BRCA1 and *BRCA2* sequence variations detected with next-generation sequencing in patients with premature ovarian insufficiency

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Abstract

Objective: Although the association between *BRCA1* and *BRCA2* gene mutations and breast and ovarian cancer is known, there is insufficient data about premature ovarian insufficiency (POI). However, several studies have reported that there might be a relationship between POI and *BRCA1* and *BRCA2* gene mutation. Therefore, in the present study, we aimed to investigate the role of *BRCA1* and *BRCA2* gene mutations in the etiology of POI in a Turkish population.

Material and Methods: The cohort was classified into two groups: a study group, consisting of 56 individuals diagnosed with premature ovarian insufficiency (and who were younger than 40 years of age, had an antral follicle count <3-5, and FSH levels >12 IU/I), and a control group, consisting of 45 fertile individuals. A total of 101 individuals were analyzed by next-generation sequencing to detect *BRCA1* and *BRCA2* gene mutations.

Results: We detected four new variations (p.T1246N and p.R1835Q in BRCA1 and p.I3312V and IVS-7T>A in BRCA2) that had not been reported before.

Conclusion: We did not find an association between the BRCA1 and BRCA2 gene mutations and premature ovarian insufficiency. However, larger, functional studies are needed to clarify the association. (J Turk Ger Gynecol Assoc 2016; 17: 77-82)

Keywords: Premature ovarian insufficiency, *BRCA1*, *BRCA2*, next generation sequencing, *in vitro* fertilization

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Introduction

Premature ovarian insufficiency (POI), characterized by a loss of function of the ovaries before the age of 40 years (1), is a health problem in women that affects 1% of the population (2). The spontaneous pregnancy rate is low in POI, and although approximately 5–10% of women with POI are still able to conceive, most women with POI have a permanent loss of fertility. The association between POI and rare genetic disorders, some autoimmune and viral diseases, chemotherapy, and radiotherapy are well known. However, the underlying mechanism is still unknown. It has been speculated that there might be a relationship between POI and *BRCA1* and *BRCA2* gene mutations (3–5). There are a limited number of studies in the literature on single cases or related to small patient groups investigating *BRCA1* and *BRCA2* mutations in patients with premature ovarian insufficiency.

BRCA1 and *BRCA2* genes are responsible for DNA repair. Mutations in these genes result in unrepaired DNA damage, leading the cell to apoptosis (6). Loss of function in *BRCA* genes in oocytes also leads them to the apoptotic pathway and may cause early depletion of ovarian reserves (4). Although the association between *BRCA1* and *BRCA2* gene mutations and breast, ovarian, and prostate cancer is clearer, there is insufficient data concerning POI. Recently, it has been speculated that there might be a relationship between POI and *BRCA1* and *BRCA2* gene mutations (5). Although *BRCA1* and *BRCA2* genes have been analyzed in several studies in Turkish population, these studies only analyzed the relationships of *BRCA1* and *BRCA2* gene variations and breast cancer (7-9).

Next-generation sequencing is a valuable tool that analyzes up to gigabases of DNA reads at a high speed and with a low cost per base. This method has also been used in worldwide collaborative projects, such as the International Genome Consortium (ICGC) (10) and The Cancer Genome Atlas (TTGA) (http://cancergenome.nih.gov). Because of the large size of *BRCA1* and *BRCA2* genes (5592 bp and 10257 bp, respectively) and lack of mutation hot spots, these genes need useful prescreening strategies, such as next-generation

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sequencing; therefore, we used the MiSeq Illumina sequencer (MiSeq, Illumina Inc.; San Diego, CA, USA) to detect the variants of *BRCA1* and *BRCA2* genes.

There are only a limited number of studies about the importance of *BRCA1* and *BRCA2* gene mutations in the etiology of POI in the literature. Therefore, the association of POI and *BRCA1* and *BRCA2* gene mutations is unclear. In the case of detecting variations related to breast-ovarian cancer, these patients might be referred for screening and follow-up programs for breast/ovarian cancer before the age of 40 years. Hence, we aimed to investigate the role of *BRCA1* and *BRCA2* gene mutations in the etiology of POI. According to our knowledge, this study is the first study in Turkish patients to assess the genetic predisposition to premature ovarian failure. Also, it is the first study to analyze the whole *BRCA1* and *BRCA2* genes by next-generation sequencing in Turkish patients.

Material and Methods

To determine the mutations and variants in the target exon sequences of *BRCA1* and *BRCA2*, we sequenced and analyzed these genes using next-generation sequencing technology in Turkish patients with premature ovarian failure and in control subjects.

Patients

We enrolled 101 individuals referred to the Zekai Tahir Burak Women's Health Training and Research Hospital who fulfilled the exclusion and inclusion criteria and who accepted to participate in our study. The study group consisted of 56 individuals who had been referred to the IVF unit due to infertility problems and who were younger than 40 years of age with an antral follicle count <3-5 and FSH levels >12 IU/I. The control group consisted of 45 individuals who had been referred to the Family Planning Unit due to contraception and who had spontaneous pregnancies before. None of the individuals had a history of internal systemic disease, pelvic-ovarian surgery, or familial breast/ovarian cancer. Informed consent was obtained from all the patients and controls. This study was approved by Baskent University Institutional Review Board and Ethics Committee (Project No: KA13/297) and was supported by Baskent University Research Fund and Turkish German Gynecology Education and Research Foundation.

Next-generation sequencing analyses

Genomic DNA was obtained from 200 μL peripheral blood samples from each individual using the QIAamp DNA Blood Mini Kit (Qiagen Inc.; Hilden, Germany) according to the manufacturer's instructions.

Primer design was performed for the coding regions of *BRCA1* and *BRCA2* genes. These primers were used to construct a library containing the essential nucleotide sequences. Thirty-eight primers for *BRCA1* and 40 for *BRCA2* were used to amplify 19 and 20 amplicons, respectively. The sizes of the amplicons varied between 299 and 5504 bps. PCRs were performed on isolated DNA samples, using the designed primers, and the reactions were checked by 2% agarose gel electrophoresis. PCRs belonging to each individual were mixed to obtain PCR pools, which had all the amplicons of each individual in one tube. While mixing, the amplification efficiency and the length of the amplicons were taken into consideration; the volume for each PCR was directly proportional to the length of the amplicon and inversely proportional to the efficiency of the reaction, which was estimated by gel electrophoresis. The PCR pools for each individual were purified using the NucleoFast® 96 PCR kit (MACHEREY-NAGEL GmbH; Düren, Germany). The purified pools were quantified using a ND1000 (Thermo Fisher Scientific Inc.; Wilmington, DE, USA) micro volume spectrophotometer and standardized to 0.2 ng/μL, which was needed for the sample preparation step. The samples were prepared for next-gene sequencing using the NexteraXT sample preparation kit (Illumina Inc.; San Diego, CA, USA). Sequencing was performed using the Next Generation Sequencing MiSeq Illumina sequencer (Illumina Inc.; San Diego, CA, USA). Obtained sequences were aligned to the reference genome (GRCh37/hg19) using MiSeq Reporter software (Illumina Inc.; San Diego, CA, USA).

Analysis of the variants

The data were analyzed on IGV 2.3 software (Broad Institute; Cambridge, MA, USA). The clinical outcomes of the variations found on the samples were estimated using the following databases: Ensembl (http://www.ensembl.org/index.html) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) for minor allele frequencies; SIFT (http://sift.jcvi.org/), Mutation Taster (http://www.mutationtaster.org/), and Polyphen II (http://genetics.bwh.harvard.edu/pph2/) for the effects of amino acid changes on the protein; HSF (http://www.umd.be/HSF3/) for the mutations that affect the splicing pattern.

The established variants were cross-checked with Align GVGD (http://agvgd.iarc.fr/) and the breast cancer databases UMD-*BRCA1*/*BRCA2* (http://www.umd.be/*BRCA1*/, and http://www.umd.be/*BRCA2*/).

Statistical analysis

To examine the association between *BRCA* variations and POI, Fisher's-Exact Test and Student's t-test were used. The outcome was considered statistically significant when the p value was below 0.05.

Results

Of the 101 women included in our study, 56 were in the study group and 45 in the control group. The mean ages were 33.4 years (±4.5) and 29.4 years (±6.1), respectively. The difference in ages between the control and study groups was statistically significant. The mean E2 level in the study group was 52.3 pg/mL (±82.1), and the FSH level was 23.1 IU/mL (±10.1).

Next-generation analysis results

We identified a total of 11 *BRCA1* and 13 *BRCA2* sequence variants in the study group. Two of the variants detected in the study group have not been reported in the BIC and UMD-*BRCA1*/*BRCA2* databases previously. Of these novel variants, c.3737C>A was in *BRCA1* and c.9934A>G were in *BRCA2*

Table 1. Variants in the *BRCA1* and *BRCA2* genes in the study and control groups

Exon	cDNA	Protein	SIFT (0-1)	Variation class (UMD)	Polyphen–HumDiv	Polyphen–HumVar	GVGD	Domain	Control group % (n:45)/ Study group % (n:56)
BRCA1									
7	c.536A>G	p.Y179C	0	1	-	0.85	Class45	-	2 (1)/0 (0)
10	c.1067A>G	p.Q356R	-	1	-	-	Class0	*BRCTassoc*	16 (7)/14 (8)
10	c.2077G>A	p.D693N	-	1	-	-	Class0	*EIN3*	20 (9)/9 (5)
10	c.2612C>T	p.P871L	-	1	-	-	Class0	*EIN3*	67 (30)/55 (31)
10	c.3113A>G	p.E1038G	-	1	-	-	Class0	-	60 (27)/54 (30)
10	c.3119G>A	p.S1040N	-	1	0.01	-	Class0	-	2 (1)/2 (1)
10	c.3541G>A	p.V1181I	0.26	1	-	0.01	Class0	-	0 (0)/2 (1)
10	c.3548A>G	p.K1183R	-	1	-	-	Class0	-	62 (27)/54 (30)
10	c.3737C>A	p.T1246N	0	Not reported	-	-	Class0	-	0 (0)/2 (1)
10	c.1456T>C	p.F486L	0.22	-	-	-		*BRCTassoc*	2 (1)/0 (0)
10	c.1648A>C	p.N550H	0.01	-	0.99	0.88		-	2 (1)/0 (0)
12	c.4342A>G	p.S1448G	0.01	3	0.33	-	Class0	-	0 (0)/2 (1)
14	c.4535G>T	p.S1512I	0.01	1	-	0.13	Class0	-	2 (1)/0 (0)
15	c.4837A<G	p.S1613G	-	1	-	-	Class0	-	64 (29)/54 (30)
15	c.4883T>C	p.M1628T	-	1	-	-	Class0	-	2 (1)/0 (0)
15	c.4956G>A	p.M1652I	-	1	-	-	Class0	*BRCT*	4 (2)/2 (1)
23	c.5504G>A	p.R1835Q	0.02	Not reported	*BRCT* domain	-	Class0	*BRCT*	2 (1)/0 (0)
BRCA2									
3	IVS2-7T>A	-	-	Not reported	-	-	-	-	2 (1)/0 (0)
10	c.865A>C	p.N289H	-	1	-	-	Class0	-	9 (4)/13 (7)
10	c.1114A>C	p.N372H	-	1	-	-	-	-	47 (19)/45 (26)
10	c.1368G>C	p.E456D	0.04	-	0.85	0.32	Class0	-	2 (1)/0 (0)
11	c.2971A>G	p.N991D	-	1	-	-	Class0	-	9 (4)/11 (6)
11	c.5744C>T	p.T1915M	-	1	-	-	Class0	-	4 (2)/4 (2)
11	c.4258G>T	p.D1420Y	0	1	0.03	0.01	-	-	4 (2)/2 (1)
11	c.6853A>G	p.I2285V	0.12	2	0.61	0.14	Class25	-	0 (0)/2 (1)
11	c.6100C>T	p.R2034C	-	1	-	-	Class0	-	0 (0)/2 (1)
11	c.3318C>G	p.S1106R	0	-	1	1	-	-	2 (1)/0 (0)
11	c.2919G>A	p.S973S	-	-	-	-	-	-	2 (1)/0 (0)
18	c.8187G>T	p.K2729N	0.07	3	1	0.93	Class35	*BRCA2DBD_OB1*	2 (1)/2 (1)
22	c.8851G>A	p.A2951T	-	1	-	-	Class55	*BRCA2DBD_OB2*	2 (1)/4 (2)
26	c.9581C>A	p.C3194Q	0.12	3	1	0.95	-	-	0 (0)/2 (1)
27	c.9934A>G	p.I3312V	0.88	Not reported	0	0	Class0	-	0 (0)/2 (1)
27	c.10234A>G	p.I3412V	-	1	-	-	Class0	-	0 (0)/2 (1)
27	c.9976A>T	p.K3326X	-	1	-	-	-	-	4 (2)/2 (1)

Classification UMD database: 1 - Neutral, 2 - likely neutral or contradictory neutral/UV, 3 - UV, 4 - likely causal or contradictory deleterious/UV, 5 - Causal. Neutral variant: non-causal variant in terms of disease risk, present in less than 1% of the general population, designated as "less likely" for Align-GVGD, "benign" for PolyPhen, and "not clinically important" for BIC. Polymorphism: neutral variant present in more than 1% of the general population, Predicted neutral: considerable evidence for neutrality but no final GGC decision. UV: unclassified variant, designated as "unknown" for BIC. Predicted causal: considerable evidence for pathogenicity but no final GGC decision, Causal mutation: causal or pathogenic mutation in terms of disease risk, designated as "most likely" for Align-GVGD, "damaging" for PolyPhen, "pathogenic" for UMD-Predictor, and "clinically important" for BIC PolyPhen results for each variant were classified as benign (score ≤0.5), possibly damaging (0.5< score <2), probably damaging (score >2), and unknown.
C/P: P: Patient group; C: Control group, patient numbers with * indicates homozygous variant, patient number without * indicates heterozygote variant.
SIFT score: Ranges from 0 to 1. The amino acid substitution is predicted damaging is the score is <=0.05, and tolerated if the score is >0.05.
GVGD: Align GVGD scores amino acid substitutions on a 7-scale scoring system, from C0 to C65. C0: Neutral, C15-25 intermediate, as changes to protein structure or function are uncertain, and C35 scores or higher are considered as likely deleterious.
UMD: Universal Mutation Database; SIFT: Sorting Tolerant From Intolerant; GVGD: Grantham Variation Grantham Deviation; BIC: Breast Cancer Information Core; *BRCT*: *BRCA* C-terminus

genes. In contrast, in the control group, 14 *BRCA1* and 12 *BRCA2* sequence variants were detected. Two of them were novel: c.5504G>A in *BRCA1* and IVS2-7T>A in *BRCA2* genes. All the detected variants are shown in Table 1.

Each *BRCA1* variant was seen in different numbers of individuals (Table 1). For example, c.4342A>G was seen only in one individual in the study group. However, c.3113A>G was seen in 27 individuals in the control group and in 30 individuals in the study group. *BRCA2* variants were also seen in different numbers of individuals in each group. As an example, c.1368C>G was seen in only one individual in the control group; however, c.1114A>C was detected in 19 individuals in the control group and in 20 individuals in the study group.

Discussion

According to the best of our knowledge, this study was the first to perform *BRCA1* and *BRCA2* gene sequencing using next-generation sequencing methods in Turkish patients with premature ovarian insufficiency. Different variants were detected in *BRCA1* and *BRCA2* genes.

There are only a few relevant studies in the literature investigating the relationship between *BRCA1* and *BRCA2* mutations and premature ovarian failure and/or ovarian reserve or ovarian stimulation (3–5,11). For the first time, Oktay et al. (4) showed the relationship between ovarian stimulation and BRCA1 mutations and concluded that there might be a possible link between gene repair and infertility and breast/ovarian cancer risks. Then, Titus et al. (11) showed the association between *BRCA1*-related DNA double-strand break repair and ovarian aging in mice and humans. Finch et al. (3) found that women carrying a *BRCA* mutation experience menopause earlier, on average, than women who have no mutations, although the difference is small and does not affect fertility. Santoro (12) commented on Finch et al.'s (3) study that BRCA mutations appear to have normal fertility in a study group. A recent study concluded that *BRCA1* germ-line mutations may be associated with reserved ovarian reserve (5). Another study investigated the effects of *BRCA1* and *BRCA2* mutations on female infertility (13). Finally, a recent study (14) reported that patients with *BRCA* gene mutations showed a normal ovarian response in IVF compared to patients with no *BRCA* mutations. A survey reported that knowledge of *BRCA* mutations affects the marriage and childbearing decisions of the patients (15). However, most of the studies used different study groups from our study, with other studies mostly including patients who had IVF treatment with *BRCA1* and *BRCA2* mutations (14), whereas our study group consisted of women diagnosed with POI.

BRCA1 encodes an 1863 amino acid protein. It has three major domains: first, the N-terminal RING finger (amino acids 18–136); second, consisting of three nuclear localization signals in the central region; and third, the tandem of two *BRCA1* C-terminus (*BRCT*) domains (i.e., *BRCT1*: amino acids 1642–1736; *BRCT2*, amino acids 1756–1835) at the C-terminus (16). Many inherited cancer-associated *BRCA1* mutations have been found within the RING and *BRCT* domains, indicating that both domains are involved in suppressing breast and ovarian cancer (17).

We detected two variations in *BRCA1* and two in *BRCA2* that have not been reported before. The first one in *BRCA1* was p.T1246N and was detected only in one patient with POI but was not detected in the control group. Although it does not correspond to any domain, the SIFT score was 0, which means the amino acid substitution can be predicted to be damaging. The GVGD score shows that amino acid substitution is not deleterious. However, our results are not sufficient to conclude whether the variation involves a polymorphism.

The other variation detected in *BRCA1* was p.R1835Q, which corresponds to the *BRCT* domain and was seen in only one individual in the control group but none in the study group. According to the SIFT score of 0.02, the amino acid substitution can be predicted to be damaging. The *BRCT* (*BRCA1* carboxyl terminal domain) domain is an evolutionary conserved module that exists in a large number of proteins, from prokaryotes to eukaryotes. Most of the proteins that contain the *BRCT* domain participate in DNA damage checkpoint or DNA repair pathways. However, the function of the domain is still controversial. It is known that germ-line mutations in the *BRCT* domain lead to 50% of familial breast cancers (18). Most *BRCT* domain mutations cause a truncated *BRCA1* protein. It has been shown that loss of the *BRCT* domain leads to tumor formation in mice (19). Therefore, the *BRCT* domain has an important role in the cellular process of DNA damage. Because *BRCT* repeats are found in different proteins associated with the regulation of the DNA damage response, such as *BARD1*, *53BP1*, and *MDC1*, this individual in the control group did not show any clinical signs although she had the mutation in the *BRCT* domain. Other proteins that have a *BRCT* domain might function properly to protect tumor formation in this individual. The risk of developing breast cancer by the age of 70 years for *BRCA1* mutation carriers is between 57% and 65% and between 45% and 57% for *BRCA2*. The risk of developing ovarian cancer by the age of 70 years for *BRCA1* mutation carriers is between 39% and 59% and between 11% and 18% for *BRCA2*. However, the overall risk for younger age (<40) is reported to be lower for ovarian cancer in *BRCA1* and *BRCA2* mutation carriers (20-22). It is also known that *BRCA* mutations in oocytes may lead to early depletion of the ovarian reserve (4). There are also other factors that affect breast-ovarian cancer, such as age, gender, family history of breast-ovarian cancer, and mutations other than *BRCA1* and/or *BRCA2* genes (*ATM, TP53, CHEK2, PTEN, CDH1, STK11, PALB2*) (23). Because we did not have other clinical data, such as family history or mutations in other genes of this individual, it was not possible to predict the clinical outcome.

We detected two new variations in *BRCA2*. The first one was in the intergenic sequence IVS-7T>A, which was detected only in one patient in the control group but none in the study group. Because the intergenic sequence is a non-coding region, there might not be an effect on a gene or a protein, thus resulting in no clinical signs in the patient. The second new variation p.I3312V was detected in only one case in the study group (1/45). The SIFT score was 0.88, which means that the amino acid substitution is tolerated. Although the new variation was detected in a patient with POI, this might not be related with the disease because the amino acid substitution is tolerated

and does not correspond to any domain. Therefore, the variation might be only a rare polymorphism seen in the Turkish population.

We detected 17 different variations in *BRCA1* and 17 in *BRCA2*. Six of 17 *BRCA1* variations corresponded to a domain, whereas only 2 of 17 variations corresponded to a domain in *BRCA2*. Oktay et al. (4) found nine variations in *BRCA1* and *BRCA2* that might be associated with premature ovarian failure and/or ovarian reserve. Of those, only one of them corresponded to the *BRCT* domain; however, another five variations did not correspond to any domain. Wang et al. (5) detected 13 different variations in *BRCA1* and 10 in *BRCA2*. Five of them correspond to *BRCA* domains. Of them, only one corresponded to the *BRCT* domain.

Our study has a limited number of individuals in both the study and control groups. In addition, our study lacks the confirmation of the detected variations by Sanger sequencing. However, the depth of coverage of our study was >100x in 98% of the patients. We repeated the results when the depth of coverage was <20x. Current next-generation sequencing guidelines for inherited disorders do not define quality parameters to provide concrete guidance for confirmatory analysis. In a recent next-generation sequencing laboratory standards paper, the College of American Pathologists justifies that "Sufficient depth of coverage and quality parameters should not expect false positives in their filtered data" (24). Implementing NGS-based tests according to diagnostic standards is a challenge for individual laboratories. To facilitate the implementation of NGS into routine laboratory practice several studies done such as the Dutch Society for Clinical Genetic Laboratory Diagnostics (VKGL) working group. And also, in a recent paper researchers have been emphasized that the necessity of Sanger confirmation of next-generation sequencing variants lower than 30x depth of coverage might need to be explored (25).

As a conclusion, we did not detect an association between POI and the *BRCA1* and *BRCA2* gene variations. However, functional studies are needed to clarify the variations of *BRCA1* and *BRCA2* genes because there are conflicting results about the association of *BRCA1* and *BRCA2* variations. In case of detecting variations that are related to breast-ovarian cancer, these patients might be referred for screening and follow-up programs for breast-ovarian cancer before they reach the age of 40 years. We also detected new variations in *BRCA1* and *BRCA2* genes both in the study and control group, which have not been reported before. Therefore, next-generation sequencing is a valuable tool to detect gene variations of large genes in a fast and cost-effective way.

Ethics Committee Approval: *Ethics committee approval was received for this study from the ethics committee of Başkent University (Project No: KA13/297).*

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