original article

Two mutations of BRCA2 gene at exon and splicing site in a woman who underwent oncogenetic counseling

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Background: Although most BRCA sequence variants are clearly deleterious and unequivocally pathogenetic, several are still classified as variants of unknown significance.

Patients and methods: We followed families undergoing oncogenetic counseling from risk identification to risk definition by genetic testing and risk management.

Results: We identified two germline mutations in the BRCA2 gene in a woman with breast and ovarian cancer. One sequence alteration was 859/G>A in exon 7 (V211I). The other second sequence alteration (IVS13-2A>T) affected the splicing site in intron 13. The latter alteration is not yet listed in the Breast Cancer Information Core database. RT-PCR resulted in transcription of a sequence lacking exon 7 and a subsequent anomalous stop codon in exon 9 thereby confirming altered messenger RNA (mRNA) maturation. Amplification of the mutation in intron 13 resulted in transcription of a sequence lacking exon 14 and an anomalous stop codon in exon 15 thereby confirming altered mRNA maturation. Both mutations led to a truncated BRCA2 protein in its carboxy-terminal region.

Conclusion: The two BRCA2 mutations identified affect mRNA splicing fidelity and play a pathogenetic role in breast and ovarian cancer.

Key words: BRCA2 germline mutation, cancer genetic counseling, hereditary breast cancer, intron mutations, unknown significance variation

Introduction

Progress in the molecular genetics of cancer has led to the identification of predisposing hereditary breast/ovarian cancer (HBOC) genes. About 84% of hereditary breast cancers derive from BRCA1 and BRCA2 mutations that sustain the HBOC syndrome [1]. Approximately 3400 sequence variants, listed in the Breast Cancer Information Core (BIC) database, have been identified in BRCA1/2 genes by extensive mutational analysis since they were cloned in the mid-1990s [2–5].

The HBOC syndrome is characterized by clustering of tumors at sites other than the breast in the same family. BRCA1 mutation carriers have an increased relative risk for prostate, pancreas, endometrium and cervix cancer, whereas BRCA2 mutation carriers have an increased relative risk for prostate, pancreas, ductal gall, stomach and skin cancer, such as melanoma [6–9].

Few data are available about the correlation between the site of the mutation and a specific cancer spectrum [2, 10]. Risch et al. [11] reported an increased risk of breast cancer associated with mutations downstream the BRCA1 coding sequence and a peak in ovarian cancer risk associated with mutations in the middle of the coding sequence. Several studies reported a higher risk of ovarian cancer for carriers of BRCA2 mutations located in the ovarian cancer cluster region (OCCR) [12], whereas an increased risk of breast cancer seems to be restricted to non-OCCRs, particularly those in region 3' of the OCCR [10]. Lastly, mutations of uncertain significance, such as intronic or missense alterations, have been implicated in the HBOC syndrome, and studies are underway to determine whether they have polymorphic or pathogenetic role [2, 13, 14].

Given the complexity of issues related to hereditary/familial breast cancer and clinical implications of genetic testing, oncogenetic counseling is, at present, the most suitable approach to the management of high-risk subjects [15, 16].

Here, we report a case in which mutational analysis was carried out, within oncogenetic counseling, and revealed two mutations in the BRCA2 gene, one of which was not listed in the BIC database. Written informed consent was obtained for publication of this case.

Patients and methods

Oncogenetic counseling

Patients or disease-free subjects were referred to Screening and Follow-up for Hereditary and Familial Cancer Unit at Federico II University in Naples,
Cancer genetic counseling was led by the oncologists of the multidisciplinary team, according to the multistep model designed and validated within the Italian Network for ‘Hereditary Breast and/or Ovarian Cancer’ [16–18]. At proband intake, the family history of at least three generations was acquired by pedigree construction and the clinical history registered. For each subject, we defined the risk profile (hereditary, familial and personal) by widely used predictive models [19–21]. Hereditary and familial risks were clinically defined according to the Modena criteria [22, 23].

We assessed the a priori genetic risk of BRCA mutations by the Frank criteria, and the BRCAPro Italia model [24–28]. When an a priori hereditary risk was identified, genetic testing for the BRCA1 and BRCA2 genes was offered to affected subjects according to the American Society of Clinical Oncology policy statement [29, 30].

Subjects had sessions with the psycho-oncologist of the team in order to assess their psychological functioning and empower them to make informed and aware decision about genetic testing and cancer prevention [15]. Preventive measures are offered to subjects on the basis of their risk profile and genetic test results [9, 31–33].

**mutational analysis of BRCA1 and BRCA2 genes**

Mutational analysis was carried out at the Section of Genetic Oncology, Division of Surgical, Molecular and Ultrastructural Pathology, University of Pisa. Genomic DNA was extracted from peripheral blood lymphocytes according to a standard protocol. Mutational screening of the BRCA1/2 genes was carried out by direct sequencing. DNA sequencing was carried out directly on PCR purified products using the BigDye terminator v 3.1 sequencing kit (Applied Biosystems, Foster City, CA) and different primers (primer sequences available upon request). Capillary gel electrophoresis and data collection were carried out on an automated DNA sequencer (ABI PRISM 3100, Applied, Norwalk, CT). Sequence analyses were carried out with Seq-Scape Software (Applied Biosystems). Mutation nomenclature for the BRCA1 and BRCA2 genes is as used in the BIC database according to recommendations of GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) [34].

To verify that the mutations identified affected messenger RNA (mRNA) maturation, total RNA was isolated from peripheral blood lymphocytes (TriReagent; Molecular Research Center, Cincinnati OH, USA) and analyzed by RT–PCR using SuperScript spl (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocols. PCR amplification was extended to adjacent exons. Amplified fragments were visualized on a 2% agarose gel. Anomalous bands were excised from the gel and sequenced using internal primer pairs to the ones used for template PCR products. The primer sequences are available upon request.

**results**

**oncogenetic counseling**

A 59-year-old disease-free woman requested counseling because of a strong familial clustering of breast cancers. Pedigree analysis revealed six cases of breast cancer in first- and second-degree family members and cancers at other sites, namely ovary, prostate and uterus (see Figure 1). The family was classified as being affected by HBOC according to the Modena criteria [23, 24]. According to the Frank [25, 26] and BRCAPro Italia [29] models, the a priori genetic probability of a BRCA mutation in the proband was 5.2% and 33.6%, respectively.

**Figure 1.** Pedigree of the family with the two BRCA2 mutations. The arrow indicates the disease-free proband. Filled symbols indicate subjects affected by cancer. The numbers after cancer sites indicate the age at diagnosis. The age of death is reported if known. Subject (IV 11) is a BRCA2 mutation carrier. CSU, cancer site unknown; D, deceased.
Genetic testing was offered to the family. The affected proband’s sister underwent genetic testing after psychological session. She was 57 years of age with breast and ovarian cancer.

The clinical and pathological characteristics of the breast and ovarian cancers are reported in Table 1. Benign morphological alterations as ductal fibrocystic ectasia, apocrine metaplasia, classic and florid adenosis and hyperplasia coexist with malignant features.

The subject was found to be a BRCA2 mutation carrier. In posttest counseling, the mutation carrier was informed about the genetic test results and clinical implications and was advised to share her test results with her family members. Genetic testing was extended to the unaffected proband. Other family members, including the proband’s brother with prostate cancer, decided not to undergo genetic testing.

**BRCA1 and BRCA2 genetic testing**

Direct automatic sequencing of DNA extracted from lymphocytes showed two sequence alterations in the BRCA2 gene (Figure 2). One alteration (IVS13-2A>T) affected the splicing site in intron 13 and entailed an alteration in the maturation of mRNA. The other sequence alteration was 859/G>A, which corresponded to the last base of exon 7. It entails the substitution of valine (Val) to isoleucine (Ile) in position 211 of the amino acid sequence and is reported in BIC database as a splice site mutation. No mutation was found in the BRCA1 gene sequence.

The mutation at the splicing site in intron 13 showed altered mRNA maturation that determined the transcription of a sequence lacking exon 14 and an anomalous stop codon in exon 15. The variation in exon 7 led to altered mRNA maturation and to the transcription of a sequence lacking exon 7 and the subsequent anomalous stop codon in exon 9. Both mutations led to the expression of a truncated nonfunctioning BRCA2 protein in its carboxy-terminal region (Figure 2). The analysis of a second blood sample confirmed both mutations.

The DNA sample of a healthy sister was analyzed by direct sequencing for the two mutations and both mutations were found. To evaluate if the two mutations were present in the same BRCA2 allele (cis), we carried out a retrotranscription analysis using a gene-specific primer in exon 14 and PCR amplification of the complementary DNA obtained using a forward primer in exon 6 and a reverse primer in exon 10. Two bands were obtained: 0.9 and 0.8 kb. The former corresponds to a product containing exons 6–10, and the small one corresponds to a product lacking exon 7. If the mutations were present in cis, we should obtain only the 0.9-kb band corresponding to the wild-type allele; if the mutations were present in trans, we should obtain only the 0.8-kb band corresponding to the allele carrying the Val 211 Ile (mRNA lacking of exon 7). Other family members were died or refused genetic testing.

**discussion**

Since the BRCA genes were cloned in the mid-1990s, much progress has been made in translating molecular findings into clinical practice. The characterization of the BRCA genes is relevant in a prevention setting and for the clinical management of hereditary breast cancer patients. We have identified a new BRCA2 mutation and have considered its implications in clinical management within oncogenetic counseling.

Genetic testing in our sample showed two sequence alterations. One (IVS13-2A>T) affected the splicing site in intron 13 and the other (859/G>A) affected exon 7. The BIC database contains at least 30 different alterations (distinct mutations, polymorphisms and variant sequences) of exon 7 of the BRCA2 gene [2]. The IVS13-2A>T mutation found in our patient is not reported as a polymorphism or as an unknown variant in the BIC database.

Most BRCA sequence variants are clearly deleterious and known to be unequivocally involved in the pathogenesis of breast cancer. A large number of genetic alterations are still classified ‘variants of unknown significance’ [13]. Introns variants must be evaluated in order to understand their

| Table 1. Clinical and pathological characteristics of breast and ovarian cancer in the woman tested for BRCA1 and BRCA2 genes |
|---------------------------------|-----------------|---------------------------------|---------------------------------|------------------------------|
| **Breast Cancer**              | **Ovarian Cancer** |
| Age at diagnosis               | 55 years        | Age at diagnosis               | 55 years        |
| Surgery                        | Right total mastectomy plus axillary lymphadenectomy | Surgery                        | Bilateral hysterectomy |
| Histology                      | Infiltrating ductal carcinoma with apocrine features associated with ductal carcinoma in situ with solid and comedo features; high intra- and peritumoral lymphatic infiltration; vascular invasion | Histological report            | Serous and transitional carcinoma |
| ER and PgR                     | Both negative   | Stage<sup>a</sup>              | pT2 G3 N1a Mx  |
| Mib-1                          | 30%             |                                 |                  |
| p53                            | Negative        |                                 |                  |
| Erb-B2                         | 1+              |                                 |                  |
| Stage<sup>b</sup>              | pT3 G3 N0 (IIIC) |                                 |                  |

<sup>a</sup>According to the tumor–node–metastasis staging system.

<sup>b</sup>According to the International Federation of Gynecology and Obstetrics staging system.

ER, estrogen receptor; PgR, progesteron receptor.
pathogenetic or polymorphic effects on the mRNA splicing process. Classifying these variants of unknown clinical significance as neutral or disease causing is important for genetic counseling and for the implications in terms of cancer risk [35]. We show that the two BRCA2 mutations identified in our patient are involved in the splicing mechanisms and affect mRNA splicing fidelity and expression. The RT–PCR analysis confirmed that both mutations lead to a nonfunctioning BRCA2 protein. The lack of the carboxy-terminal region gives rise to a truncated BRCA2 protein unable to carry out its transcriptional function.

The likelihood of developing multiple primary malignancies depends on the gene in which the mutation occurs and also on the location of the mutation within the gene [10, 11, 36]. For example, an increased risk of breast cancer is associated with mutations outside the OCCR [10]. The two mutations detected outside the OCCR in our patient could explain the development of breast cancer besides ovarian cancer.

The breast cancer in our patient showed a complex pathological phenotype and various morphological alterations in the remaining breast tissue. This phenotype could be correlated with the specific BRCA2 genotype. Moreover, estrogen and progesterone receptors were negative, although these are typically expressed in breast cancers affecting BRCA2 mutation carriers [37, 38]. The ovarian cancer showed a serous histology, which is a typical feature of the BRCA2 genotype. It also showed transitional features, which are usually less frequent in BRCA2 tumors [37, 38].

BRCA1 and BRCA2 mutation carriers are known to be at an increased risk of cancer in different body sites. Thompson et al. [10] reported that the cumulative risk of prostate cancer by age 80 years was 33.6% higher for non-OCCR mutations than for OCCR mutations. In the family we describe, genetic testing could have been useful for the brother of the BRCA2 mutation carrier, who was affected by prostate cancer. It would be useful to know if the two BRCA2 gene mutations we identified play a disease-causing role in prostate cancer. This finding could help the oncologist to design a surveillance program and oncological follow-up for male BRCA2 mutation carriers focusing on body sites at increased risk for primary cancer.

The two BRCA2 mutations identified affect mRNA splicing fidelity and play a pathogenetic role in breast and ovarian cancer. We are still far from anticipating the tumor phenotype and planning the patient’s management on the basis of the mutation. Knowledge of a specific cancer spectrum and phenotype for each mutation could be relevant in oncological prevention and management.

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references