BRCA2 Gene Mutations in Greek Patients with Familial Breast Cancer

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Family history is a well-recognized risk factor for the development of breast cancer. The isolation of BRCA1 and BRCA2 genes, the two major predisposing genes in familial and early onset breast and ovarian cancer, has resulted to the identification of a large number of families with mutations in these two genes. Despite the large number of distinct mutations detected in both genes, several mutations have been found to recur in unrelated families of diverse geographical origin. We have analyzed 27 Greek patients with familial breast cancer with a majority of those having one first and one second degree relatives affected and 28 patients with sporadic breast cancer for BRCA2 germline mutations. The techniques used were single-strand conformation polymorphism analysis (SSCP) followed by sequencing. Furthermore, the clinical presentation and prognosis of BRCA2 associated breast cancer cases was compared to 20 adequately matched for age and date of diagnosis (within one year) sporadic breast cancer patients. We identified three novel BRCA2 mutations (3058delA, 6024delTA, and 4147delG) in the ovarian cancer cluster region (OCCR) and one already known (2024del5) germline BRCA2 gene mutation in five different breast cancer families. The 4147delG mutation was detected in two unrelated patients. BRCA2 germline mutations were correlated with early-onset breast cancer RR=4.77 (95% CI: 0.666-34.463). Although patients with BRCA2 germline mutations did not have a distinct histological phenotype they had an improved overall survival (100% vs 65%). Our findings suggest that there is a cluster of novel mutations in exons 10 and 11 in Greek patients with familial breast cancer. These mutations appear to have a milder clinical phenotype when compared to the rest of the study group. © 2001 Wiley-Liss, Inc.

KEY WORDS: BRCA2; breast cancer; germline mutations; screening; survival; Greek

INTRODUCTION

Breast cancer is the most common disease affecting the women population with a lifetime risk of 10% in the general population (Easton et al., 1993; Roswell et al., 1994). BRCA1 (MIM# 113705) and BRCA2 (MIM# 600185) genes are the two major breast cancer genes identified in individuals with hereditary predisposition. Women with mutations in either of these genes have a lifetime risk of breast cancer of 60-85% and a lifetime risk of ovarian cancer of 15-40% (Strewing et al., 1997; Ford et al., 1998) Germline mutations in BRCA2 gene (GI:
4502450) account for the genetic predisposition and increased risk for breast cancer in almost 35% of the families with inherited predisposition to this cancer (Gauthier-Villars et al., 1999). Thus far more than 500 germline mutations have been identified within the BRCA2 gene that, by and large, are unique to each high-risk family (Information provided by the Breast Cancer Information Core, BIC www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). Most of the mutations that are associated with breast cancer are truncation mutations leading to premature termination of the protein (Strewing et al., 1997; Ford et al., 1998). A number of disease-associated missense mutations have also been described, most of these altering an amino acid involved in the BRC structures in exon 11 of the BRCA2 gene (Breast Cancer Information Core, BIC). For all other missense alterations a clear disease association is difficult to establish. Population-based studies have defined high- and low-risk subsets for developing breast cancer based on ethnic origin. Several large studies were performed to analyze breast cancer families from different populations for BRCA2 mutations. These have shown that specific mutations can be found in specific populations and a variable number of novel mutations are found in different populations (Gayther et al., 1997).

The breast cancer incidence in Greece is similar to that in other western countries. There is no evidence that familial breast cancer is more frequent in Greece than elsewhere. So far the type and prevalence of BRCA2 mutations have not been reported in Greek families. In the current study, we describe the results of germline mutational analysis of the BRCA2 gene, in twenty-seven Greek breast cancer patients with at least one first-degree relative affected by the disease (moderate risk group for BRCA2 germline mutations) and 28 Greek patients with sporadic breast cancer (low risk group for BRCA2 germline mutations). A survival analysis with adequately matched for age and date of diagnosis (within one year) controls was also performed in order to determine the clinical phenotype of patients with BRCA2 germline mutations.

MATERIALS AND METHODS

Patients

Fifty-five consecutive patients were recruited from the breast cancer clinics of two University Hospitals in Athens, Greece (Hippokrateion Hospital and Laikon Hospital). All the patients included in our registry were operated in the above surgical clinics from 1981 to 1998. All participants gave informed consent to the use of their questionnaire data and blood samples for studying the genetic basis of their breast cancer. Out of the 55 breast cancer cases included in the mutation analysis, 27 were breast cancer patients with one first-degree relative affected by the disease (moderate risk group for BRCA2 germline mutations) and 28 Greek patients with sporadic breast cancer (low risk group for BRCA2 germline mutations). A survival analysis with adequately matched for age and date of diagnosis (within one year) controls was also performed in order to determine the clinical phenotype of patients with BRCA2 germline mutations. The rest 28 were sporadic breast cancer patients. Furthermore, for every patient carrying a definite BRCA2 germline mutation, four patients with sporadic breast cancer (absence of family history) were matched for age and date of diagnosis (within one year). These patients were also selected from the sporadic breast cancer registries of the above institutions. We gathered data on age at onset, surgical procedure, tumor-node-metastasis status, grade and the presence or not of contralateral breast cancer from hospital records and pathology reports. Histological grading was done according to the Nottingham scheme (Elston and Ellis, 1991) (Table 1). In the three groups of patients studied (27 moderate risk breast cancer patients with at least one affected first degree relative, 28 low risk sporadic breast cancer cases and 20 patients with sporadic breast cancer adequately matched for age and date of diagnosis), breast cancer was diagnosed between 1981 and 1996. Follow up data was collected from medical records provided by the health care givers of the patients involved and our national cause of death registry. The mean age at diagnosis was 55.67 for the low risk sporadic breast cancer cases (median 56.5, range 30-86) and 57.96 for the 27 moderate risk patients who had a family history for breast cancer (median 60.5, range 31-80).

Modified radical mastectomy was applied to forty-three of the fifty-five breast cancer patients included in our mutation analysis (78.2%) (Twenty-two of the 27 moderate risk breast cancer patients with a positive family history (81.5%), and 21 of the 28 low risk sporadic breast cancer cases (75%)). A breast conserving therapy (lumpectomy plus irradiation) was administered in twelve of the 55 patients (21.8%) (Seven low risk sporadic breast cancer cases and five moderate risk breast cancer patients with a positive family history). Twenty-three of the 27 moderate risk breast cancer patients (85.2%), and 25 of the 28 low risk sporadic cases (89.3%) received adjuvant therapy (chemotherapy and/or radiotherapy). Seven patients (12.7%) did not receive any type of adjuvant treatment (Three low risk sporadic breast cancer patients and four moderate risk breast cancer patients).
Table 1: Tumor stage and grade distribution in BRCA2 germline mutation carriers and non-carriers.

<table>
<thead>
<tr>
<th>Feature</th>
<th>BRCA2 associated breast cancer patients (n=5)</th>
<th>Non BRCA2 associated breast cancer patients (n=70)</th>
<th>Total (n=75)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>19</td>
<td>20</td>
</tr>
</tbody>
</table>

Mutation analysis

Mutation analysis was obtained by PCR, SSCP and sequencing. Exons and adjacent intron sequences of the BRCA2 gene were analyzed by PCR. The primers used have been previously described (Miki et al., 1996).

PCR-SSCP analysis

Each PCR reaction took place in 25 µl containing 15 ng genomic DNA, 1X PCR buffer, 2-3mM MgCl2, 25mM dNTPs. The PCR conditions for exons 2-9, 12, 13-21, 24 and 25 consisted from: 1 cycle at 94°C for 5min, 35 cycles 94°C for 30sec, 58°C for 30sec, 72°C for 30sec and 1 cycle at 72°C for 7 min. The first cycle (annealing 94°C for 5min) as well as the last cycle (elongation 72°C for 7min) remained constant in each case. For exons 22, 23 and 26 the conditions were similar to the previous ones apart from the fact that the extension was carried out for 40 sec. In the case of exon 10 the PCR reaction was carried out for 35 cycles at 94°C for 40 sec, 55°C for 30sec, and 72°C for 30 sec. Exon 11 due to its large size was divided into 4 pieces of about 1000bp each (8 PCR primers were used). The conditions used for all the fragments were 35 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 2 min. Similar were the conditions used for exon 27. Finally the conditions for exon 18 were 35 cycles at 94°C for 40sec, 50°C for 40 sec and 72°C for 2 min. (The PCR thermocyclers used were the Perkin Elmer 2400 and the Techne Progene).

In each case apart from exons 10, 11A, B, C, D, 14, 18 and 27 the PCR products were examined for possible mutations by electrophoresis in 6% non denaturing acrylamide gel at 200C. Due to their large size, the products of exons 10, 11, 14, 18 and 27 had to be digested with various combinations of restriction enzymes as previously described by Miki et al. (1996). These enzymic digest products were electrophoresed in 10% polyacrylamide gels. In all the cases the gels were visualized by silver staining (Promega).

Sequencing of PCR products

The PCR products of the samples with SSCP differences were sequenced bidirectionally (5' to 3' and 3' to 5'), directly using an ABI PRISM di-Deoxy Terminator Cycle Sequencing kit and an ABI 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems) according to manufacturers instructions.

Statistical analysis

The differences in the prevalence of BRCA2 germline mutations between familial and sporadic breast cancer cases were examined by the McNemar’s test. The same test was applied in order to estimate the correlation between BRCA2 mutations and the clinicopathological characteristics of the patients studied. Kaplan Meier survival probabilities and differences were explored by the log rank test. The simultaneous effects of different prognostic factors on disease-free and overall survival were analyzed by Cox’s proportional hazards method. All tests were two-sided and differences were considered non-significant when p values were greater than 0.05.

RESULTS

Description of families

The 55 patients included in the mutation analysis came from two series of patients: The first one with 27 breast cancer patients, the majority of those with one first degree relative affected by the disease; and the second one with
28 sporadic breast cancer cases with no previous family history of breast cancer. The first series of patients included two patients with two first-degree affected relatives and one patient with two first-degree and two second-degree affected relatives. The first group also included one case of breast/ovarian cancer. In this family there was one affected member with concurrent breast and ovarian cancer and one first-degree relative with ovarian cancer alone.

**Mutations and phenotype/genotype correlation**

Each patient was examined more than twice for the presence of mutations and/or common polymorphisms in all the BRCA2 gene exons by SSCP analysis. Four different mutations were detected in the group of patients with moderate risk of developing breast cancer. Mutations were not detected in the group of sporadic breast cancer patients. Furthermore we did not detect any common polymorphisms in any of the patients participated in this study.

Patients presenting one BRCA2 germline mutation from those identified in this study, had a RR=9.025 (95% CI:1.459 – 51.815) to develop familial breast cancer. None of the sporadic breast cancer cases examined had any pathogenic BRCA2 mutation (p=0.023, McNemars test). In all the cases the mutation was present as a heterozygote with the wild-type allele. Out of the four mutations determined, only one was in exon ten whereas the other three were detected in exon eleven. All the mutations identified, were small deletions of either one or more nucleotides (Table 2).

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Phenotype Affected relatives*</th>
<th>Mutation</th>
<th>Age of onset</th>
<th>exon</th>
<th>a.a. change</th>
<th>effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>78</td>
<td>Breast cancer 1 first degree</td>
<td>2024del5</td>
<td>67</td>
<td>10</td>
<td>Frameshift</td>
<td>Truncation signal at codon 599</td>
</tr>
<tr>
<td>43</td>
<td>Bilateral breast cancer 2 first, 1 second degree</td>
<td>3058delA**</td>
<td>62</td>
<td>11</td>
<td>Frameshift</td>
<td>Truncation signal at codon 957</td>
</tr>
<tr>
<td>44</td>
<td>Breast cancer 1 first, 1 second degree</td>
<td>6024delTA**</td>
<td>32***</td>
<td>11</td>
<td>Frameshift</td>
<td>Truncation signal at codon 1943</td>
</tr>
<tr>
<td>46</td>
<td>Breast cancer 3 first degree</td>
<td>4147delG**</td>
<td>31***</td>
<td>11</td>
<td>Frameshift</td>
<td>Truncation signal at codon 1334</td>
</tr>
<tr>
<td>41</td>
<td>Breast and ovarian cancer 1 first degree</td>
<td>4147delG**</td>
<td>59</td>
<td>11</td>
<td>Frameshift</td>
<td>Truncation signal at codon 1334</td>
</tr>
</tbody>
</table>

* with breast cancer; **novel mutation; *** early-onset breast cancer cases

The mutation detected in exon ten was the 2024del5, which resulted in the ceasing of the translation of the BRCA2 protein at amino acid 599, leading to the production of truncated BRCA2 protein. The particular mutation is not very common since it has only been previously reported twice (BIC). In exon eleven, we detected three novel, deletion mutations, each one of them results in the shifting of the reading frame of the BRCA2 gene. These mutations were the 3058delA, the 4147delG and the 6024delTA (Table 1b). The mutation 3058delA resulted in the truncation of the BRCA2 protein at codon 599, the mutation 4147delG at the codon 1334 and the mutation 6024delTA at the codon 1943. The mutation 4147delG was observed in two unrelated breast cancer patients (Table 2). The BRCA2 germline mutations detected were confirmed by sequencing a second PCR product.

The mean age of disease presentation did not differ substantially among patients of moderate risk and low risk sporadic breast cancer cases (low risk) (57.96 and 55.67 years, respectively). Although BRCA2 germline mutation carriers presented earlier onset of breast cancer compared to those who did not carry a BRCA2 germline mutation (age range 31-67 vs. 30-86 years, respectively), the difference did not reach statistical significance (p=0.408). Early-onset breast cancer (age of diagnosis < 42 years) (Neuhausen et al., 1996) was presented in three patients with moderate risk to develop breast cancer. Two of them had BRCA2 germline mutations. The 3058delA novel BRCA2 gene mutation was identified in a patient with bilateral breast cancer and positive family history (Table 2). Although the three novel mutations were detected in the OCCR region, only the mutation 4147delG, seems to be associated with ovarian cancer in one out of the two unrelated patients presenting it.

Overall survival at 4 years was 100% for the BRCA2 mutation carriers and 65% for the age-matched sporadic breast cancer patients (Fig. 1). The twenty-two moderate risk patients who did not carry a BRCA2 germline mutation had an 85% survival rate, while the twenty-eight low-risk sporadic cases had a 92% 4-year survival rate. Disease-free survival at 4 years was 33% for the BRCA2 mutation carriers, 45% for the age-matched sporadic...
breast cancer patients, 57% for the moderate risk patients who did not carry a BRCA2 germline mutation and 44% for the sporadic breast cancer patients with no family history for breast/ovarian cancer. The adjusted for tumor stage, type of surgery and age of onset hazard ratios for recurrence and mortality among the BRCA2- mutation-associated cases were 0.54 (95% CI, 0.04-7.3) and 1.05 X 10^-7 respectively, compared to sporadic patients (Table 3). The operation criteria as well as the criteria for the administration of any adjuvant treatment where the same for the five BRCA2 germline mutation carriers and the twenty age matched controls. From the five BRCA2 mutation carriers four (80%) received a modified radical mastectomy and one (20%) a lumpectomy plus irradiation. Two patients from the 20 age-matched sporadic breast cancer control group (10%) received a breast conserving therapy while the other 18 patients (90%) were submitted to a modified radical mastectomy. Furthermore four BRCA2 germline mutation carriers (80%) received adjuvant therapy compared to 16 sporadic age-matched controls (80%).

Figure 1: Survival curves of the individuals with BRCA2 associated breast cancer and of the sporadic breast cancer patients of the control group (20 in total sporadic breast cancer patients age and stage matched were compared with the five patients with the BRCA2 associated breast cancers, four sporadic breast cancer cases per BRCA2 associated breast cancer case).
Table 3: Crude and adjusted hazard ratios for patients with BRCA2 mutations versus age-matched sporadic breast cancer cases.

<table>
<thead>
<tr>
<th></th>
<th>Death from disease</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>0.0386 (4.73 x 10^{-6} - 314.63)</td>
<td>0.4788</td>
</tr>
<tr>
<td>Adjusted for tumor stage, age and type of treatment</td>
<td>1.051 x 10^7 (CI not calculated)</td>
<td>0.9924</td>
</tr>
</tbody>
</table>

DISCUSSION

Twenty-seven Greek familial breast cancer patients (moderate risk) and twenty-eight sporadic breast cancer cases (low risk) were analysed for mutations in the BRCA2 gene. We found four mutations in five moderate risk familial breast cancer patients.

A common ancestor is likely to explain recurrent mutations in a definite geographical region. This has clearly been shown in a number of defined ethnic and geographical populations: 24% of such families in Israel (Strewing et al., 1999), 21% in Italy (Santarosa et al., 1999), and 17% in Iceland (Thorlacios et al., 1998). Of our families, 18.51% had mutations suggesting that in Greece almost a similar proportion of breast cancer families to that internationally reported have identifiable BRCA2 mutations. From the five patients of our study with BRCA2 mutations two developed breast cancer before the age of 32. Their mutations were the 6024delTA and the 4147delG.

Two sisters, one with breast cancer and a history of endometrial cancer and the other with breast and ovarian cancer had the 2024del5 mutation. This implies that the particular mutation may be related not only to breast cancer but to ovarian cancer as well. Additionally, there was a patient with the novel mutation 3058delA that developed bilateral breast cancer. This patient had two first-degree and one second-degree relative with bilateral breast cancer and one first-degree relative with prostate cancer. The 3058delA mutation may be associated with bilateral breast cancer in the Greek population. The 4147delG mutation occurred in 2 unrelated breast cancer cases (3.6%), suggesting an increased frequency for this mutation in the Greek population.

Three out of the four mutations detected were novel and the fourth (the 2024del5) is a very rare one. Furthermore there were all small deletions detected in exons 10 and 11. This indicates that the Greek population constitutes a unique pool of BRCA2 mutations differing from the populations already studied. This finding may reflect a geographical clustering of mutations in specific regions of the BRCA2 gene.

We did not detect any BRCA2 mutations in the randomly selected sporadic breast cancer group of patients included in our study. No BRCA2 mutations were identified in any of the five early onset sporadic breast cancer cases. This has not been the case in the study of Neuhausen et al. (1996) that reported an 8% incidence of a recurrent BRCA2 6174delT mutation in patients with early-onset sporadic breast cancer. Although the number of sporadic breast cancer patients in our study is rather small, the absence of BRCA2 germline mutations throughout its entire coding region underlines the decreased effect of these particular mutations in the development of sporadic breast cancer in Greece.

BRCA1 associated tumors have consistently presented differences in histological phenotype, compared with nonhereditary tumors. Commensurate phenotypes though, have not been documented for BRCA2-associated tumors. BRCA2-associated breast carcinomas were not correlated with a different histological phenotype compared to the control group in our study.

The survival analysis revealed that survival was higher among breast cancer patients carrying a BRCA2 mutation compared to sporadic breast cancer cases matched for age and date of diagnosis. Verhoog et al. (1999) have previously reported that BRCA2 mutation carriers presented a similar overall survival with non-mutated age-matched sporadic breast cancer controls (74% vs. 75%, respectively). Similar results have also been reported by Gaffney et al. (1998). On the contrary, Lee et al. (1999) and Loman et al. (2000) pointed out that BRCA2 mutation carriers bear an unfavorable clinical outcome compared to their control groups. A borderline improvement in the overall survival of BRCA2 mutation carriers, has been reported by van den Berg et al. (1996), by Phillips et al. (1999), and by Gaffney et al. (1998).
So far at least 500 mutations with a deleterious effect have been identified in the BRCA2 gene and a clear phenotype-genotype correlation has not yet been deduced. This indicates the need for a rational strategy that will take into account not only the family history, but will also define the mutation most frequently seen in a population within a given geographical area, the clinicopathological characteristics and the clinical course of the disease. Regional testing for common mutations would provide us relatively inexpensive first line of analysis that should detect the majority of mutations present and could be carried out with ease in any regional molecular biology lab. We reported a unique pool of novel mutations in the Greek population. All the mutations were small deletions occurring in exons 10 and 11. Patients bearing these mutations had an excellent five-year survival.

It is necessary to establish the simultaneous effects of environmental factors and of other tumour-associated genes in modifying the impact of inherited cancer predisposition genes in breast cancer patients. Studying the available genotype-phenotype correlations will allow us to improve not only the clinical management of these patients, but their quality of life as well.

ACKNOWLEDGMENTS

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REFERENCES


