Characterization of the c.190T>C missense mutation in BRCA1 codon 64 (Cys64Arg)

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Abstract. In the Milan area (Northern Italy), we identified a family characterized by a high prevalence of ovarian and breast cancer cases (5 out of 6 subjects, over 3 generations), and a predominant prevalence of ovarian lesions (4 out of 5 patients). Analysis of BRCA1 and BRCA2 genes allowed the identification of the missense c.190T>C mutation in codon 64 (Cys64Arg) of BRCA1. The aims of the present investigation were to characterize the functional implications of the c.190T>C mutation at the molecular level, and to search whether additional polymorphisms might be linked to the peculiar phenotypic features observed in the Italian pedigree. Molecular modelling studies suggested that substitution of the cysteine 64 with an arginine likely disrupts the architecture of the BRCA1 RING finger domain, responsible for the interaction with BARD1, essential for the tumor-suppressor activity of the BRCA1-BARD1 complex. By splicing site information analysis, exonic splicing enhancer site characterization, and analysis of transcript fragment length and sequence, we showed that the c.190T>C mutation was able to modulate the splicing of exon 5 in a fashion opposite to the c.190T>G transversion, responsible for the functionally-related Cys64Gly amino acid substitution. Genotyping of BRCA1 and BRCA2 in the Italian family revealed the presence of two significant polymorphisms: the cancer-associated c.2612C>T SNP in BRCA1, and the c.-26G>A SNP in the BRCA2 gene, acting as an ovarian cancer risk modifier in carriers of deleterious BRCA1 mutations. Analysis of these SNPs in a genotypically-unrelated Polish family, characterized by prevalent breast neoplasms in carriers of the c.190T>C mutation, revealed a genetic profile consistent with the hypothetic role of both polymorphisms.

Introduction

Hereditary breast cancer accounts for 5-10% of all breast cancers worldwide. Prevalence of BRCA1 and BRCA2 mutations among high-risk cancer patients may vary by ethnicity, study inclusion criteria and mutation detection techniques. Since their early characterization, inherited BRCA1 mutations were found to be responsible for ~45% of inherited early-onset breast cancers, and for >80% of inherited breast and ovarian cancers (1). In Europe, BRCA1 mutation frequencies in breast cancer populations show considerable variability and range between 0.4% in Finland and 7% in Sweden (2). Meta-analytic mean cumulative cancer risks for mutation carriers at age 70 years are 57% for BRCA1 and 49% for BRCA2 mutation carriers; ovarian cancer risk is 40% for BRCA1 and 18% for BRCA2 mutation carriers (3). In Italy, breast cancer penetrance, estimated in a series of 568 families, was found to be 27% at age 50 and 39% at age 70 in BRCA1 mutation carriers, and 26% at age 50 and 44% at age 70 in BRCA2 mutation carriers (4). Ovarian cancer penetrances in Italy were estimated to be 14% at age 50 and 43% at age 70 in BRCA1 mutation carriers, and 3% at age 50 and 15% at age 70 in BRCA2 mutation carriers (4).

BRCA1 is a gene with a coding sequence spanning over 23 exons, including the large exon 11, that holds more than half of the coding potential of the gene (5). The major BRCA1 product comprises 1863 amino acids and can be subdivided into three major domains: a RING domain adjacent to the amino-terminus, tandem-repeated C-terminal BRCT domains,
and a central domain, including the large region encoded by exon 11.

The function of the poorly conserved central domain is not fully understood, although some evidence suggests interaction with RAD51 (6). The structure of BRCT motifs is similar to C-terminal domains found in a number of proteins involved in cell cycle checkpoints and DNA repair (reviewed in ref. 7). BRCT motifs were shown to activate gene transcription when linked to a DNA-binding domain (8).

The RING domain is probably the better-characterized structure of BRCA1. It encompasses amino acid residues 24-64 and contains a Cys3-Hys-Cys4 (C3HC4) motif, which has been shown to allow interaction with proteins involved in cell-cycle regulation, such as cyclins and cyclin-dependent kinases (9), and with estrogen receptors (10). A major binding partner is the BRCA1-associated RING domain protein 1 (BARD1). Originally identified due to its interaction with the RING domain of BRCA1 (11), BARD1 is required for S phase progression, contact inhibition and normal nuclear division. BRCA1 acquires significant ubiquitin ligase activity when bound to BARD1 as a RING heterodimer, and catalyzes Lys-6-linked polyubiquitin chains, which are recognized in vitro by the 26S proteasome for deubiquitination as opposed to degradation (12).

Pathogenic alterations in BRCA1 include mutations in the regulatory regions of the gene, splice site mutations, nonsense/frameshift/missense mutations, and large chromosomal rearrangements, estimated to account for ~19% of all BRCA1 mutations in Italian patients (13).

In Northern Italy, we identified a family characterized by a high prevalence of neoplasia: of a total of 6 women in a 3-generation maternal lineage, 4 were affected by ovarian cancer, and one by early-onset bilateral breast cancer associated with ovarian dysplasia and abnormalities. The only non-diseased subject showed breast dysplastic lesions. In two patients belonging to the youngest generation and treated with a modern approach, a good prognosis and long-term survival was observed. Although family history was collected at diagnosis or hospitalization, patients were not subjected to genetic analysis, nor were treated as members of a HBOC pedigree.

To identify the molecular determinants of the hereditary breast and ovary cancer (HBOC) syndrome in this family, we analyzed the 

**Materials and methods**

Detection of BRCA1 and BRCA2 mutations and polymorphisms

High-resolution melting analysis. DNA was extracted from peripheral blood leukocytes of Patient 1 (Fig. 1), and the entire coding sequence and exon-intron boundaries were amplified using the polymerase chain reaction (PCR). Thirty-six PCR products (size range: 150-437 bp for BRCA1) and 49 PCR products (179-500 bp for BRCA2) were amplified (14). PCR was performed in 10 μl reaction mixture containing template DNA, dNTPs (Roche, Mannheim, Germany), FastStart Taq DNA Polymerase (Roche), fluorescent dye LCGreen Plus (Idaho Technology) and forward and reverse primers for each gene segment, in Roche LightCycler capillaries and amplified in an adapted RapidCycler2 instrument (Idaho Technology). PCRs were performed using appropriate sets of primers as described (14), with slight modifications.

PCR conditions were optimized to Tm between 49 and 68°C for each segment. After 40 cycles of amplification, PCR products underwent an additional cycle of 1 min at 98°C, followed by 5 min at 40°C to promote heteroduplex formation. Reaction products were then transferred to a high-resolution melting instrument (HR-1, Idaho Technology) for high-resolution melting and curve analysis. Samples were melted at 0.2°C/sec ramp rate. Melting profiles were analyzed with the HR-1 software using fluorescence normalization, temperature shift and conversion to difference and derivative plots. Fragments with melting patterns different from the wild-type were sequenced to determine the exact sequence alterations.

Nucleotide numbering was according to BIC database, where position 1 represents the first nucleotide of exon 1. Reference sequences used were NG_005905.1 for BRCA1, and NW_001838072.1 for BRCA2.

Before sequencing, PCR products were purified with ExoSAP-IT (USB, Cleveland, USA), and then sequenced in both directions using the Big Dye terminator 1.1 cycle sequencing kit (Applied Biosystems, Foster City, USA). Sequencing analysis was performed on an automatic sequencer ABI PRISM 310 genetic analyzer (Applied Biosystems).

RNA-based sequencing. In the Polish pedigree, detection of the c.190T>C mutation in BRCA1 was performed by sequencing RT-PCR products, amplified from RNA purified from peripheral blood lymphocytes of patients and relatives, as previously described (15).
Table I. PCR primers for genotyping the c.190T>C (Cys64Arg) mutation in BRCA1, and SNaPshot primers for genotyping the c.2612C>T (Pro871Leu) SNP in BRCA1 and the c.-26G>A SNP in BRCA2.

<table>
<thead>
<tr>
<th>Mutation/polymorphism</th>
<th>PCR primers</th>
<th>SNaPshot primer</th>
</tr>
</thead>
</table>
| BRCA1: c.190T>C (Cys64Arg) | F - GCCTTTTGTAGTTCCCTTCTAC  
R - TCCTACTGTTGTTGTTCCC | // |
| BRCA1: c.2612C>T (Pro871Leu) | F - GGAGGCAGAAAACAGAAC  
R - CCAACCCAGGAGGACTT | GTTCAAAGCAGGTCATTGGCTC |
| BRCA2: c.-26G>A | F - TGTGCCTCCAGGAGATG  
R - AGCAACCTGAGCTGACGTACTG | CATTCTACCTAGATATCTCCAGT |

F, forward; R, reverse.

**PCR-RFLP and SNaPshot analysis.** To detect the c.190T>C (Cys64Arg) and c.2612C>T (Pro871Leu) variants in BRCA1, and the c.-26G>A non-coding variant in BRCA2 in Italian and Polish patients, i) PCR combined with restriction fragment length polymorphism (RFLP) analysis, or ii) SNaPshot techniques were used.

First, PCR products were amplified using 100–200 ng DNA in a 25 μl reaction containing 0.5 mM dNTPs (Amersham Biosciences), 1X PCR buffer (Invitrogen), 1.5 mM MgCl2, 1 μl of each primer (forward and reverse), and 0.3 μl LIZ-120 size standard (Applied Biosystems). Results were visualised on the Peak scanner program version 1.0 (Applied Biosystems).

**In silico structural and functional analysis of the Cys64Gly mutation.** The three-dimensional NMR structure of the BRCA1-BARD1 RING finger heterodimer [PDB ID 1JM7; (16)] was downloaded from the Protein data bank (http://www.rcsb.org). Structural models were drawn with the Swiss-PdbViewer (17).

Analysis of splice sites was performed using the Information analysis tools at Schneider’s lab, Molecular Information Theory Group, Center for Cancer Research Nanobiology Program, National Cancer Institute, Frederick, MD (http://www.lecb.ncifcrf.gov/~toms/delila.html). Exonic splicing enhancer analysis (18,19) was performed using the ESEfinder 2.0 program, Cold Spring Harbor Laboratories (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home).

**Immortalization of peripheral blood lymphocytes from patients and healthy individuals.** EBV-immortalized cell lines from healthy individuals, homozygous (T/T) at the 109 locus, were obtained from the cell bank of Professor A. Vral, (University of Gent, Belgium). The EBV cell line BC1 was generated from peripheral blood lymphocytes of the Italian patient 1 (Fig. 1), heterozygous for the c.190T>C mutation in BRCA1. Immortalization was achieved by exposure of lymphocytes to the supernatants of the B95-8 EBV producer cell line, according to a standard protocol (20). EBV cell lines were used to amplify transcript sequences of the BRCA1 gene by RT-PCR, and for radiosensitivity testing with the cytokinesis-block micronucleus (MN) assay, as previously described (20). The EBV cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin with 10% FBS and cultured at 37°C, 5% CO₂, in a humidified incubator.

**Analysis of BRCA1 transcripts by RT-PCR.** Total RNA was isolated from 10⁷ EBV-immortalized lymphoblastoid cells using the SV Total RNA isolation system (Promega Italy), according to manufacturer directions. A 2-3 μg aliquot of total RNA was used to generate first-strand cDNA and to amplify transcript fragments with a RobuS² II RT-PCR kit (Finnzymes, Italy) at the following conditions: 48°C for 30 min, followed by 94°C for 2 min for first-strand cDNA synthesis, followed by 35 amplification cycles (94°C, 30 sec; 60°C, 60 sec; 72°C, 1 min) and a final extension step at 72°C for 7 min. To detect normal and/or alternative transcript fragments
of BRCA1, exon 5, we designed a forward primer (5'-ATT TATCTGTCTTTCGCGTTG-3'), annealing with a specific sequence on exon 5, and a reverse primer (5'-CACTGAG ACTGGTTTCCTGC-3'), annealing on exon 6. In normal conditions a fragment of 456 bp would be amplified by RT-PCR. In case of alternative splicing, as described in cells harboring the c.190T>C mutation (21), a 434-bp fragment would be detected. For transcript fragment length analysis, RT-PCR products were run on 2.5% agarose gels; fragments were analyzed by restriction digestion (presence of T>C mutation was screened with SnaBI) and automated sequencing.

Results and Discussion

Clinical and genetic analysis of the Italian HBOC pedigree. Fig. 1 shows the HBOC pedigree from Italy analyzed in this study. Patient 1 was born in 1931; at the age of 27 an undefined ‘adenomatous nodule’ was surgically removed from her left breast. At age 47 she was diagnosed with bilateral breast cancer; a right radical mastectomy and a left quadrantectomy were performed to remove a T3N1b+ and a T1N0- lesion, respectively; surgery was followed by chemotherapy. Ten years later she was again hospitalized for removal of a left T1N0- lesion by radical mastectomy. On the same occasion,
diffuse ovarian dysplastic lesions were reported. The patient underwent post-surgery standard chemotherapy treatment. The patient is in long-term remission since 1989. Patient 2 was diagnosed with breast dysplasia over 15 years ago, and is presently disease-free. Patient 3 was diagnosed with ovarian cancer (pT N G2) in 1988 at the age of 58, underwent surgical removal and chemotherapy, and is presently in remission. Patients 1, 2 and 3 are daughters of patient 4, who died of ovarian cancer at the age of 46. Patients 5 and 6, sister and mother of patient 4, also died of ovarian cancer, both around the age of 60.

All patients gave their informed consent for genetic analysis and publication of anonymous data. Patient 1 gave her consent for thorough BRCA1 and BRCA2 analysis. Genomic DNA was purified from peripheral blood lymphocytes and subjected to high-resolution melting analysis. Table II lists the mutations and polymorphisms identified in this patient. The c.190T>C mutation in exon 5 of BRCA1, generating the Cys64Arg amino acid substitution, was detected (Fig. 2). Moreover, nine BRCA1 and four BRCA2 known sequence variants, detected with high allele frequencies (19.57-38.26%) in diverse European control populations (e.g., 23), were found. All sequence variants were
cross-referenced with the Breast Cancer Information Core (BIC; http://research.nhgri.nih.gov/projects/bic/) (Table II), and all except two were classified as not clinically significant by BIC. c.6841+80_83delTAAA was the only variant not classified as non-significant; it is reported only once and classified as unknown. This is not unusual, since it is located 80 bp away from exon junction, and the variants commonly reported in BIC are up to 50 bp away from exon junctions. This variant is present in a high percentage of healthy women with no family history of breast cancer [allele frequency in a central-European population: 38.26%; (23)], thus it can be classified as non-significant.

Together with the missense Cys64Gly substitution, Cys64Arg is listed as being of unknown clinical significance in the BIC database. However, the Cys64Tyr substitution is defined as clinically important.

The functional relevance of the Cys64Arg substitution is described in the following section. Analysis of the polymorphisms detected in patient 1, showed the presence of the c.2612C>T (Pro871Leu) missense polymorphism in BRCA1, and of the c.-26G>A noncoding variant in BRCA2 (Table II). The significance of these mutations will be discussed in the following chapter.

**Structural and functional analysis of the c.190T>G missense mutation**

**Functional role of the Cys64Arg amino acid substitution.** Cys61Gly and Cys64Gly are among the most prevalent BRCA1 missense mutations, and are hypothesized to be responsible for hereditary breast cancer. These substitutions target specific metal-binding residues of BRCA1, and alter zinc coordination within the RING domain by presumably disrupting its three-dimensional coordination and in turn resulting into the impairment of BRCA1 interaction with its major functional ligand, BARD1 (reviewed in ref. 5).

The Cys61Gly mutation was shown to abolish metal binding to Site II of the RING domain of BRCA1, and disrupt BRCA1 homodimer formation (24). Moreover, the mutation resulted in increased proteolytic susceptibility of the carboxy-terminal portion of the amino-terminal domain of BRCA1. The Cys61Gly mutation was also shown to abolish the in vitro ubiquitin-ligase activity displayed by the BRCA1-BARD1 complex (25).

The Cys64Gly mutation was demonstrated in a large African-American pedigree, characterized by early-onset breast/ovarian cancer, and subsequently in other African-American families (1). Similarly to Cys64Gly and Cys61Gly, the Cys64Tyr mutation was shown to abolish the ubiquitin protein kinase activity of BRCA1, and to cause hypersensitivity to ionizing radiation (26). The same role can be hypothesized for the more rare Cys64lys mutation (27).

So far, the Cys64Arg mutation has only been reported in Italy and in a single unrelated family in Poland (15,28,29). Similarly to Cys61Gly, Cys64Gly and Cys64Tyr (26), the Cys64Arg substitution may cause disruption of BRCA1-BARD1 interactions, thus affecting the ubiquitin-ligase activity of this heterodimeric complex.

The N-terminal region of BRCA1 shows the typical RING finger structure, belonging to the C3HC4 RING finger domain family. In this family, two zinc ions are tetrahedrally coordinated by three cysteines and one histidine residue, and by four cysteine residues, respectively (see Fig. 3, upper panel, for a model). BRCA1 and BARD1 interact via an extensive anti-parallel 4-helix bundle interface formed by helices that flank the central RING motifs. From the analysis of the NMR structure of the BRCA1-BARD1 RING finger heterodimer (PDB ID: 1JM7), it appears that the Cys64 residue, belonging to the C,H Zn coordination motif, is in close proximity to the Asp67 residue on the beta strand flanking the Zn environment.

In the case of the Cys64Arg mutation, Arg64 is able to rotate towards Asp67, possibly forming a salt bridge (Fig. 3, lower panel). In turn, Zn is no longer stabilized by the tetradedral coordination field formed by Cys39, His41, and Cys61. Alternatively, the Zn ion might form a complete coordination cage involving the Arg64 Nε atom. Interestingly, in the wild-type protein the beta strand opposite to the mutation (e.g., Cys39, Asp40, and His41) interacts with BARD1, with a hydrogen bond between Asp40 of BRCA1 and Asn98 of BARD1 (Fig. 3). It might therefore be suggested that the Cys64Arg mutation determines a rearrangement of the BRCA1 39-41 residues and destabilizes the BRCA1(Asp40)-BARD1(Asn98) interaction (Fig. 3).

A similar hypothesis for the destabilization of the complex can be formulated for the Cys64Gly and Cys64Tyr mutants, described in other BRCA1 pedigrees (24-26). In the first case, the zinc atom should clearly dissociate from its coordination site. In the latter case, Tyr does not appear to be able to rotate towards the Zn ion, unless a dramatic rearrangement of the BRCA1 39-41 strand would take place.

**BRCA1 involvement in DNA double-strand break repair.** By likely disrupting the RING domain binding capacity, the Cys64Arg substitution might decrease the capacity of BRCA1 to stimulate DNA double-strand break repair in response to genotoxic agents such as ionizing radiation. This was shown for the Cys64Gly, Cys64Tyr and Cys61Gly substitutions (26).

To test this hypothesis, we generated an EBV-immortalized lymphoblastoid cell line from peripheral blood lymphocytes of patient 1 from the Italian HBOC family, denominated BC1. Since BC1 cells showed an unexpected loss of heterozygosity during immortalization (Fig. 6, under investigation), they represented an optimal model for unbiased analysis of DNA DSB repair capacity. Cells were exposed to a dose of 2-Gy γ-rays, and subjected to the cytokinesis-block micronucleus (MN) assay. Radiosensitivity of BC1 cells (109±28 micronuclei/500 binucleated cells) was compared to radiosensitivity of 23, randomly selected control EBV cell lines, T/T homozygous at locus 190 (codon 64). Fig. 4 shows the frequency distribution of MN scores of control EBV cell lines. Sixteen cell lines were more resistant to the genotoxic damage of ionizing radiation, whereas only 7 EBV control cell lines were found to be more radiosensitive. Thus, BC1 cells, carriers of the homozygous c.190T>C mutation, were amongst the most radiosensitive cell lines investigated.

**Effect of the c.190T>C mutation on BRCA1 mRNA splicing.** The c.190T>G mutation was shown to generate aberrant splicing products of the BRCA1 RNA (21). This occurred by two main mechanisms. First, the T>G transversion activated a cryptic splicing donor site located within the coding region of exon 5, adjacent to the boundary with intron 5. Activation of the cryptic splicing donor site in the T>G mutant results in...
Figure 5. (a) Lister map, sequence walkers and information content of natural and cryptic splice donors of \textit{BRCA1} exon 5. Exonic sequences are capitalized. (A) information content of the natural splicing donor (in dark grey). The position of the cryptic splicing donor is highlighted in light grey. (B) information content of the cryptic splicing donor (in light grey), in the absence of an activating mutation. The c.190T base is represented in dark grey. (C) cryptic splicing donor (in light grey) activated by the c.190T>G transversion (in dark grey). (D) putative cryptic splicing donor containing the c.190T>C mutation (in dark grey). The Schneider's lab tools (http://www.lecb.ncifcrf.gov/~toms/delila.html) were used to produce lister map and sequence walkers, and to calculate the information content of these splicing sites. (b) Effect of the c.190T>G and c.190T>C mutations on high-score SRp55-binding motifs, in \textit{BRCA1} exon 5. SR motif analysis was performed using ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home). The c.190T>G mutation results in disruption of the SRp55 motif shown by the arrow. Conversely, the c.190T>C mutation increases the score of the SRp55 motif from ~3.6 to ~5.1. SR motif types are listed in the legend.
the deletion of 22 bases from exon 5, and in the generation of an aberrant, frame-shifted transcript, predicted to create an UGA premature termination codon at position 80 within exon 6 (21).

Surprisingly, in addition to the activation of a cryptic splicing site, the c.190T>G mutation was shown to disrupt a high-score SRp55-binding exonic splicing enhancer (ESE) motif (position 189-193), likely involved in the normal splicing mechanism of intron 5 (21).

To investigate the influence of the c.190T>C mutation in BRCA1 splicing, we compared by information analysis the information content (IC) of the natural BRCA1 exon 5 splicing donor with the corresponding value of the putative splicing sites generated by the c.190T>G and c.190T>C mutations. Fig. 5 shows that the natural splicing donor of BRCA1 exon 5 is characterized by an information content (IC) of 6.0 bits. Interestingly, the wild-type cryptic donor (c.190T), located 22 bases upstream the exon 5-intron 5 boundary, shows a slightly higher information content (6.2 bits, Fig. 5a). The choice of the natural splicing donor over the cryptic one, is likely determined by the presence of splicing modulators, like the SRp55 motif described above, disrupted by the c.190T>G mutation (21).

In the presence of the c.190T>G mutation, the score of the cryptic splice donor increases markedly, to a value of 9.5 bits. Since the likelihood of a splicing event has been shown to correlate with the amount of information contained in competing, alternative splice sites (30), our information-content analysis confirms the data described by Yang and coworkers (21), and shows that the c.190T>G mutation, by increasing the IC of a cryptic splice donor, is responsible for the generation of alternatively-spliced, aberrant transcripts of the BRCA1 gene.

In striking contrast with this evidence, information analysis showed that the c.190T>C mutation decreases the information content of the cryptic splicing donor from 6.2 to a value of 5.5 bits, thus diminishing the likelihood of aberrant splicing of the BRCA1 RNA.

This hypothesis was strongly supported by analysis of the ESE motifs likely concurring to exon 5 splicing. Fig. 5b shows that, as shown by Yang et al (21), the c.190T>G mutation results in disruption of a high-score SRp55-binding ESE motif. However, the c.190T>C mutation not only preserves the binding capacity of the ESE motif, but also further increases its score from ~3.6 to ~5.1.

In summary, whereas the c.190T>G mutation increases the information content of the competing, cryptic splice site located 22 nt upstream of the natural donor of exon 5, and simultaneously disrupts a high-score SRp55 binding site, working in normal conditions to the advantage of normal splicing of intron 5, the c.190T>C mutation preserves and even strengthens the natural splicing scenario of BRCA1 intron 5, by decreasing the information content of the cryptic splice donor, and concurrently increasing the strength of the SRp55 ESE motif.

To confirm these in silico data, we analyzed by RT-PCR the splicing pattern and transcript sequence of the exon 5-exon 6 boundary, using total RNA purified from the BC1 cell line. RT-PCR performed with the same primers, annealing with specific sequences in exon 5 and exon 6, used by Yang et al in their splicing study (21), yielded the expected fragment of 456 bp (Fig. 6). The experiment was repeated 6 times with varying stringency conditions, but no fragments indicative of aberrant splicing were detected. Sequencing of the 456-bp fragment amplified from BC1 cells confirmed a correct splicing pattern of BRCA1, at the level of exons 5 and 6 (Fig. 6).

Possible role of single nucleotide polymorphisms in BRCA1 and BRCA2, in the generation of the phenotype observed in the Italian HBOC family. In the literature, the typically Italian
c.190T>C mutation has to our best knowledge only been reported abroad in Poland, in a large family without Italian ancestry (15). This family is characterized by 3 cases of breast cancer (with two long-term survivors), one case of ovarian cancer (deceased) and one case of leukemia in a male proband (Fig. 7). For tentative comparison with a pedigree showing a potentially unrelated haplotype, we analyzed a total of 8 healthy or diseased probands from this Polish family.

As shown in a previous paragraph, analysis of the BRCA1/BRCA2 genotype of patient 1 from the Italian HBOC family (Table II), showed the presence of two heterozygous polymorphisms that were reported in the literature to be significantly linked to breast or ovarian cancer.

c.2612C>T, BRCA1. The c.2612C>T missense variant in exon 11 of BRCA1, responsible for the Pro871Leu amino acid substitution, was shown to be the only polymorphic variant, out of a panel of 19 SNPs detected in BRCA1, whose frequency was significantly higher in patients (42%) than in control individuals (28%) (31). This was described in a population of 200 patients with breast/ovarian cancer screened for BRCA1 mutations, drawn primarily from high-risk families and from isolated early-onset breast cancer cases. Comparison was made with a population of control individuals of diversified haplotypes.

Interestingly, the heterozygous c.2612C>T transition was detected in patients 1 and 3 from the Italian pedigree, but not in the cancer-free proband 2 (Table III), sister of these two patients. In breast cancer patients 7 and 8 from the Polish pedigree, the Pro871Leu substitution was found in a heterozygous and homozygous expression, respectively (Table III). All other healthy younger family members express this mutation in homo- or heterozygous forms. It would be of interest to extend these observations to larger patient populations and pedigrees, to ascertain the role of this polymorphism in the phenotypical expression of neoplasia (age of onset, intra-pedigree prevalence, penetrance and influence on prognosis).

c.-26G>A, BRCA2. It has been demonstrated that the risk of ovarian cancer is higher for carriers of BRCA1 mutations in the 5' portion of the gene, (e.g., Cys64Arg), although larger excesses of ovarian cancers were also observed near the 3'-end of the gene (32). In Italian patients carrying the Cys64Arg mutation, the ratio between breast and ovarian lesions was shown to be ~10-fold (28). In contrast, the Italian HBOC pedigree analyzed in this study showed a high prevalence of ovarian cancers (80%; Fig. 1). Moreover, in breast cancer patient 1, ovarian precancerous lesions were detected. In families showing highly predominant ovarian lesions, the presence of modifier factors influencing the tumor localization may be hypothesized.

The c.-26G>A transition within the 5'UTR of BRCA2 was initially identified as a polymorphism able to suppress the

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Table III. Expression of significant polymorphisms in the BRCA1 or BRCA2 genes, in Italian and Polish pedigrees.a

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Pt. no.</th>
<th>BRCA1, c.2612C&gt;T</th>
<th>BRCA2, c.-26G&gt;A</th>
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<tbody>
<tr>
<td>Italy</td>
<td>1</td>
<td>C/T</td>
<td>G/A</td>
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<tr>
<td></td>
<td>2</td>
<td>C/C</td>
<td>G/G</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>C/T</td>
<td>G/A</td>
</tr>
<tr>
<td>Poland</td>
<td>7</td>
<td>C/T</td>
<td>G/G</td>
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<tr>
<td></td>
<td>8</td>
<td>T/T</td>
<td>G/G</td>
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<td>G/A</td>
</tr>
</tbody>
</table>

*Cancer patients are emphasized with bold italic fonts.*
expression of the RNA transcribed from its carrier allele, without affecting transcription of the allele carrying the c.-26G variant (33). This allele-specific BRCA2 suppression was demonstrated only in patients harboring a pathogenic mutation in BRCA2, and not in individuals heterozygous for the c.-26G>A SNP, but lacking an additional deleterious mutation (33).

The c.-26G>A polymorphism in BRCA2 was subsequently characterized as an ovarian cancer risk modifier in carriers of deleterious BRCA1 mutations. In a study performed on 778 women carrying a BRCA1 germline mutation belonging to 403 families, a significant association of the c.-26A allele with increased risk of ovarian cancer was observed (Hazard ratio: 1.43, 1.01-2.00, 95% CI) (34). The authors postulated for the c.-26G>A variant of BRCA2 a role as modifier of BRCA1-associated ovarian cancer risk in BRCA1-mutation carriers, but not in the general population (34).

In the Italian pedigree shown in Fig. 1, characterized by predominant ovarian lesions, heterozygous expression of the c.-26G>A polymorphism was documented in cancer patients 1 and 3, showing bilateral breast cancer and diffuse ovarian dysplasia, and ovarian cancer, respectively (Table III). Cancer-free proband 2, sister of patients 1 and 2, shows a homozygous G/G genotype. Interestingly, the patients of the Polish pedigree (Fig. 7), characterized by predominant breast cancers, showed a homozygous G/G profile.

In summary, the c.2621C>T and the c.-26G>A common variants seem to segregate in the Italian and Polish pedigrees in a fashion coherent with their hypothesis. However, the data shown in this study, analyzed in only two pedigrees, are only indicative of the potential association of the studied polymorphisms with disease penetrance and phenotypical modulation. It would be of interest to substantiate these preliminary observations with extensive studies performed on a large number of related and unrelated pedigrees.

In conclusion, the c.190T>C (Cys64Arg) mutation analyzed in this study is presently described in the BIC database as a mutation of unknown clinical significance. Our study is indicative of a pathogenic role of this mutation, as concurred to the peculiar phenotypic characteristics of the pedigrees examined in this preliminary study.

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References


