groups were evaluated using the Chi-square test or the Fisher exact test, when appropriated. All analyses were performed using STATA v.13. Differences in gene expression levels between groups were evaluated using the T-test. Pearson's correlation was used to investigate the association between gene expression levels. This analysis was performed using Prism version 5.0 software (GraphPad Software). P-values (p) ≤ 0.05 were considered statistically significant.

Chapter 4

Results

4.1 Hereditary breast cancer population

We retrospectively screened a total of 366 patients. We identified a total of 73 patients (20%) who tested positive for *BRCA1/2* mutational status and 61 patients who tested negative but had at least two first degree relatives affected with breast and/or ovarian cancer. Therefore, a total of 134 patients were enrolled in the case-control study. All patients were Caucasian females. The clinical and histopathological characteristics are described in Table 4.1. Specifically, control patients were more frequently affected with DCI (73.3%) and the triple negative histotype was diagnosed only in 4 patients (8.5%). Fifty-three patients (86.9%) were stage 0-I-II, 6 patients (9.8%) were stage III and no patient was stage IV. Fifty-five patients (90.2%) underwent at least one adjuvant treatment: 42 patients (67.2%) underwent adjuvant radiotherapy, 44 (72.1%) hormonal therapy and 25 (41%) chemo/immunotherapy. One patient (1.6%) had ipsilateral disease, 2 (3.2%) had contralateral disease, 3 (4.9%) had distant relapse and were treated according to the Institutional guidelines.

In the case arm of the study, patients have been grouped according to the mutated gene and the type of mutation (Table 4.2). All mutations and VUS were detected by Sanger sequencing and are described in Table 4.3 and Table 4.4, respectively. We identified 38 unique mutations and 28 unique VUS, with 6 VUS co-occurring with a known mutation. Thirty-four patients (46.5%) were positive for *BRCA*1 mutation and 15 patients (20.5%) were positive for *BRCA2* mutation. Two patients (3%) tested positive for both *BRCA1* and *BRCA2* mutations. Six (8.2%) and 16 (21.9%) patients tested positive for VUS in *BRCA1* and *BRCA2* genes, respectively.

Table 4.1 Global population's clinical and histopathological characteristics *(to be continued)*

Characteristics[a]	N	%
Age at diagnosis		
Median 43.4 (range 27.4-71.8)		
< 45	71	53.0
≥ 45	63	47.0
Family history of breast cancer		
Negative	17	12.7
Positive	117	87.3
Family history of ovarian cancer		
Negative	111	82.8
Positive	22	16.4
NA	1	0.7
Risk factors		
Negative	37	27.4
Positive	98	72.6
Side		
Right	61	45.5
Left	66	49.3
Right and left	7	5.2
Histology		
ILC	11	8.2
IDC	105	78.4
Other	16	11.9
NA	2	1.5
Stage at diagnosis		
0 1 2	59	44.0
3	70	52.2
NA	5	3.7
Grading		
1	12	9.0
2	47	35.1
3	64	47.8
NA	11	8.2
Estrogen Receptors		
Negative	36	26.9
Positive	90	67.2
NA	8	6.0
Progesteron Receptors		

Table 4.1 (*continued)*

Characteristics[a]	N	%
Negative	47	35.1
Positive	79	59.0
NA	8	6.0
HER2		
0 1 2	102	76.1
3	16	11.9
NA	16	11.9
Ki-67		
< 20	52	38.8
≥ 20	71	53.0
NA	11	8.2
Histotype		
Luminal A	44	32.8
Luminal B	36	26.9
HER2 positive	4	3.0
Triple negative	33	24.6
NA	17	12.7
Surgery		
Quadrantectomy	85	63.4
Mastectomy	44	32.8
Other	5	3.7
Adjuvant treatment		
Radiotherapy	94	70.2
Hormonal therapy	83	61.9
Chemo/immunotherapy	82	61.2
Relapse of disease		
Ipsilateral	8	6
Contralateral	10	7.5
Ipsilateral and contralateral	2	1.5
Distant metastases	8	6

[a] Some data for each parameter were not available

ILC, Invasive Lobular Carcinoma

IDC, Invasive Ductal Carcinoma

Table 4.2 Distribution of *BRCA* mutations among case arm of *BRCA* mutation carriers

Carriers	N	%
BRCA1 mutation*	34	46.5
BRCA2 mutation*	15	20.5
Mutation in *BRCA1* + *BRCA2*	2	3.0
VUS in *BRCA1* or/and *BRCA2*	22	30.1
VUS in *BRCA1*	6	8.2
VUS in *BRCA2*	16	21.9
Mutation in *BRCA1* + VUS in *BRCA1*	3	4.1
Mutation in *BRCA1* + VUS in *BRCA2*	3	4.1

* They include carriers of both mutations in *BRCA1* and VUS in *BRCA1*/*2* with mutations having a predominant role for breast cancer risk.

Table 4.3 *BRCA1* and *BRCA2* mutations identified in breast cancer patients (*to be continued*)

Gene	Exon (Intron)	Mutation (HGVS)	Mutation type	Protein change (HGVS)	N[a]	Concomitant mutation/VUS[b]	Ref[c]
BRCA1							
	2	c.65T>C	Missense	p.Leu22Ser	1	c.8331+1G>A	19543972
	3-4(3)	c.134+3_134+6delAAGT	Splice	p.Cys27*	1		23451180
	5	c.181T>G	Missense	p.Cys61Gly	1		18824701
	5	c.190T>C	Missense	p.Cys64Arg	1		15235020, 24516540
	5-6(5)	c.213-11T>G	Splice	p.Cys64Arg	1		23451180, 24516540
	11	c.835delC	Frameshift	p.His279Metfs*19	1	c.8195T>G	
	11	c.981_982delAT	Frameshift	p.Cys328*	1		
	11	c.1088delA	Frameshift	p.Asn363Ilefs*11	4		
	11	c.1612C>T	Non sense	p.Gln538*	1		
	11	c.1687C>T	Non sense	p.Gln563*	2	c.9649-6dupT	
	11	c.2331T>G	Non sense	p.Tyr777*	2		
	11	c.2350_2351delTC	Frameshift	p.Ser784Valfs*5	1		
	11	c.2389_2390delGA	Frameshift	p.Glu797Thrfs*3	1	c.5152+20T>A	
	11	c.2727_2730delTCAA	Frameshift	p.Asn909Lysfs*90	2		
	11	c.2960dupA	Frameshift	p.Ser988Valfs*4	1		
	11	c.3013G>T	Non sense	p.Glu1005*	1		
	11	c.3514G>T	Non sense	p.Glu1172*	1	c.3491G>T	
	11	c.4117G>T	Non sense	p.Glu1373*	1		
	16	c.4688dupA	Frameshift	p.Tyr1563*	1	c.6443C>A	
	16	c.4964_4982del19	Frameshift	p.Ser1655Tyrfs*16	2		
	17	c.5030_5033delCTAA	Frameshift	p.Thr1677Ilefs*2	2		
	17	c.5062_5064delGTT	In frame deletion	p.Val1688del	1	c.9118-12T>C	

Table 4.3 **(***continued)*

Gene	Exon (Intron)	Mutation (HGVS)	Mutation type	Protein change (HGVS)	N[a]	Concomitant mutation/VUS[b]	Ref[c]
BRCA1							
	20	c.5266dupC	Frameshift	p.Gln1756Profs*74	4	c.206_207delinsTG	
BRCA2							
	7	c.631G>A	Splice	p.Gly173Serfs*19	1	c.7008-2A>T	23451180, 24516540
	10	c.1796_1800delCTTAT	Frameshift	p.Ser599*	1		
	11	c.2623_2624delGT	Frameshift	p.Val875Glnfs*5	1		
	11	c.2651C>G	Non sense	p.Ser884*	1		
	11	c.2979G>A	Non sense	p.Trp993*	1		
	11	c.4131_4132insTGAGGA	Frameshift	p.Thr1378*	1		
	11	c.5851_5854delAGTT	Frameshift	p.Ser1951Trpfs*11	1		
	11	c.6081delA	Frameshift	p.Glu2028Lysfs*12	1		
	11	c.6468_6469delTC	Frameshift	p.Gln2157Ilefs*18	1		
	12-13 (12)	c.7008-2A>T	Splice	p.Thr2337Phefs*17	1	c.631G>A	23451180, 24516540
	14	c.7180A>T	Non sense	p.Arg2394*	1		
	18	c.8195T>G	Non sense	p.Leu2732*	1	c.835delC	
	18-19 (18)	c.8331+1G>A[d]	Splice	Not precisely defined[d]	1	c.65T>C	
	22	c.8878C>T	Non sense	p.Gln2960*	3		
	27	c.9676delT	Frameshift	p.Tyr3226Ilefs*23	1		

Mutation and protein change designation according to Human Genome Variation Society (HGVS)

[a] Number of breast cancer patients carrying the specific mutation

[b] Concomitant mutations identified in one patient

[c] ID number of Pubmed (PMID) of references on mutations with verified deleterious effect on splicing

[d] This mutation has been classified as likely pathogenic (Class 4)

Table 4.4 *BRCA1* and *BRCA2* variants of unknown clinical significance identified in breast cancer patients (*to be continued*)

Gene	Exon (Intron)	Mutation (HGVS)	Mutation type	Protein change (HGVS)	N[a]	Concomitant mutation/VUS[b]	Ref (PMID)[c]
BRCA1							
	5	c.206_207delinsTG	Missense	p.Thr69Met	1	c.5266dupC	18680205
	5-6(5)	c.212+17T>C	Intervening Sequence	Not defined	1		
	11	c.3082C>T	Missense	p.Arg1028Cys	1		
	11	c.3491G>T	Missense	p.Ser1164Ile	1	c.3514G>T	
	11	c.4031A>G	Missense	p.Asp1344Gly	1		
	15	c.4654T>C	Missense	p.Tyr1552His	1		
	18	c.5152+20T>A	Intervening Sequence	Not defined	1	c.2389_2390delGA	
	24	c.5509T>C	Missense	p.Trp1837Arg	2		15235020, 15689452, 16969499, 20516115
BRCA2							
	2-3(2)	c.68-7T>A	Intervening Sequence	Not defined	1		21939546, 22505045, 20215541, 21673748, 21702907, 22144684, 18284688, 24607278
	3-4(3)	c.316+12A>G	Intervening Sequence	Not defined	1		
	7	c.521G>A	Missense	p.Arg174His	1		
	8	c.681+14A>G	Intervening Sequence	Not defined	1	c.7976+18C>T (VUS)	
	10	c.1244A>G	Missense	p.His 415Arg	1		
	10	c.1433C>T	Missense	p.Thr478Ile	1		
	11	c.2396A>G	Missense	p.Lys799Arg	1		

Table 4.4 *(continued)*

Gene	Exon (Intron)	Mutation (HGVS)	Mutation type	Protein change (HGVS)	N[a]	Concomitant mutation/VUS[b]	Ref (PMID)[c]
BRCA2							
	11	c.2970G>C	Missense	p.Met990Ile	1		
	11	c.3668A>G	Missense	p.His1223Arg	1		
	11	c.5345A>C	Missense	p.Gln1782Pro	1		
	11	c.6332A>C	Missense	p.Lys2111Thr	1		
	11	c.6443C>A	Missense	p.Ser2148Tyr	1	c.4688dupA	
	15	c.7505G>A	Missense	p.Arg2502His	1		
	16	c.7649T>C	Missense	p.Ile2550Thr	1		
	17-18(17)	c.7976+18C>T	Intervening Sequence	Not defined	1	c.681+14 A>G (VUS)	
	19	c.8486A>G	Missense	p.Gln2829Arg	1		
	24	c.9118-12T>C	Intervening Sequence	Not defined	1	c.5062_5064delGTT	
	26	c.9586A>G	Missense	p.Lys3196Glu	1		
	26	c.9502-12T>G	Intervening Sequence	Not defined	1		
	27	c.9649-6dupT	Intervening Sequence	Not defined	1	c.1687C>T	

Mutation and protein change designation according to Human Genome Variation Society (HGVS)

[a] Number of breast cancer patients carrying the specific mutation

[b] Concomitant mutations identified in one patient

[c] ID number of Pubmed (PMID) of references on mutations with verified deleterious effect on splicing

As shown in Table 4.5, statistically significant associations emerged from our analysis of the case arm of the study compared to the control arm. Breast and ovarian cancer family history was found significantly associated with mutations in both *BRCA1* and *BRCA2* genes ($p < 0.001$ and $p = 0.045$, respectively). Mutations in both *BRCA1* and *BRCA2* genes were also significantly associated with early onset of disease (< 45 year old) (86.1% and 64.7% vs 27.9%, $p < 0.001$ and $p = 0.005$, respectively). VUS in *BRCA2* were reported to be significantly associated with breast cancer family history ($p = 0.001$) and early onset of disease too ($p = 0.033$). Overall, median age at diagnosis was 38.2 (range 28.2-68.1). No significant associations were found between *BRCA* mutations and other risk factors (diet, smoke, absence of regular physical activity, reproductive history, and contraceptive use) in comparison with the control arm. Patients with *BRCA1* mutation were more frequently affected with DCI (94.4%; $p = 0.032$). Furthermore, grading 3 and high Ki-67 were significantly associated with *BRCA1* mutations compared with the control arm ($p < 0.001$ for each comparison). Triple-negative histotype was significantly associated with *BRCA1* mutations ($p < 0.001$). Interestingly, *BRCA1* VUS carrier patients were affected with triple negative (2) or luminal B tumors (4), only. Among all patients positive for *BRCA1* mutations, 94.3% were stage 0-I-II and 5.7% were stage III. Among all patients with *BRCA2* mutations, 88.2% were stage 0-I-II and 11.8% were stage III. Similarly to control group, almost all patients (97.3%) underwent at least one adjuvant treatment: 52 patients (71.2%) received adjuvant radiotherapy, 39 (53.4%) hormonal therapy and 57 (78.1%) chemo/immunotherapy. During follow up 7 patients (3 with *BRCA1* mutation, 2 with *BRCA2* mutation and 2 with VUS in *BRCA2*) had ipsilateral disease, 8 patients (4 with *BRCA1* mutation, 1 with *BRCA2* mutation, 2 with VUS in *BRCA1* and 1 with mutations in both *BRCA1* and *BRCA2* genes) developed contralateral disease. Two patients with *BRCA1* mutation had both ipsilateral and contralateral disease and another patient with *BRCA1* mutation developed contralateral disease and subsequently metastatic disease. Three patients among *BRCA2* mutation carriers developed metastatic disease.

Table 4.5 Significant associations between *BRCA* mutation status and clinical-pathological features in hereditary breast cancer patients (*to be continued*)

	BRCA1			***BRCA2***			***BRCA2* VUS**			**Controls**	
Characteristics[a]	**N**	**%**	***p* value**	**N**	**%**	***p* value**	**N**	**%**	***p* value**	**N**	**%**
Age at diagnosis											
< 45	31	86.1	**<0.001**	11	64.7	**0.005**	9	56.3	**0.033**	17	27.9
≥ 45	5	13.9		6	35.3		7	43.7		44	72.1
Family history of breast cancer											
No	8	22.2	**<0.001**	2	11.8	**0.045**	4	25.0	**0.001**	0	0.0
Si	28	77.8		15	88.2		12	75.0		61	100.0
Family history of ovarian cancer											
No	23	65.7	**0.001**	14	82.4	0.171	13	81.2	0.152	57	93.4
Si	12	34.3		3	17.6		3	18.8		4	6.6
Histology											
ILC[a]	1	2.8		3	17.6		2	12.5		5	8.3
IDC[b]	34	94.4	**0.032**	12	70.6	0.537	11	68.8	0.811	44	73.3
Other	1	2.8		2	11.8		3	18.7		11	18.3
Grading											
1	0	0.0		0	0.0		3	21.4		9	16.1
2	4	12.1	**<0.001**	7	46.7	0.200	6	42.9	0.856	28	50.0
3	29	87.9		8	53.3		5	35.7		19	33.9

Table 4.5 (*continued*)

	BRCA1			*BRCA2*			*BRCA2* VUS			Controls	
Characteristics[a]	**N**	**%**	***p* value**	**N**	**%**	***p* value**	**N**	**%**	***p* value**	**N**	**%**
Estrogen receptors											
Negative	23	65.7	**<0.001**	2	11.8	1.000	2	13.3	1.000	8	14.5
Positive	12	34.3		15	88.2		13	86.7		47	85.5
Progesteron receptors											
Negative	26	74.3	**<0.001**	2	11.8	0.327	4	26.7	1.000	14	25.4
Positive	9	25.7		15	88.2		11	73.3		41	74.6
HER2											
0 1 2	33	94.3	**0.035**	16	100.0	**0.029**	12	85.7	0.716	37	75.5
3	2	5.7		0	0.0		2	14.3		12	24.5
Ki-67											
<20	5	14.7	**<0.001**	10	58.8	1.000	8	57.1	0.862	30	54.6
≥20	29	85.3		7	41.2		6	42.9		25	45.4
Histotype											
Luminal A	4	11.4	**<0.001**	10	58.8	0.839	8	57.1	0.864	23	48.9
Luminal B	5	14.3		5	29.4		4	28.6		5	38.3
HER2 positive	1	2.9		0	0		1	7.1		1	4.3
Triple negative	25	71.4		2	11.8		1	7.1		25	8.5

Subgroups of patient carriers of *BRCA-1* VUS were not included in the analysis for the small number of cases

[a] Some data for each parameter were not available

ILC, Invasive Lobular Carcinoma

IDC, Invasive Ductal Carcinoma

4.2. *In silico* analysis of Variant of Unknown Significance

We identified 8 unique *BRCA1* VUS (2 intervening sequence (IVS), 6 missense) and 20 unique *BRCA2* VUS (7 IVS, 13 missense) (Table 4.4). Among them, 6 missense variants (1 *BRCA1* and 5 *BRCA2* VUS) were located in *BRCA1* and *BRCA2* regions corresponding to functional protein domains, suggesting their potential damaging role in protein functionality. These 6 missense VUS were considered as potentially deleterious variants (Fig. 4.1). Interestingly, among identified VUS, 6 different variants were identified in co-occurrence with *BRCA1* or *BRCA2* mutations (Table 4.4).

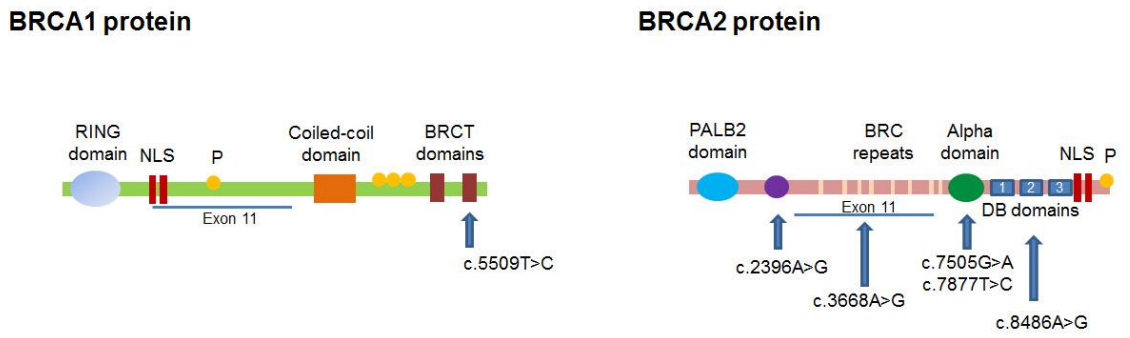

Figure 4.1 Localization of potential deleterious missense *BRCA1* and *BRCA2* VUS occurring in gene regions correspondent to functional domains of BRCA proteins. The most potential pathogenic *BRCA1* and *BRCA2* VUS relative to functional domain are indicated, 1 for *BRCA1* gene and 5 for *BRCA2* gene. BRCA1 C-terminal domain (BRCT), and alpha and OB2 folds of BRCA2 DNA binding (DBD_OB2) domains are the regions where mainly our VUS are located. NLS, Nuclear Localization Site; NPM1, Nucleophosmin.

Missense variants were analysed by bioinformatics tools in order to evaluate their possible functional implications (Table 4.6). Prediction of amino acid substitutions with Align-GVGD, PolyPhen and PROVEAN platforms concordantly supported a deleterious effect only for the *BRCA1* VUS c.5509T>C (BIC entry: W1837R), whose location is importantly within the *BRCA1* C-terminal (BRCT) domain of BRCA1 protein. In the *BRCA1* CIRCOS this variant resulted to have a functional impact in different in vitro assays related to protease sensibility, phosphopeptide binding activity, embryonic stem cell viability and interestingly to *in vitro* cisplatin response. In our cohort of hereditary breast cancer patients this variant has been identified in two cases who have developed

triple negative breast cancer. Human Splicing Finder was also used to investigate the role of *BRCA1* and *BRCA2* IVS VUS in determining potential splicing defects. Specifically, only *BRCA2* VUS c.316+12A>G, occurring in the late intronic positions, was predicted to create a new acceptor site by the disruption of the wild type acceptor site resulting most probably in the affection of splicing. However, this VUS has been reported in co-occurrence with the *BRCA1* c.1687C>T mutation in one patient of our cohort.

Table 4.6 Potential deleterious effect of *BRCA* missense VUS according to different *in silico* prediction tools

	Functional domain	POLYPHEN[a]	Align-GVGD[b]	PROVEAN[c]
BRCA-1[*]				
T69M		Probably damaging	C65	Neutral
R1028C		Possibly damaging	C15	Deleterious
S1164I		Possibly damaging	C15	Deleterious
D1344G		Possibly damaging	C0	Neutral
Y1552H		Benign	C0	Neutral
W1837R	BRCT	Probably damaging	C65	Deleterious
BRCA-2[*]				
R174H		Benign	C0	Neutral
H415R		Possibly damaging	C0	Neutral
T478I		Benign	C0	Neutral
K799R	NPM1 binding domain	Benign	C0	Neutral
M990I		Benign	C0	Neutral
H1223R	BRCA-2 repeat	Benign	C0	Neutral
Q1782P		Benign	C0	Deleterious
K2111T		Benign	C0	Neutral
S2148Y		Benign	C0	Neutral
R2502H	BRCA2_helical	Benign	C0	Deleterious
I2550T	BRCA2_helical	Possibly damaging	C25	Neutral
Q2829R	BRCA2DBD_OB2	Possibly damaging	C35	Neutral
K3196E		Possibly damaging	C0	Neutral

[*] Variant designation according to BIC nomenclature

[a] PolyPhen-2 calculates the naive Bayes posterior probability that a given mutation is damaging and reports estimates of false positive (the chance that the mutation is classified as damaging when it is in fact non damaging) and true positive (the chance that the mutation is classified as damaging when it is indeed damaging) rates. A mutation is also defined qualitatively, as benign, possibly damaging or probably damaging

[b] A-GVGD scores amino acid substitutions on a 7-scale scoring system, from C0 to C65. An amino acid substitution with a C0 score is considered to be neutral, amino acids with C15 and C25 scores are considered intermediate, as changes to protein structure or function are uncertain, and C35 scores or higher are considered as likely deleterious

[c] If the PROVEAN score is equal to or below a predefined threshold (-2.5), the protein variant is predicted to have a "deleterious" effect, otherwise the variant is predicted to have a "neutral" effect

BRCT, *BRCA-1* C-terminal domain

BRCA-2_helical, BRCA2 helical domain

BRCA2DBD_OB2, OB2 folds of BRCA-2 DNA binding domain

BRCA-2 repeat, internal repeats of BRCA-2

4.3. Protein structural modeling

Protein modeling prediction of *BRCA1* c.5509T>C (BIC:W1837R) variant has been performed. This variant was predicted to induce different destabilizing effects on protein structure (Fig. 4.2). First, Trp1837 is positioned in proximity of the aromatic side chains of Phe1761 and Tyr1853; stacking interactions among aromatic side chains are a known stabilizing effect, which is loss when the aromatic side chain of Trp1837 is substituted by an Arginine. Secondly, the side chain of Trp1837 is not accessible to the solvent, as results by specific evaluation of solvent exposure, and therefore the amino acidic substitution may modify the solvent-accessible surface area of the protein. This was also confirmed by other visualization techniques (data not shown). Thirdly, the stability of the protein structure is expected to be compromised also by the substitution of a non-polar side chain (Tryptophan) positioned into the hydrophobic core with a positively charged and, therefore, polar chain (Arginine).

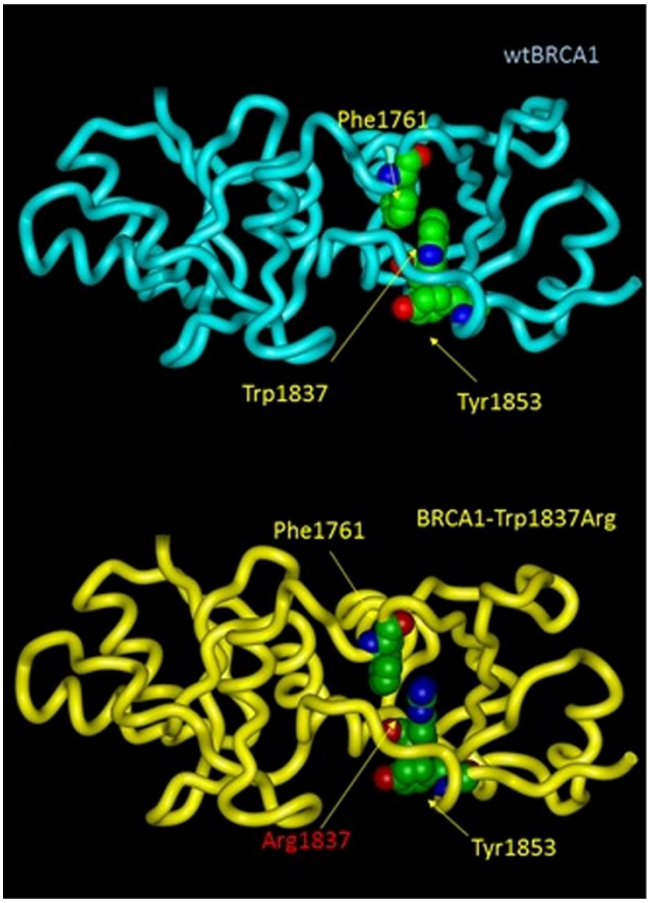

Figure 4.2 Molecular models of proteins derived from wild-type *BRCA1* (wt*BRCA1*) and *BRCA1*-Trp1837Arg variant. The upper image represents wtBRCA1 protein, with in evidence the possible interactions of Trp1837 with close side chains. The lower panel shows BRCA1 protein derived from Trp1837Arg variant. Backbone is drawn as solid ribbon, colored in cyan for wt*BRCA1* and yellow for the variant. The side chains of interest are evidenced in CPK coloring representations, colored by atom standards colors, and labeled by type and number of the amino acid. Red labels highlight the modified amino acids.

4.4. *BRCA1/2* expression and DNA damage levels

We observed aberrant nuclear expression of BRCA1 and BRCA2 protein of cancer cells in tumors from *BRCA1* and *BRCA2* mutation carriers, respectively (Fig. 4.3 and Fig. 4.4, respectively). In particular, *BRCA1*-related tumors showed an irregular pattern of BRCA1 expression in terms of focalization into discrete foci or positive signal limited to the periphery of nucleus, compared to those of *BRCA* mutation non carriers. Of note even tumors with high percentage of positive cells showed patchy staining with moderate to negative intensity (Fig. 4.3, panel A), in contrast to diffuse and intense nuclear staining of cells in tumors from the control group (Fig. 4.3, panel B). In some cases, cytoplasmic staining was present. Normal breast tissue, when present adjacent to the malignant one, showed exclusively intense and diffuse nuclear staining.

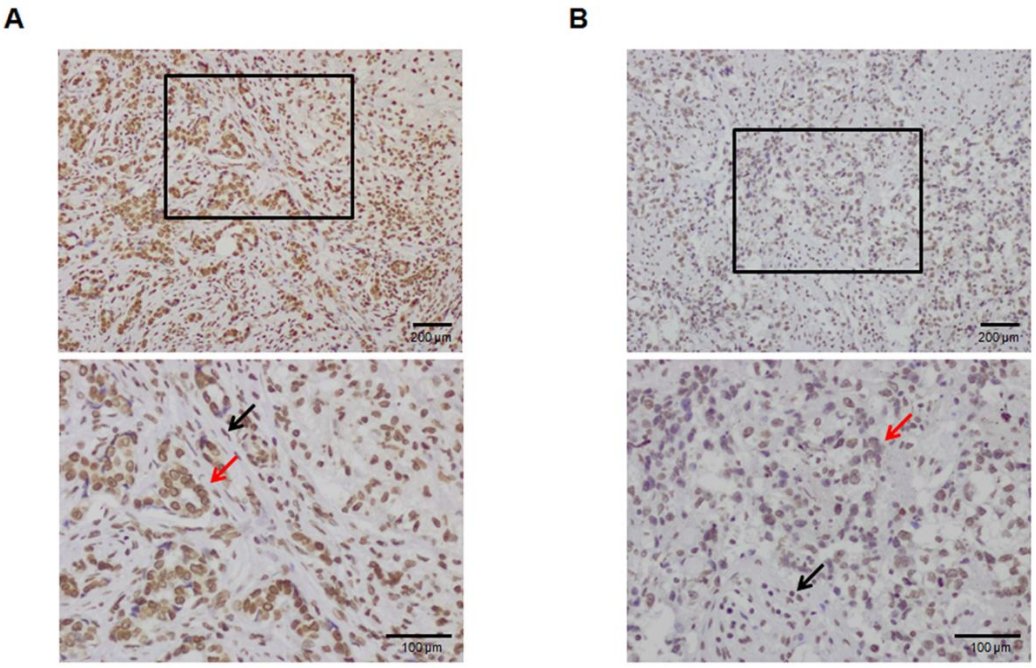

Figure 4.3 Expression of BRCA1 protein in tumors derived from familial breast cancer patients. **A-B** Expression pattern of BRCA1 protein in two IHC stains showing breast tumors from a non carrier (A) and a *BRCA1* mutation carrier (B). **A** Low (10x) and high (20x) power images of strong diffuse intensity staining of tumor cell nuclei (red arrows) with presence of positive internal controls (black arrows). **B** Low and high power images of moderate to negative intensity staining of tumor cell nuclei (red arrows) with presence of positive internal controls (black arrows). Of note, the patchy pattern of BRCA1 expression at tumor cell nuclei. In both cases, stromal cells and tumor infiltrating lymphocytes (black arrows) were used as positive internal controls.

Accordingly, *BRCA1* mRNA level was found significantly reduced in *BRCA1* ($p = 0.028$) and *BRCA1/2*-related tumors ($p = 0.01$) compared to that from non carrier patients (Fig. 4.5). No statistically significant difference was found for *BRCA2* mRNA level between

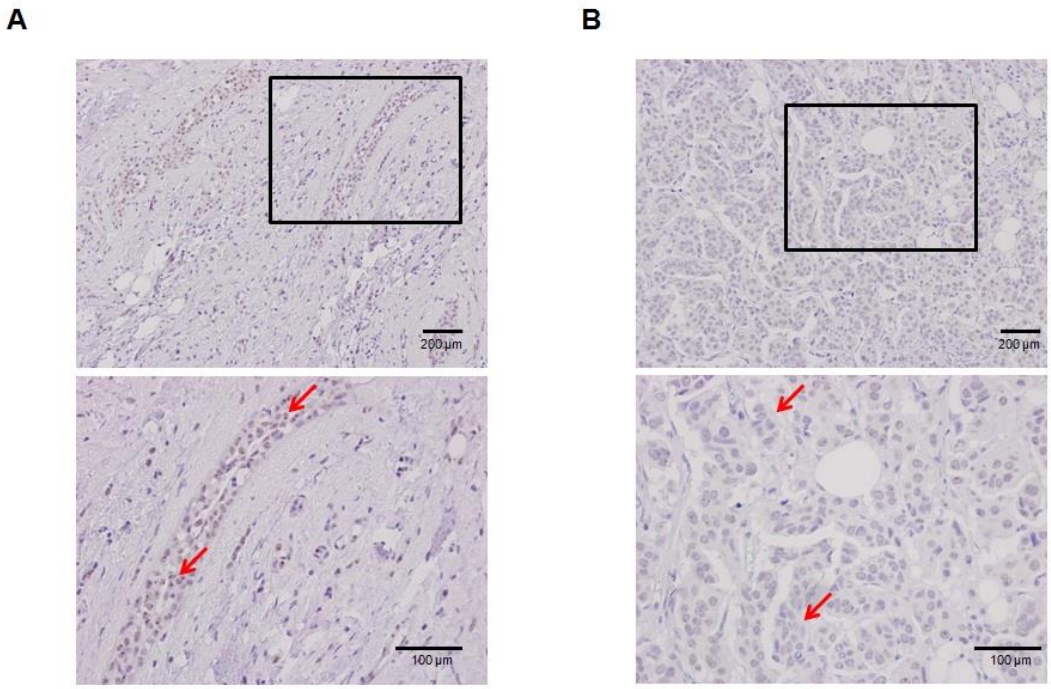

Figure 4.4 Expression of BRCA2 protein in tumors derived from familial breast cancer patients. **A-B** Expression patterns of BRCA2 protein in two representative IHC stains of breast tumors derived from a *BRCA2* mutation non carrier (A) and a *BRCA2* mutation carrier (B). **A**. Low (10x) and high (20x) power images of strong intensity staining of tumor cell nuclei (red arrows). **B**. Low and high power images of rare positive tumor cell nuclei (red arrows). Positive staining is characterized by focal foci distribution at nucleus.

tumors derived from *BRCA2* mutation carrier and non carrier groups. Interestingly, tumors related to *BRCA2* mutations more frequently showed reduced expression of BRCA2 protein, in contrast with differential expression among tumors of *BRCA2* VUS carriers (Fig. 4.6). In addition, qRT-PCR analysis showed a lower mean value of *BRCA2* expression levels in *BRCA2*-related tumors compared to those derived from *BRCA2* VUS carriers (0.25 vs 0.53).

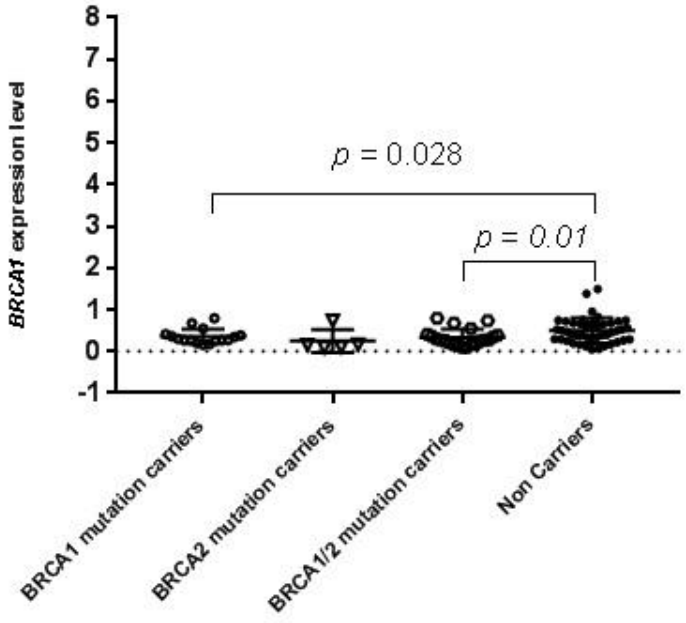

Figure 4.5 Differential relative expression levels of *BRCA1* according to patient groups defined by the presence or the absence of germline *BRCA1* mutation. In the scatter plot, median values are depicted as horizontal lines with relative standard deviations. *P* values < 0.05 were considered statistically significant.

Overall, we observed different detectable levels of DNA damage marker in tumors associated with *BRCA1* mutations. (Fig. 4.7 panel A-B). Spearman's correlation analysis between mRNA expression levels (*BRCA1* vs *H2AX*, *BRCA2* vs *H2AX*, *BRCA1* vs *BRCA2*) did not reveal any statistically significant correlations either in the group of *BRCA1*-related tumors or in the group of *BRCA2*-related tumors (Fig. 4.8 panel A). In contrast, tumors derived from non carrier patients resulted to show statistically significant correlations between *BRCA1* vs *H2AX* (r = 0.64, $p < 0.0001$), *BRCA2* vs *H2AX* (r = 0.68, $p < 0.0001$) and *BRCA1* vs *BRCA2* (r = 0.72, $p < 0.0001$) (Fig. 4.8 panel B). Among *BRCA1* VUS carriers, we observed the presence of negative staining for γ-H2AX protein in 3 tumors associated with missense VUS (c.3082C>T, c.4031A>G, c.4654T>C) not located in specific functional domains (Fig. 4.7 panel C). Interestingly, 2 tumors derived from carriers of potentially deleterious c.5509T>C VUS located within BRCT domain, and one tumor from carrier of c.212+17T>C VUS showed detectable levels of DNA damage. Of note, patient with c.212+17T>C VUS underwent neoadjuvant therapy.

Specifically, tumors associated with *BRCA1* c.5509T>C VUS showed concurrent aberrant expression of BRCA1 and higher levels of γ-H2AX proteins overlapping the level associated with *BRCA1* mutations (Fig. 4.7 panel D).

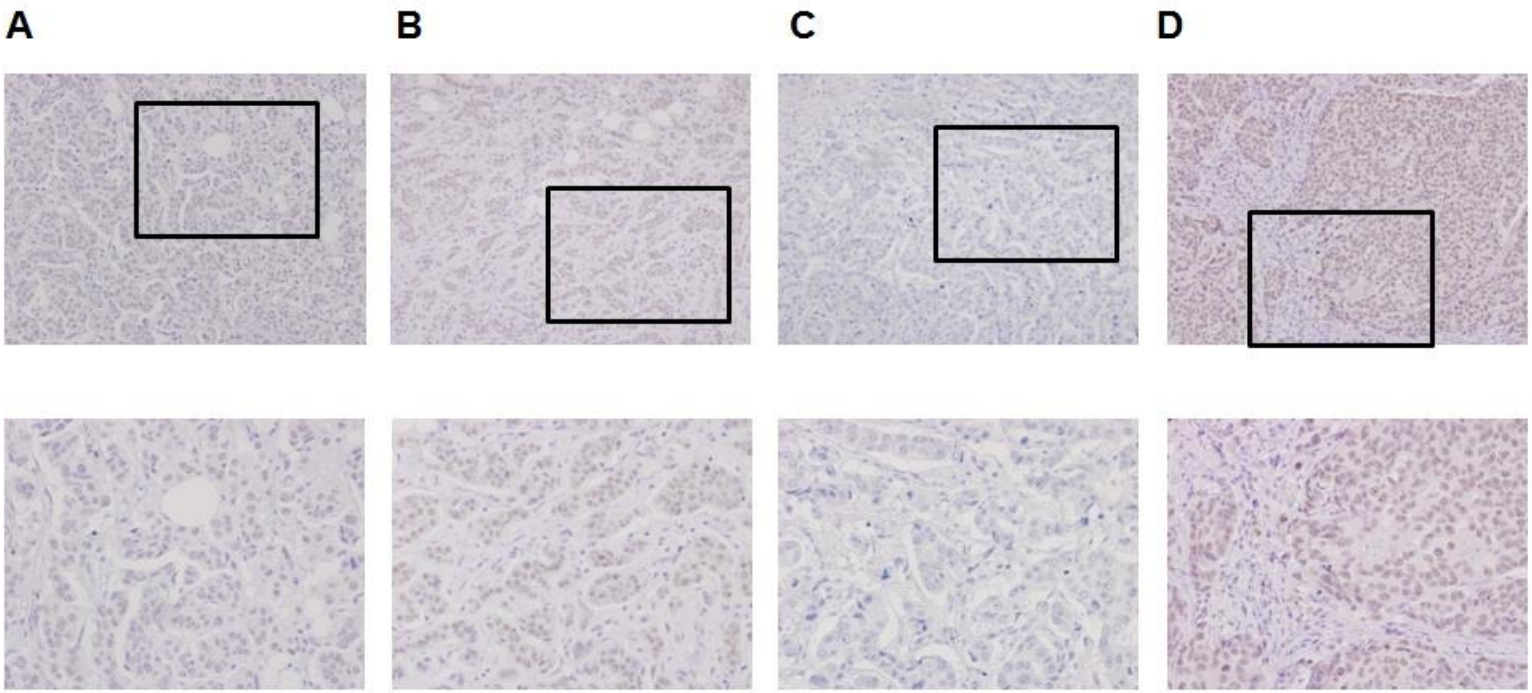

Figure 4.6 Expression levels of BRCA2 protein in four representative IHC stains of breast tumors derived from two *BRCA2* mutation carriers (A and B) and two *BRCA2* VUS carriers (C and D). A-B. Tumors derived from two *BRCA2* mutation carriers. **C**. Tumor related to a *BRCA2* VUS located in BRCT Helical domain. **D**. Tumor related to a *BRCA2* VUS not located in a specific functional domain. Differentially expression of BRCA2 may be due to different effects of germline mutations on BRCA2 protein.

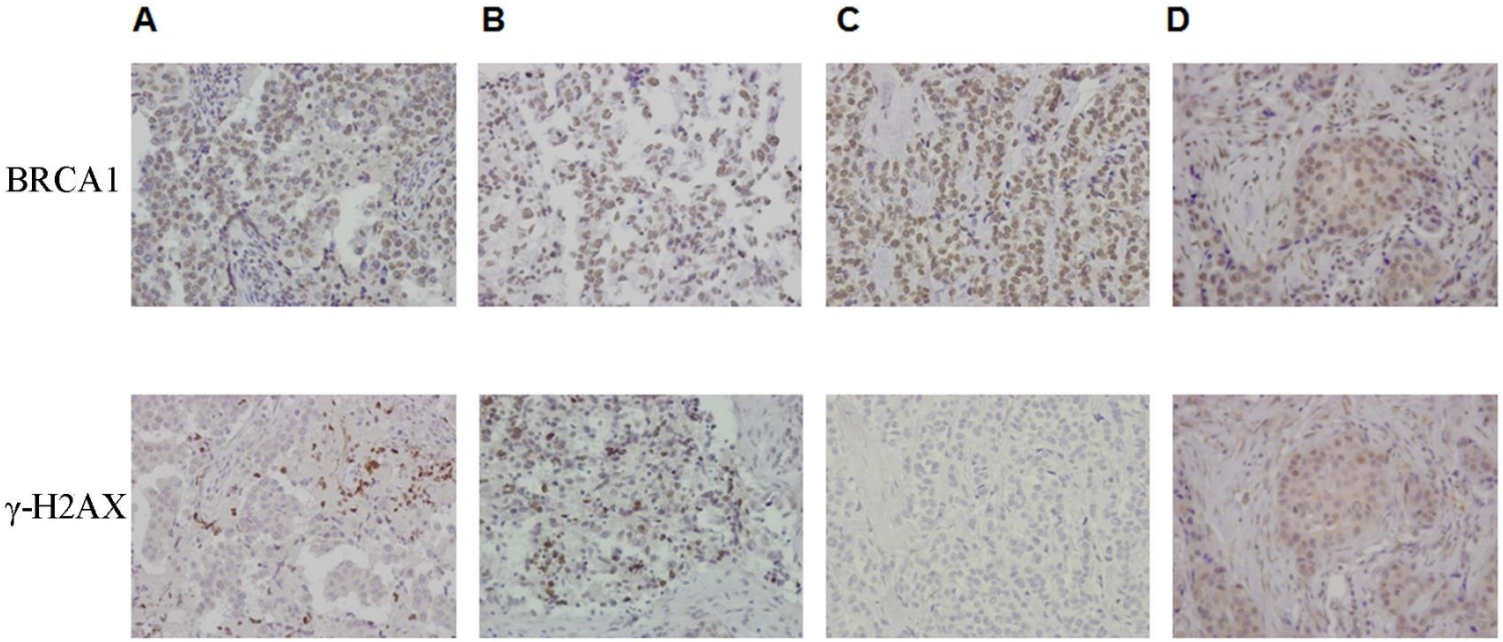

Figure 4.7 Expression patterns of BRCA1 and γ-H2AX proteins in four different IHC stains of breast tumors derived from two *BRCA1* mutation carriers (A and B) and two *BRCA1* VUS carriers (C and D). **A** Tumor related to the *BRCA1* mutation c.4964_4982del19 (p.Ser1655Tyrfs*16) located within BRCT domain. **B** Tumor related to the *BRCA1* mutation c.1687C>T (p.Gln563*) located in NLS region. **C** Tumor related to the *BRCA1* VUS c.4654T>C (p.Tyr1552His) not located in a specific functional domain. **D** Tumor related to the *BRCA1* VUS 5509T>C (p.Trp1837Arg) located within BRCT domain.

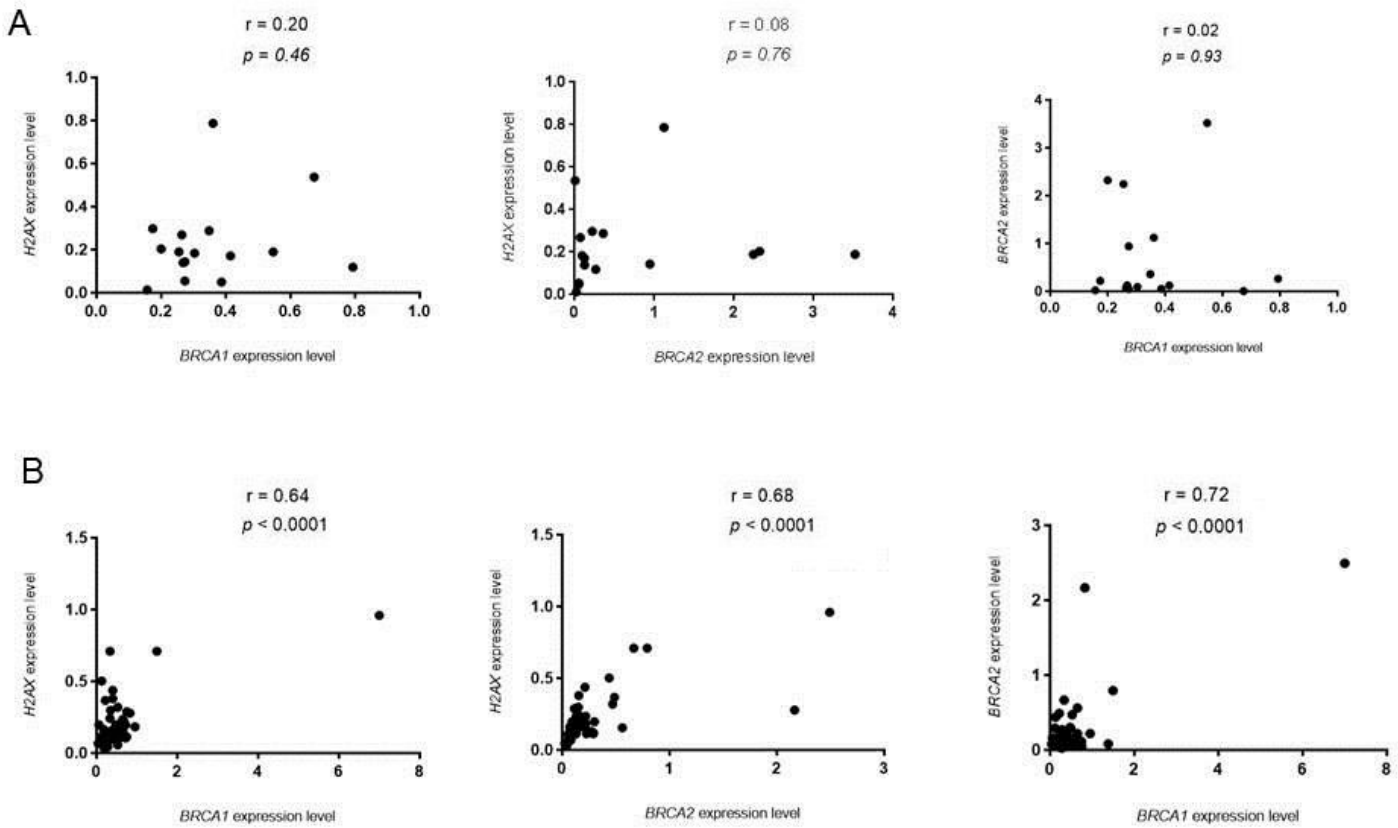

Figure 4.8 Correlation analysis between *BRCA1, BRCA2* and *H2AX* expression levels in the groups of *BRCA1* mutation carrier (A) and non carrier patients (B). Scatter plots show results for *BRCA1*/*H2AX*, *BRCA2*/*H2AX* and *BRCA1*/*BRCA2* correlations. Pearson's correlation coefficients (r) and relative P-values (*p*) are included in each graph. P values < 0.05 were considered statistically significant.

4.5. CRISPR/Cas9-mediated transfection experiments

The design of gRNAs and of DNA oligonucleotides was based on the selection of target DNA sequence within the region of interest containing the desired point mutation in *BRCA1* gene (Table 4.7). Target DNA is unique compared to the rest of the genome and is present immediately upstream of a Protospacer Adjacent Motif (PAM). Table 4.7 shows the sequences of the two selected gRNAs and of the donor dsDNA template resulting from the annealing of DNA oligonucleotides.

Table 4.7 gRNAs and donor dsDNA sequences selected for transfection experiments

Target DNA	AGGCACCTGTGGTGACCCGAGAGTGGG
gRNA1 (F)	ACCTGTGGTGACCCGAGAGT
gRNA2 (RC)	TCACACATCTGCCCAATTGC
Donor	TGTCTCCAGCAATTGGGCAGATGTGTGAGGCACCTGTGGTGACCCGAGAG CGGGTGTTGGACAGTGTAGCACTCTACCAGTGCCAGGAGCTGGACACCTA C

gRNA1 and gRNA2 represent two guides with forward (F) and reverse (RC) orientation, respectively. The donor dsDNA contains the desired point mutation T>C (highlighted in red). Target DNA is present immediately upstream of a Protospacer Adjacent Motif (PAM, highlighted in yellow).

The optimization procedure of transfection experiment has led to the identification of a pCas-Guide/donor DNA/TransIT-X2 ratio of 1 µg/1 µg /6 µl as the best condition to ensure > 60% of cell viability and the strongest GFP signal at visual monitoring of transfected cells (Fig. 4.9). In particular, GFP signal resulted to increase from 24 to 72 hours' post-transfection, with a maximum value at 72-hour time point (Fig. 4.9). Visual GFP-related signal of pCas-gRNA1- and gRNA2-mediated transfections were compared, and pCas9-gRNA1 was found to show more efficient plasmid delivery compared to pCas9-gRNA2 (Fig. 4.9). FACS analysis confirmed the higher absolute number of GFP positive cells sorted out from transfection experiments with pCas9-gRNA1 compared to pCas9-gRNA2 (Fig. 5).

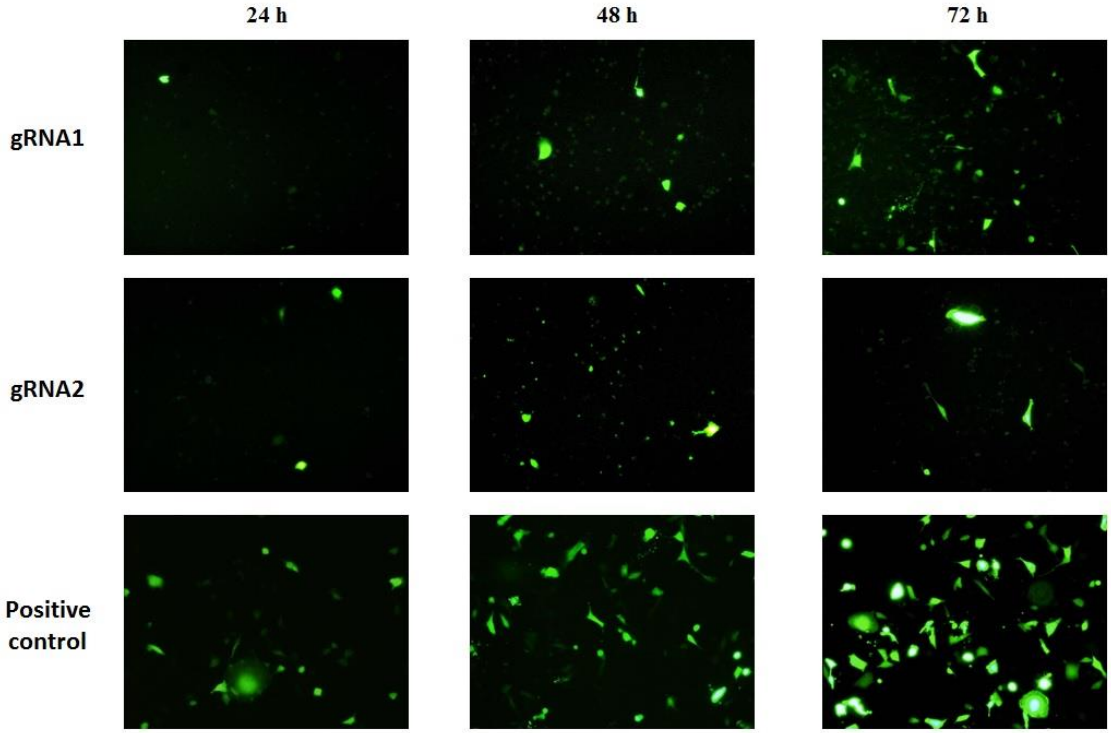

Figure. 4.9 Representative images of transfection experiments using pCas-Guide vectors gRNA1 or gRNA2. Cells were transfected with pre-incubated mixture containing 1.5 µg of pCas-Guide vector and 9 µl of TransIT-X2 transfection reagent. GFP-related signal has been monitored at 24, 48 and 72 hours (h) post-transfection. GFP-plasmid was used as positive control for transfection.

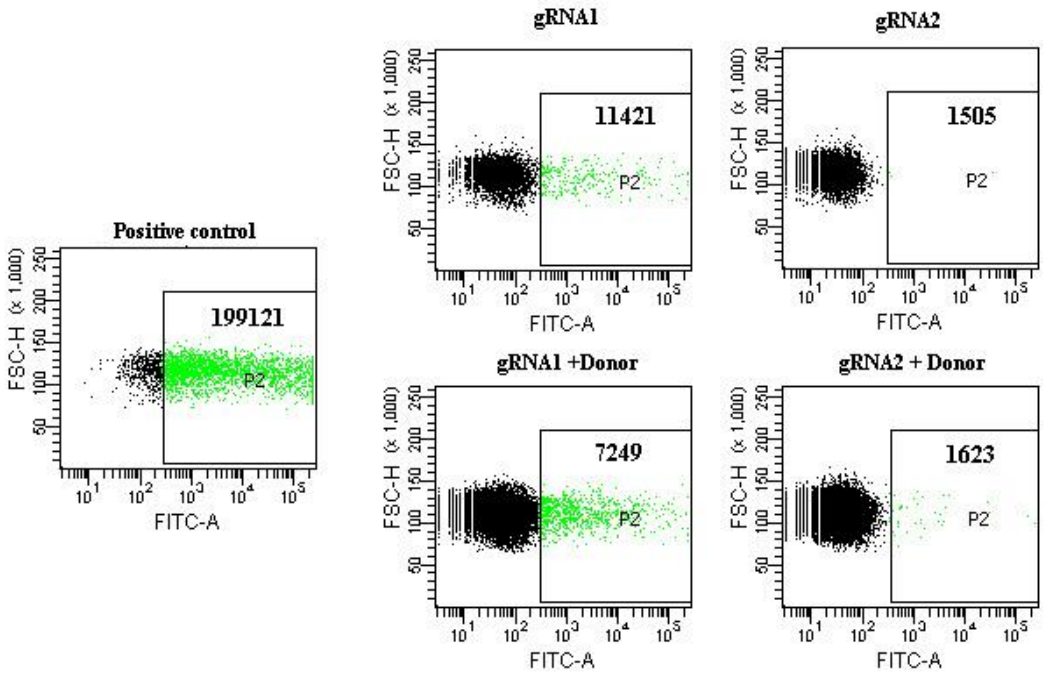

Figure 5 Counting and enrichment of transfected cells by FACS analysis. Each diagram is obtained from analysis of cells transfected with pCas9-gRNA1, pCas9-gRNA2, pCas9-gRNA1 + Donor, pCas9-gRNA2 + Donor and control plasmid. In each diagram the number of sorted out GFP positive cells is shown.

After 72-hour transfection, GFP positive cells were sorted out and ten individual cells were plated into each 96-well plate to ensure growth and clonal expansion for at least three weeks. Single colonies were further expanded in 24-well plates for mutational analysis. Overall, PCR product of target DNA from 41 clonal cell line were sequenced and analysed for sequence variation compared to control wild type sequence. The c.5509 T>C point mutation in *BRCA1* gene was identified in few clones (Fig. 5.1A). Sequencing analyses showed the presence of the heterozygous mutation (Fig. 5.1B).

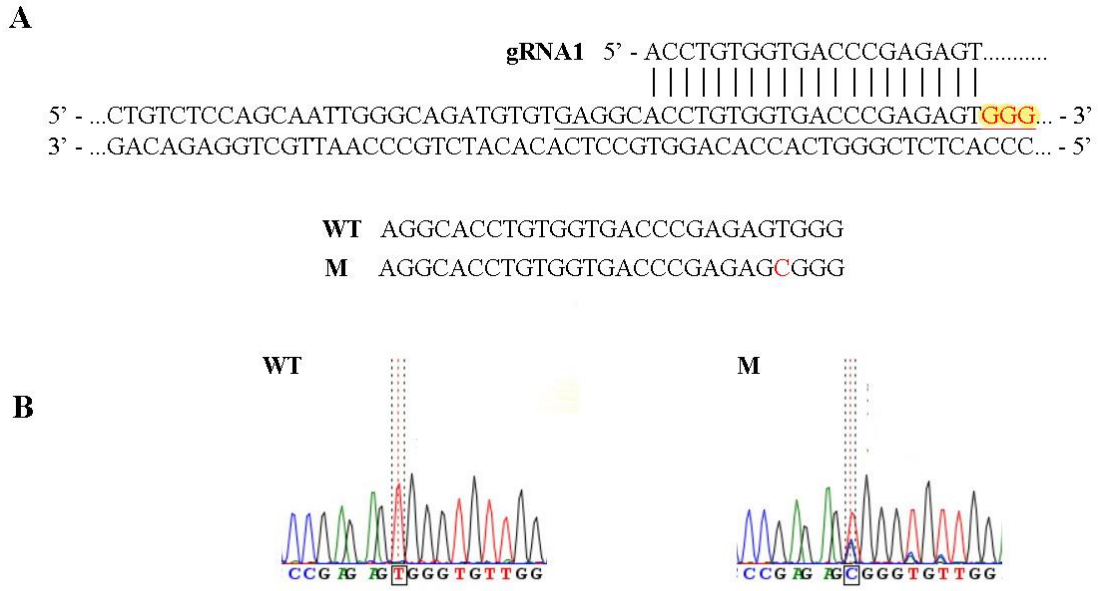

Figure 5.1 Identification of clonal cell line carrying the c.5509 T>C point mutation in *BRCA1* gene. **A** Schematic representation of localization of gRNA1 on target DNA sequence (underlined) upstream of a PAM (highlighted in yellow). Sequences from wild type (WT, control experiment of non transfected cells) and *BRCA1* c.5509 T>C mutant cells (M, transfected cells) are shown. **B** Sequence chromatograms showing wild type (WT) and *BRCA1* c.5509 T>C mutant (M) sequences.

4.6. Cisplatin and Olaparib treatment response

MCF-7, MDA-MB-231 and HCC1937 breast cancer cell lines were selected as representative models of *BRCA1* gene status: MCF-7 (luminal subtype) and MDA-MB-231 (triple-negative subtype) for wild type *BRCA1* and HCC1937 for mutated *BRCA1* gene. Cells were exposed to different concentration of Cisplatin and Olaparib for 72 hours, and proliferation rates were compared. HCC1937 were found to be significantly more sensitive to Cisplatin and to Olaparib compared to MDA-MB-231 (p=0.02 and p=0.05, respectively) and MCF-7 (p<0.001 and p=0.005, respectively) cells (Fig. 5.2), confirming that *BRCA1* status affects the response to DNA damaging agents. Of note, HCC1937 cell line harbors the pathogenic *BRCA1* c.5266dupC mutation which is located within C-terminal BRCT domain [215].

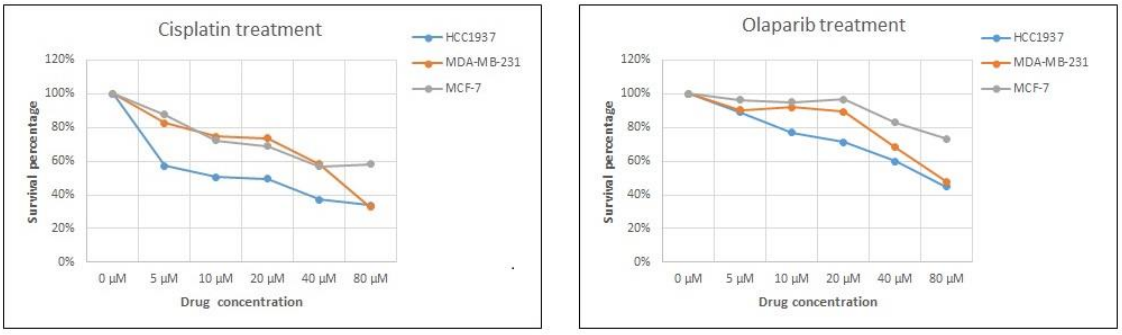

Figure 5.2 Survival response to Cisplatin and Olaparib treatment in breast cancer cell lines. MCF-7, MDA-MB-231 and HCC1937 were treated with 5, 10, 20, 40 and 80 µM Cisplatin or Olaparib for 72 hours. Each survival point represents the mean of three experimental replicates. HCC1937 shows significant higher sensitivity compared to MCF-7 and MDA-MB-231 cells.

Apoptosis was determined on wild type and *BRCA1* c.5509 T>C mutated MDA-MB-231 cells after 72 hours of treatment with 6 µM of Cisplatin and Olaparib. The analysis showed that the total percentage of apoptotic cells was significantly increased in *BRCA1* mutated cells (81%) compared with wild type cells (25%) after treatment with Olaparib (p<0.0001) (Fig. 5.3). Interestingly, the treatment with Cisplatin did not induce a significative increase of cell death in *BRCA1* mutated cells (34%) compared with wild type cells (46%).

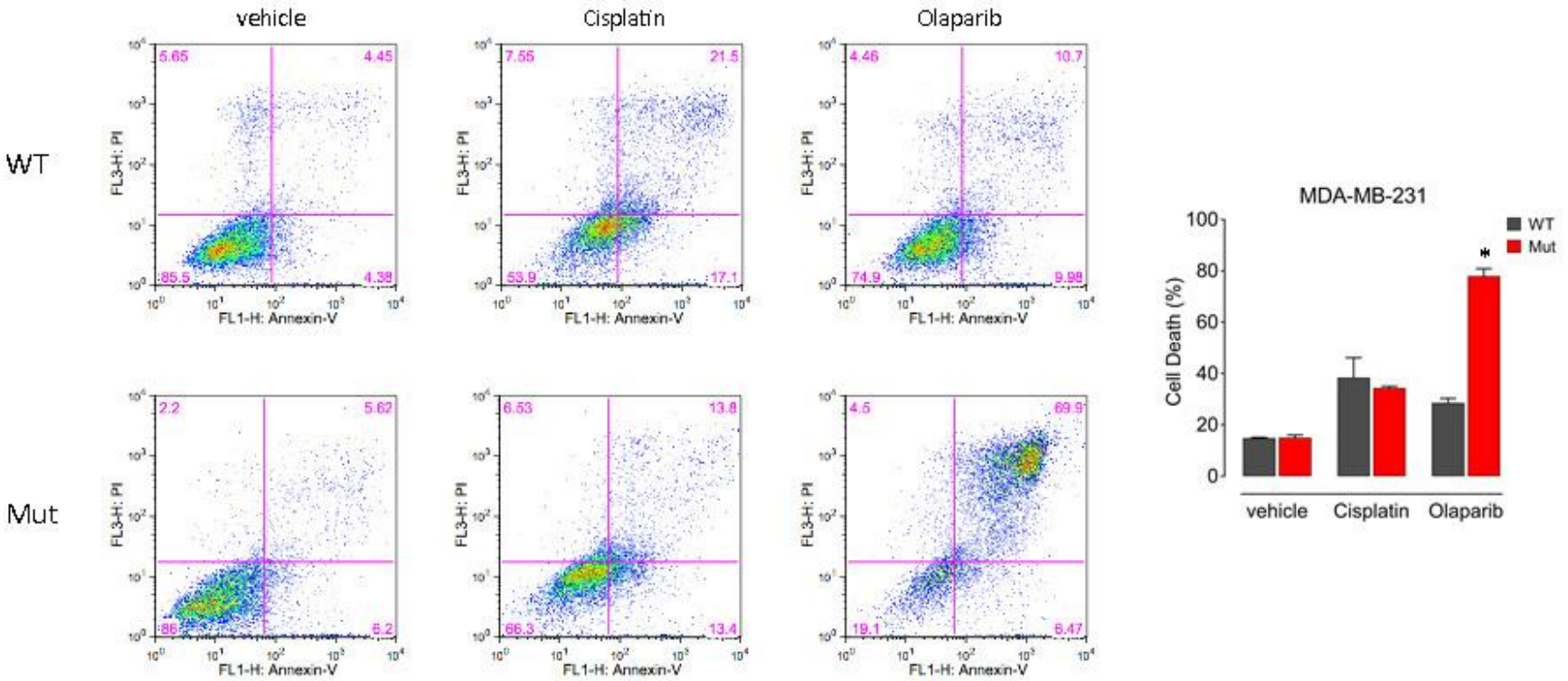

Figure 5.3 Cell apoptosis assays of wild type and mutated MDA-MB-231 cells after treatment with DNA damage agents. Wild type (WT) and mutated (Mut) MDA-MB-231 cells were treated with 6 µM Cisplatin or Olaparib for 72 hours. Representative flow cytometry scatter plots indicate the percentage of apoptotic cells (early phase, late phase and death phase) for the type of cell (WT and Mut) at each condition (vehicle, Cisplatin and Olaparib). P values < 0.05 were considered statistically significant.

Chapter 5

Discussion and Conclusions

The assessment of the biological relevance of *BRCA* VUS represents an important step for the prognosis and the clinical management of individuals carrying these variants. In general, VUS include missense mutations, small in-frame deletions, exonic and intronic variants potentially affecting pre-mRNA splicing, and variants in regulatory sequences, whose effects on the protein structure cannot be immediately predicted [202]. In this study, we aimed to describe *BRCA* alterations and to investigate the pathobiological significance of several VUS identified in a consecutive series of hereditary breast cancer patients. The frequency of *BRCA* mutations among our cohort was approximately 20%. We found that the majority of mutations detected was already classified as pathogenic, whereas up to 27% is currently considered as VUS in the main International databases of variants (BIC and LOVD). *In silico* analysis and protein prediction modeling allowed us to identify *BRCA1* c.5509T>C as a potential deleterious VUS in hereditary breast cancer patients. The c.5509T>C variant has been already reported as VUS in the BIC database in number of 8 times in populations of different ethnicity (Caucasian and Western Europe populations) [216], suggesting the need to improve the knowledge of its biological and clinical relevance for breast cancer risk. Importantly, this missense variant is located within the BRCT domain of BRCA1 protein, and consists in the substitution of a tryptophan to an arginine at 1837 amino acid position. This region is involved in binding of important BRCA1 interactors, such as corepressor CtIP and the helicase BACH1 [217], and possesses a transcription-activation function [218]. Mutations occurring within BRCA1 BRCT domain are the most frequent published alterations and specific VUS of this type have been already reported to functionally compromise the domain structure of BRCA1 protein [207]. In our analysis, the modeling for *BRCA1* c.5509T>C variant clearly predicted general destabilizing effects on the stability of the protein in terms of potentially compromised electrochemical interactions between specific amino acid chains and a different solvent exposure area. These structural changes affect the stability of the BRCT domain resulting in a potential lack of the interaction between BRCA1 and important proteins of the HR DNA repair pathway, such as CtIP and BACH1. The distribution of BRCA1 and BRCA2 protein among *BRCA* mutation carriers and non

carriers demonstrated that the deficiency of these proteins affect their functionality in terms of protein expression and localization in tumor tissue. Importantly, BRCA deficiency has been demonstrated to impact the expression levels of γ-H2AX as marker of DSB DNA damage [219]. Accordingly, our analysis identified aberrant BRCA1 and γ-H2AX levels in cancer tissue derived from *BRCA1* c.5509T>C VUS carriers, and we confirmed the effect of this variant in terms of deficiency of BRCA1 protein and impairment of the correct resolution of DNA DSB occurring in cancer cells. Although further experiments of co-localization with interacting proteins are warranted, this study suggests that the identification of this variant should be taken into account to define an efficient therapeutic approach. Our experiments supported the findings that *BRCA1* status affects the response to DNA damaging agents [170; 220]. Accordingly, our *in vitro* data confirmed the greater sensitivity in terms of cell death conferred by *BRCA1* c.5509 T>C mutation to Olaparib treatment compared to Cisplatin. This effect can be explained by the specificity of Olaparib mechanism of action, which is based on the blocking of BER process through the specific inhibition of PARP1 enzyme. This represents a deleterious event in cancer cells with already defective HR pathway and induces selective cell death due to the principle of synthetic lethality [221]. In contrast, Cisplatin interferes more generally with DNA replication through its direct binding to DNA molecules and chemoresistance to platinum compounds remains a very significant problem [222-223]. In line with our results, previous data from different functional assays have reported the most likely damaging effects of *BRCA1* c.5509T>C variant (protease sensibility, phosphopeptide binding activity, ES viability) [224-227]. Interestingly, this variant has been shown to have a functional impact in embryonic stem cells response to cisplatin in *in vitro* assay, sustaining the predicted impairment of HR DNA repair pathway [228].
Overall, we observed a decreased and a peripheral pattern of BRCA1 expression in terms of intensity and nuclear localization of the staining among tumors from *BRCA* carriers compared to tumors from the group of *BRCA* non carriers, and also to internal positive control. Moreover, gene expression analysis of *BRCA1*, *BRCA2* and *H2AX* highlights the lack of correlation between these two genes in *BRCA* mutation carriers, that contributes to impair the correct mechanism of DNA repair after DSB damage. Our data support the hypothesis that potential pathogenic VUS in *BRCA1* gene might contribute to the potential disruption of correct nuclear recruitment of this protein and other related-ones,

including BRCA2, to γ-H2AX foci during the cellular S phase and following DNA damage [229-231]. We have demonstrated that high levels of Ki-67 were significantly correlated with *BRCA1* mutations compared with the control-arm, supporting the notion that damaged DNA affecting genomic integrity unrestrains the proliferation of cancer cells [232]. Accordingly, in our series of breast cancer patients, all c.5509T>C VUS carriers showed a more aggressive histopathological phenotype (triple negative histology, grading 3 and high Ki-67). Finally, our results of γ-H2AX staining in *BRCA* mutation carrier tumors showed differential levels of DNA damage in relation to the location of mutation in *BRCA1* gene, suggesting the importance of the prediction of affected protein domain for the definition of the pathogenicity of identified VUS. In agreement with this data, we indirectly demonstrated that all potentially deleterious VUS showed levels of DNA damage similar to those tumors from carriers of *BRCA1* mutations. The evaluation of status of different proteins involved in DNA HR repair pathway could further help us to build a protein signature for the identification of individuals carrying VUS of high risk of breast cancer. However, the genetic basis of the majority of hereditary breast cancers remains unexplained. Recently, advanced genome sequencing and microarray genotyping technology has led to the identification of mutations within genes with lower penetrance than *BRCA1*/*2* [48]. In particular, they include genes (*PALB2, RAD51C, CHEK2, ATM* and *NBS1*) coding for proteins that interact with BRCA1 or BRCA2 or act in the same DNA repair pathway. Therefore, studies for the evaluation of additional mutations within these different breast cancer susceptibility genes are warranted.

In conclusion, our study confirms the more aggressive histopathological phenotype of tumors associated with *BRCA1* mutation carriers. Our data supports that the c.5509T>C VUS in *BRCA1* gene is a potentially deleterious alteration suggesting an intense surveillance and a less toxic targeted therapeutic approach with Olaparib as single agent for patients who are carriers of this variant. These results are in line with the ongoing clinical trial OlympiA which is directed to support the efficacy and safety of Olaparib as more efficacious treatment for breast cancer patients with germline *BRCA1*/*BRCA2* mutations drastically reducing the comorbidities induced by current chemotherapies. Moreover, Olaparib has been already approved by FDA as oral drug for *BRCA*-related patients with advanced ovarian cancer who were unsuccessfully treated with three or more lines of chemotherapy [174].

However, since there is no international consensus on the system of classification of VUS, the definition of an integrated multimodal approach is necessary for a better characterization of the biological effects of *BRCA1/2* VUS in hereditary breast cancer patients. This particular setting of patients might be in fact stratified according to the functional effects of *BRCA* alterations on HR pathway in associations with different predicted levels of response to DNA damaging agents, leading to the improvement of treatments.

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