Analysis of *BRCA1/BRCA2* genes' contribution to breast cancer susceptibility in high risk Jewish Ashkenazi women

**Tal Distelman-Menachem · Tal Shapira ·
Yael Laitman · Bella Kaufman · Frida Barak ·
Sean Tavtigian · Eitan Friedman**

Abstract *Background* Three mutations in *BRCA1* (185delAG 5382InsC) and *BRCA2* (6174delT) can be detected in a substantial proportion of Jewish Ashkenazi breast/ovarian cancer families. Family-specific pathogenic mutations in both genes can be detected in up to 5% of high risk Ashkenazim. The contribution of major gene rearrangements and seemingly pathogenic missense mutations to inherited breast cancer predisposition has never been systematically evaluated in Ashkenazim. *Material and methods* High risk, Jewish Ashkenazi women, non-carriers of the predominant Jewish *BRCA1/BRCA2* mutations, were genotyped for major gene rearrangements in *BRCA1/ BRCA2* using Multiplex ligation-dependent probe amplification (MLPA), and for the occurrence rate of 6 seemingly pathogenic missense mutations in *BRCA1* (R866C, R331S, R841W, Y179C, C61G, M1008I) using a modified restriction enzyme assay. *Results* Overall, 105 Jewish Ashkenazi high risk women, participated in the study: 104 with breast cancer [age at diagnosis (mean ± SD) 51.05 ± 11.13 years], one was affected with ovarian cancer (61 years). Two were found to carry the M1008I mutation in *BRCA1* and none harbored any of the other missense mutations. MLPA reveled four changes (amplifications of exons 5, 17, 19 and 21) in *BRCA1* in five patients, and six patients exhibited 4 MLPA-detectable abnormalities in *BRCA2* (amplifications in exons 1b, 2, and deletions in exons 11a and 25). None of these abnormalities could be confirmed using quantitative PCR (qPCR) analysis. *Conclusions* Major gene rearrangements involving *BRCA1 BRCA2* contribute little to the burden of inherited predisposition of breast cancer in Ashkenazi Jews.

Keywords Genetic susceptibility · BRCA1 BRCA2 · Major gene rearrangements · Sequence variants of unknown significance

Tal Distelman Menachem and Tal Shapira have equally contributed to this manuscript.

T. Distelman-Menachem · T. Shapira · Y. Laitman ·
E. Friedman (✉)
Susanne Levy Gertner Oncogenetics Unit, The Danek Gertner Institute of Human Genetics, Chaim Sheba Medical Center, Tel-Hashomer 52621, Israel
e-mail: eitan211@netvision.net.il

B. Kaufman
Susanne Levy Gertner Oncogenetics Unit, The Oncology Institute, Chaim Sheba Medical Center, Tel-Hashomer, Israel

B. Kaufman · E. Friedman
The Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel

F. Barak
The Oncology Institute, Barzilai Medical Center, Ashkelon, Israel

S. Tavtigian
Genetic Susceptibility Group, International Agency for Research on Cancer, Cours Albert Thomas, 69372 Lyon Cedex 08, France

Introduction

In approximately 5–10% of breast cancer cases, familial clustering and other features indicative of an inherited predisposition to cancer are noted. A substantial proportion of breast-ovarian cancer families and to a lesser extent, site-specific breast cancer are accounted for by germline mutations in the *BRCA1* (MIM#113705*)* and *BRCA2* (MIM#600185) genes. While in the majority of populations, the mutational spectrum of *BRCA1* and *BRCA2* in high risk families varies from family to family, among

Jewish Ashkenazi families there are only a handful of mutations. The three predominant mutations found in this population (185delAG, 5382insC and 6174delT in *BRCA1* and *BRCA2*, respectively) can be detected in up to 12% of incident breast cancer, in 35% of incident ovarian cancer, and in 2.5% of the general Ashkenazi population [1]. These three mutations account for the majority of high risk families of Ashkenazi origin, and the prevalence of non-founder mutations in Jewish Ashkenazi women undergoing clinical testing for a family history is only ∼2% [2].

The majority of pathogenic mutations in *BRCA1* and *BRCA2* are point mutations or small deletions and insertions (BIC database). In most populations tested, the observed frequencies of *BRCA1* variations in high-risk breast and/or ovarian cancer families are lower than predicted by linkage analysis. This finding suggests that methods generally used for mutation scanning fail to detect certain types of germline defects, such as large genomic rearrangements. Most of mutation screening methods are PCR-based, and hence there is an inherent inability to detect large genomic rearrangements such as partial or complete exon loss or amplification [3] Several approaches have been reported for detecting *BRCA* germline gene rearrangements: Southern blot [4–6] long-range PCR [7], color bar coding of the *BRCA* genes on combed DNA [8, 9], semiquantitative-multiplex PCR [2], and real-time PCR [5, 6]. Multiplex ligation-dependent probe amplification (MLPA) is widely used as a highly sensitive method for detecting the relative copy number of all *BRCA1 BRCA2* exons in a high-throughput format [10].

Major gene rearrangements have been identified in *BRCA1* and *BRCA2* in several populations. Specifically, large genomic deletions in *BRCA1* were noted in the French, Italian, and Dutch populations [6, 11, 12]. More than 60 different *BRCA1* germline rearrangements with mapped breakpoints have been reported to date [13]. These are scattered throughout the whole gene, and most of them are deletions, but duplication, triplication or combined deletion/insertion events [14, 15] have also been described. The proportion of genomic rearrangements in the *BRCA1* mutation spectrum varies from 8 to 15%, depending on population and methodology, with the higher rates associated with founder mutations [1, 6, 7, 16–19]. Less is known about the occurrence rate of large genomic rearrangements in *BRCA2* in hereditary breast/ovarian cancer families. To date, few studies have been published revealing less than 20 [10, 20–23] different genomic rearrangements in *BRCA2* in hereditary breast cancer patients from diverse European populations. Testing has primarily been carried out by Southern blot analysis. Only the most recent studies have applied MLPA as a comprehensive screening method [3, 4, 7, 12, 21–38] and the value of *BRCA2* screening for such alterations is still undetermined.

Only one published study focused on the contribution of major gene rearrangements to inherited predisposition in Jewish women. Wang et al. [39] looked for major gene rearrangements by Southern blot hybridization among 47 Jewish breast cancer patients with hereditary features, 30 of whom were of Ashkenazi descent. A deletion/insertion in *BRCA2* was detected in a single family of Jewish-Turkish (non-Ashkenazi) descent. The deletion included exons 12 and 13, a track of approximately 60 adenine nucleotide residues was inserted at the breakpoint junction [39]. Notably, there were no major gene rearrangements reported among Ashkenazi patients in that study.

While the pathogenicity of inactivating, truncating *BRCA1* and *BRCA2* mutations is well established, the contribution of other *BRCA1* and *BRCA2* sequence variants to cancer risk remains largely undefined. These "variant of unknown significance" (VUSs) are mainly missense mutations. The open-access, on-line Breast Cancer Information core (BIC database) contains over 1500 distinct sequence variants that are currently reported as having unknown clinical significance. Several methodologies have been applied to assess the presumed pathogenicity of these sequence variants, but there is no consensus regarding the clinical application of any of these techniques as an aid in assigning a pathogenic role for these VUSs. A notable exception is the C61G *BRCA1* mutation, which is one of the most prevalent, clearly pathogenic, germline mutations in Polish high risk families. From the mechanistic point of view, the C61G mutation disrupts homodimer formation in the NH2-terminal BRCA1 RING finger [40], and hence is clearly pathogenic.

In the present study, 105 Ashkenazi breast/ovarian cancer patients with inherited predisposition to cancer, who tested negative for the three predominant Ashkenazi mutations in *BRCA1* and *BRCA2* were genotyped for putative pathogenic missense *BRCA1* mutations and for major gene rearrangements in both *BRCA1* and *BRCA2* using MLPA.

Patients, materials and methods

Patients and families

Patients were ascertained from one of two sources: the high risk clinic at the Oncogenetics unit, "Sheba" medical center, and an ongoing project that genetically tests and counsels consecutive Jewish Ashkenazi women diagnosed with breast cancer conducted jointly by the "Sheba" and "Shaare Tzedek" medical centers. All participants had breast or ovarian cancer, and in addition, one or more of the eligibility criteria: (1) under 40 years of age at time of diagnosis of breast cancer ($n = 16$) or (2) over 40 years of

age at time of diagnosis of breast cancer and at least one of the following: (a) bilateral breast cancer ($n = 13$) or ovarian cancer ($n = 1$); (b) at least one-first degree relative with breast cancer ($n = 20$); (c) one or more first or second degree relatives with bilateral breast cancer ($n = 21$) or ovarian cancer ($n = 7$); (d) at least two relatives of the paternal side with breast cancer ($n = 11$)and e) at least one relative diagnosed for breast cancer under the age of 40 years ($n = 8$). (3) tested negative for the three predominant "Ashkenazi" mutations (*BRCA1**185delAG, 5382insC, *BRCA2**6174delT). Inclusion criteria were not met by 8 patients who did not fit to these above mentioned criteria but had a personal and family history highly suspicious for an inherited predisposition and were also analyzed herein. The study was approved by the local and the Ministry of Health IRB, and each participant signed a written informed consent.

DNA extraction

Genomic DNA was isolated from peripheral blood leucocytes by the PUREGene kit (Gentra Inc Minenapolis MN) using the manufacturer's recommended protocol.

Selecting and assigning pathogenicity of missense mutations in BRCA1

We searched the BIC database for missense mutations in *BRCA1* gene reported in the Ashkenazi population or individuals of East European origin. Missense mutations within conserved domains (R866C; R841W), those with a proven pathogenic effect (C61G), those that scored moderately high on the align-GVGD algorithm [41] (Y179C) or are extremely rare (R331S) were selected.

Genotyping for the missense mutations

PCR primers were designed to flank the mutated area and a restriction enzyme was used to differentiate the wild type from the mutant allele. PCR was preformed in a 25 µl reaction, containing 50–100 ng genomic DNA, PCR buffer (Fisher Biotec, Australia), 2.5 mM MgCl2, 200nM dNTPs,

10 pmol of each primer and 0.2 U *FB1* DNA Polymerase (Fisher Biotec,). Amplification was carried out as follows: an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, annealing step which was different for each fragment, 72°C for 30 s, and a final extension step at 72°C for 10 min. Mutations and primers sequences are listed in Table 1. Amplification conditions for each fragment are available from the authors upon request.

Multiplex ligation-dependent probe amplification (MLPA)

MLPA analysis was performed by the Salsa P002 *BRCA1* and Salsa P045 *BRCA2* MLPA probe mix assay as described by the manufacturer (MRC-Holland Amsterdam, the Netherlands) [3]. Peak heights from each patient were then exported to an excel spreadsheet, which was designed by Dr. Andrew Wallace from the National Genetics Reference Laboratories, Manchester, to assess the ratios of each test peak relative to all other peaks for that individual. Each test and control sample's data is normalized (by summing the total control peak height and dividing each ligation product's peak height by this figure.), the Dosage quotients are calculated and the three hypotheses (normal, duplicated, deleted) are tested comparing to 5 normal controls. For normal sequence a dosage quotient of 1.0 (0.85–1.15) is expected; if a deletion or duplication is present, the dosage quotient should be 0.35–0.65 and 1.35–1.65, respectively. A dosage quotient of 0.65–0.85 and 1.35–1.65 is equivocal. In addition, the sample quality is assessed by measuring the standard deviation of all the test ligation products measured against each other. Standard deviation of less than 0.1 shows no overlap between normal, duplicated and deleted ranges [3, 4].

Quantitative PCR

Dosage changes found by MLPA analysis were re-evaluated (for ascertainment and validation) by Multiplex quantitative PCR (qPCR). qPCR was performed in the ABI7700 instrument. A set of FAM-labeled primers and

Table 1 Mutation designation, primer sequences and restriction

Mutation	Forward primer	Reverse primer	Restriction enzyme
R866C	AGAATACATTCAAGGTTTCACA	TAGACAAAACCTAGAGCCTCCT	TaaI
R331S	CCAGAAAAGTATCAGGGTAGTTC	CTTTTTTTTCTGTGCTGGGAGACC	Cfr10I
R841 W	ATTGGGACATGAAGTTAACCAGAG	CTGTCTGTACAGGCTTGATATTAG	Sch1
Y179C	TACAACCTCAAAAGACGTCTGTC	TGTGAGCCACCATGCCTGGCC	XmiI
C61G	TGGCTCTTAAGGGCAGTTGTGAGA	CTGTGGTTGCTTCCAACCTAGCAT	AvaII
M1008I	TCAGAGGCAACGAAACTGGACTCA	TGGAGCCCACTTCATTAGTACTGG	Tsp45I

probes were designed for each exon or gene fragment that showed an abnormality on MLPA analysis. The primers were designed to flank the MLPA probe, and the design of the Taqman probe was similar to the MLPA probe. A mix containing VIC-labeled primers and probe for RNaseP (Roche diagnostics, Manheim, Germany), a single copy gene, was used as an internal standard. Amplification/deletion reaction were performed in a volume of 20 µl containing 10 µl of TaqMan universal PCR mastermix (Roche diagnostics, Manheim, Germany), 1 µl of each BRCA-specific primers, 1 µl of each BRCA-specific labeled hybridization probe, 0.5 µl of the RNaseP primer-probe mix and 1 µl of genomic DNA. Thermocycling was as followed: 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 10 s.

Data analysis was carried out using ABI Prism 7700 Sequence Detection Software Since the standard curve for sequential concentrations of the two different probes was similar, a difference between the Ct of the examined BRCA exon and that of the RNaseP indicates a deletion/duplication.

Results

Patient characteristics

Overall, 105 women participated in the study: 103 were diagnosed with breast cancer [mean age at diagnosis (±SD) was 51.05 ± 11.13 years (range 28–79 years)], one with ovarian cancer (61 years) and one patient had both ovarian and breast cancers (ovarian cancer at 43 years and breast cancer at 48 years).

All participants were of Jewish Ashkenazi ancestry, and none was a carrier of any of the three predominant mutations in BRCA1 (185delAG, 5382InsC) or BRCA2 (6174DelT).

BRCA1 gene rearrangements

MLPA analysis

MLPA reaction for BRCA1 had statistically significant results ($P < 0.1$) in only 48 samples of the total 105 genotyped. In 16 of these samples, no dosage changes were found. Among the remaining 32 samples with statistically significant data, several dosage changes were found, but none in sequential exons. In only two samples, an amplification of exons 17 and 19 had significant odds ratio (1:2232, 1:421 for the first sample and 1:85, 1:74 for the second sample).The first patient displaying the abnormality is a breast cancer patient diagnosed at age 52, her sister was diagnosed with breast cancer at age 57 years, and their

paternal cousin was diagnosed with breast cancer at age 50 years. There are no other women in the family. The other patient was diagnosed with breast cancer at age 28 years and her sister was diagnosed with breast cancer at age 43 years. There are no cancer cases at their maternal family and on the paternal side there are no women relatives.

Among the remaining 57 samples, 21 samples did reach partial statistical significance ($P < 0.1$). In this set of genotyped samples, amplifications were detected in exons 5, 17, 19 and 21 in three patients.

Quantitative PCR

Due to the low incidence of statistically significant results, qPCR for confirmation of the four amplified regions detected by MLPA, was performed for the whole cohort. None of the samples showed any of the amplifications in exons 5, 17, 19 and 21, by qPCR. Specifically, the five samples that showed the MLPA abnormality did not reveal any abnormality using the qPCR probe.

BRCA2 major gene rearrangements

MLPA analysis

The MLPA reaction for BRCA2 had statistically significance ($P < 0.1$) in only 31 of the 105 samples. In 11 samples no dosage changes were detected. Among the remaining 20 samples, dosage changes were found in exons 1b, 3 (amplification) and 11a, 25 (deletion), in six patients. These dosage changes had significant odds ratio (Table 2). The MLPA kit for BRCA2 includes a probe for the Chek2*1100delC mutation. Three samples were positive for this mutation, one in a statistically significant score.

qPCR

Due to the low incidence of statistically significant results, qPCR for the search of the four dosage changes found by MLPA in the BRCA2 gene was performed for the whole cohort. None of the amplifications were detected and

Table 2 Summary of the MLPA analyses for BRCA2

Family #	Ex1b	Ex3	Ex11a	Ex25
3572	Amp 1:58361	Amp 1:23		
3965	Amp 1:102084	Amp 1:40		
4332	Amp 1:12926			
5698sz			Del 1:222	
5821sz			Del 1:1338	
5265sz				Del 1:74

validated by qPCR, including the six samples that showed the MLPA associated abnormality.

Sequencing confirmation of the 1100delT CHEK2 mutation

The three samples for which the MLPA reaction indicated the presence of the Chek2*1100delC mutation, were sequenced to ensure the existence of that mutation. Only one of the three samples was positive for the mutation and the other two samples, including the one showing the most statistically significant finding on MLPA, did not harbor that mutation.

Genotyping for missense mutations in BRCA1

All 105 samples were genotyped for 6 missense mutations in *BRCA1*: R866C, R331S, R841W, Y179C, C61G, M1008I. Two women were carriers of the I1008M mutation. The first patient displaying the abnormality is a breast cancer patient diagnosed at age 62 years, her sister was diagnosed with breast cancer at age 56 years, their mother was diagnosed with breast cancer at age 44 years, and their maternal aunt was diagnosed with breast cancer around the age of 50 years. The other patient was diagnosed with breast cancer at age 45 years and her mother was diagnosed with breast cancer and colorectal cancer at age 62 years. There are no other relatives on the maternal side. In addition to the M1008I mutation, sequencing of these two samples revealed two neutral polymorphisms: E1038G which is not clinically important, and S1040N which is of unknown significance. None of the other missense mutations was detected in any of the samples.

Discussion

In this study, MLPA analysis of Jewish women with inherited predisposition to breast cancer was suggestive of the existence of a few genomic rearrangements involving *BRCA1* and *BRCA2*. Yet, these results could not be confirmed or validated independently by another technique-quantitative PCR. Several studies reported the MLPA as a high sensitivity technique by using a positive control that was originally detected by Southern blotting, the gold standard method for dosage changes [4–6]. Other studies focusing on the presence of major gene rearrangements in *BRCA1* and *BRCA2* either used a MLPA kit with a different set of probes and primers [34] or qPCR for the validation of their findings.

Based on these studies and the present study, it seems that an approach that uses MLPA as a screening technique and as a guide for further analysis and validation of the findings by an independent technique should be recommended, at least for the set of probes applied for *BRCA1* and *BRCA2*.

The lack of finding of consistent major gene rearrangements involving *BRCA1* and/or *BRCA2* in high risk Jewish Ashkenazi families in the present study is in line with the previous study that focused on Jewish families. In that study, only one major gene rearrangement in *BRCA2* in a non-Ashkenazi high risk family [39], and notably no gene rearrangements involving Ashkenazim ($n = 30$) were reported. Similarly, two studies that have applied MLPA analysis have failed to show any *BRCA1 BRCA2* gene rearrangement in 135 French-Canadian [4] and no *BRCA2* MLPA-detectable abnormalities in 36 Finnish [32] high risk families.

In the present study one of the genotyped missense mutations in BRCA1 (M1008I) was detected in two of the study participants. The M1008I missense mutation is a conservative mutation due to a $G \rightarrow A$ transition at nucleotide 3143 in exon 11 changing codon 1008 (ATG) encoding the non-polar Met into ATA encoding Ile, another non-polar residue. This Met is changed for a Ser in the mouse BRCA1 protein [42]. The M1008I mutation was reported in ethnically diverse populations: Ashkenazim, Central/Eastern Europe, Western European population, Latin American/Caribbean, and Puerto Rico populations, (BIC database). Given these considerations and the fact that this variant was previously classified as neutral by two independent groups of investigators [43, 44] and that it could be found in trans with a clearly deleterious BRCA1 mutation [44], it seems likely that this variant does not have a deleterious effect on BRCA1 protein function and is merely a rare polymorphism.

The selection of the other five specific mutations was based on several criteria, that made it plausible that these may indeed be pathogenic. Several explanations may account for this lack of detected missense mutations: inadequate assignment of the pathogenicity of these mutations, rarity of these mutations in high risk Jewish Ashkenazi families, even if they are truly pathogenic, small sample size, inclusion of non-inherited cases among genotyped families. Indeed, at least one missense mutation tested (C61G) is a known pathogenic mutation [27]. The lack of this mutation in the present study deserves special emphasis, as a substantial proportion of Ashkenazim originated in Poland, and intuitively, one would expect to find at least some representation of this mutation (prevalent in non-Jewish individuals) among Jewish families. This expectation is based on the existence of the 5382InsC *BRCA1* mutation in Jewish and non-Jewish individuals from the same geographical region [27]. The lack of this mutation may be accounted for by a later date of origin of this mutation, or its limited geographical location to regions that were not densely populated with Jews in Poland.

The limitations of the study should be borne in mind. This is a limited study from a single medical center and the high risk families analyzed may not be representative of the spectrum of high risk families, even of Ashkenazi origin. The possibility that additional pathogenic BRCA1 BRCA2 mutations, other then the three screened predominant Jewish mutations, do exist in the sample analyzed, should also be considered a limitation. However, based on other studies performed in the Jewish Ashkenazi population, the maximum number of expected "private mutations" in both genes is less than 5% [17]. Only a subset of analyzed samples were adequately scored by the MLPA analysis, so that the actual number of analyzed individuals is even smaller regarding an assessment of the contribution of major gene rearrangement to inherited predisposition to breast/ovarian cancer among Ashkenazim. Lastly the lack of confirmation by Southern blotting (predominantly related to the lack of sufficient amounts of high quality DNA), detracts from the firmness of the conclusions.

In conclusion, no consistent major gene rearrangements involving *BRCA1* or *BRCA2* and no seemingly pathogenic missense mutations in *BRCA1* were detected in a cohort of high risk Jewish Ashkenazi women. Extension and validation of this preliminary conclusion is highly warranted.

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