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Evaluation of hereditary/familial breast cancer patients with multigene targeted next generation sequencing panel and MLPA analysis in Turkey

Elcin Bora[a,*], Ahmet Okay Caglayan[a], Altug Koc[a], Tufan Cankaya[a], Hande Ozkalayci[a], Mehmet Kocabey[a], Demet Kemer[a], Suleyman Aksoy[b], Zumre Arican Alicikus[c], Isil Basara Akin[d], Merih Guray Durak[e], Duygu Gurel[e], Tugba Yavuzsen[f], Ali Sevinc[b], Isil Somali[f], Ilknur Gorken[c], Pinar Balci[d], Aziz Karaoglu[f], Serdar Saydam[b], Ayfer Ulgenalp[a]

[a] Department of Medical Genetics, School of Medicine, Dokuz Eylul University, Izmir, Turkey
[b] Department of General Surgery, School of Medicine, Dokuz Eylul University, Izmir, Turkey
[c] Department of Radiation Oncology, School of Medicine, Dokuz Eylul University, Izmir, Turkey
[d] Department of Radiology, School of Medicine, Dokuz Eylul University, Izmir, Turkey
[e] Department of Pathology, School of Medicine, Dokuz Eylul University, Izmir, Turkey
[f] Department of Oncology, School of Medicine, Dokuz Eylul University, Izmir, Turkey

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ABSTRACT

Breast cancer, a worldwide leading cause of cancer in women, may occur in familial cases. Germline mutations in *BRCA1/2* genes are responsible for 15% of the familial cases. With the power of next generation sequencing (NGS) analysis, it is possible to analyze genes related to hereditary susceptibility to breast cancer and investigate the genetic etiology more thoroughly.

In this study, we investigated 30 genes identified frequent pathogenic alleles in Turkish population. The study includes 495 unrelated individuals diagnosed with breast cancer who are selected for genetic testing according to NCCN criteria for hereditary breast cancer. All patients were analyzed by NGS for *BRCA1/2* genes. Deletion/duplication investigation by Multiplex ligation-dependent probe amplification (MLPA) and massive sequencing of 30 breast cancer-related genes (Oncorisk Gene Panel) were performed in a stepwise manner.

BRCA1/2 variants are the most frequent pathogenic variants which are found in 45 of 495 (9.1%) patients. Four previously unreported, novel, pathogenic variants of *BRCA2* gene are identified. In four cases, exonic deletions of BRCA1/2 genes are determined and there is no duplication of these genes. NGS panel investigation involving other moderate-high risk genes contributed genetic diagnosis in an extra 39 out of 419 (9.3%) cases.

Our study presents the cost effectiveness of the gene panel approach. We suggest that gene panels should be the first-tier genetic testing for hereditary breast cancer and MLPA analysis of BRCA1/2 genes should be investigated as a complementary method of NGS analysis.

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Introduction

Breast cancer (BC) is the most frequent malignancy in women worldwide. There were about 2.1 million newly diagnosed female breast cancer cases in 2018, corresponding to 1 in 4 cancer cases among women. The incidence and mortality rates of BC are increasing in developing countries including our nation [1]. The incidence of breast cancer in Turkey was 24/100,000 in 1993, and it increased to 50/100,000 in 2010 [2].

Breast cancer is curable in up to 80% of cases with early-stage, non-metastatic disease [3]. However, advanced breast cancer with distant organ metastases is considered incurable with currently available therapies [3]. Therefore, identifying hereditary cases and their families is crucial for early diagnosis of breast cancer.

Hereditary and familial breast cancer cases are at greater lifetime risk of cancer compared to sporadic cases, but also constitute the group that is gaining greater clinical benefit from advanced genetic knowledge. Pathogenic variants in *BRCA1 and BRCA2* genes,

* Corresponding author.
E-mail address: elcin.bora@deu.edu.tr (E. Bora).

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the genes most affected in hereditary breast and ovarian cancer, are detected in ~15% of women with familial breast cancer [4,5]. The *BRCA1* pathogenic variant carriers have 59–87% lifetime risk for developing breast cancer and the risk is 38–80% for *BRCA2* [6]. The prevalence of *BRCA1* and *BRCA2* mutations among distinct ethnicities differ and the founder effect is also seen [7].

In addition to *BRCA1* and *BRCA2* genes other high (*CDH1, PTEN, TP53, STK11, PALB2),* moderate, *(CHEK2, ATM, BLM, BARD1, RAD51D, NBN),* and low-penetrance genes such as *MRE11A, BRIP1, RAD51C, MLH1* also have role in predisposition to breast cancer [8]. A polygenic risk model has been suggested for breast cancer etiology as well. Seventy Single-nucleotide polymorphisms (SNPs) are associated with breast cancer risk [9].

Although efforts have been made to identify germline genetic etiology of breast cancer, 50% of patients remain undiagnosed [10].

Many studies have been conducted since 1999 to reveal the information specific to the Turkish population [11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30]. With these studies, frequency and clinicopathologic features of pathogenic *BRCA1/2* variations in the Turkish population were investigated. Each passing year, more efficient and cost-effective molecular methods such as next generation sequencing (NGS) increase the clinical utility of genetic testing in breast cancer cases [3,9].

This study involves next generation sequencing (NGS) results of a breast cancer related multigene panel combined with multiplex ligation-dependent probe amplification (MLPA) results of *BRCA1/2* genes in 495 individuals from Turkey. We review existing literature from Turkey and compare with our results to achieve a generalization of Turkish breast cancer genetics. These results will guide genetic testing algorithms in routine clinical care and will be the basis of future research for determining clinical utility in hereditary/familial breast cancer patients in Turkey.

Materials and methods

Patient selection

The study cohort consists of breast cancer cases who were referred to the Department of Medical Genetics, Dokuz Eylul University for molecular genetic investigation, between 2016 and 2020. There were 495 individuals (2 males, 493 females). None of the study participants were found to be related by means of three generation pedigree cross-comparison to each other (i.e., only one patient per family was included). The patient evaluation was done according to NCCN Clinical Practice Guideline for breast cancer in genetic clinic [31]. The study was approved by the local ethical committee (Dokuz Eylul University, Faculty of Medicine, No: 2018/22-43, 13/09/2018).

Age at diagnosis, histological type, grade and size of the tumor, estrogen, and progesterone receptor status (ER, PR), Her2 status, Ki-67 proliferation index, the presence of lymph node and/or distant metastasis were compiled for each case. The Nottingham (Elston-Ellis) modification of the Scarff-Bloom-Richardson grading system was used to grade the tumors.

The median age of cases at admission was 47 (minimum: 24, maximum: 90) and at diagnosis 44 (minimum: 22, maximum: 87). The tumor was localized in the left breast in 42.6% (n=211), and the right breast in 37.8% (n=187) of cases. Six per cent (n=29) of the cases had bilateral breast cancer. Histologic types of breast cancer were invasive ductal carcinoma in 74.8% (n=303), invasive lobular carcinoma in 11.1% (n=45), mixed type in 11.6% (n=47) and ductal carcinoma in situ in 2.5% (n=10) of cases. Twelve per cent (n=44) of the cases were triple negative. Estrogen receptor was positive in 75.9% and PR was positive in 64% of cases. Thirty-three cases (15.1%) had distant metastasis and 108 (45.4%) had lymph node metastasis (**Table 1**).

Table 1
Characteristics of study participants.

Characteristics		Number of Patients	%
Age at Admission Median (Range)	47 (24–90)		
Age of Diagnosis Median (Range)	44 (22–87)		
Localization			
Left		211	49.4%
Right		187	43.8%
Bilateral		29	6.8%
Histology			
IDC		303	74.8%
ILC		45	11.1%
Mix		47	11.6%
DCIS		10	2.5%
Receptor Status			
TNBC		44	12%
NTNBC		323	88%
Metastasis			
Lymph node			
+		108	45.4%
		130	54.6%
Distant			
+		33	15.1%
		186	84.9%

IDC: Invasive ductal carcinoma; ILC: Invasive lobular carcinoma; TNBC: Triple negative breast cancer; NTNBC: Non-triple negative breast cancer.

Digital mammography (DM) and digital breast tomosynthesis (DBT) were used for patients older than 30 years old with *BRCA1/BRCA2* mutations. All examinations were carried out with a mammography device (Selenia, Hologic, Bedford, MA, USA). In standard DM protocol, four different images including bilateral craniocaudal (CC) and mediolateral oblique (MLO) were obtained. Afterwards, patients with dense breast tissues and any suspicious mammographic findings were evaluated with breast ultrasonography (US). All US were performed with ML 6-15-MHz transducers with an US device (LOGIQ S8; GE Healthcare, Milwaukee, WI). Magnetic resonance imaging (MRI) was also used in selected cases (Intera, software version 8.1; Philips Medical Systems, Eindhoven, The Netherlands and Gyroscan Achieva, Philips, ACS-NT, Bothell, WA, USA).

Sample collection and DNA extraction

Minimum 3 ml of peripheral venous blood samples of the subjects were collected to the EDTA tubes. DNA extraction was performed on the same day by the commercial QIAcube (Qiagen, Germany) according to manufacturer's protocol. The DNA was stored at -20 °C until molecular analysis was performed. The laboratory sample flow included the initial sequencing of *BRCA1* and *BRCA2* genes, then the MLPA study of the same genes. If the results showed no pathogenic variants, then the gene NGS panel (Oncorisk) was conducted.

Next-Generation Sequencing (NGS) of the BRCA1 *and* BRCA2 *Genes*

The coding regions of *BRCA1* and *BRCA2* genes were sequenced by custom designed, commercial test kits (Celemics, South Korea). The details of the procedure were the same with "gene panel study" and given below. When pathogenic/likely pathogenic variants identified, segregation studies were performed whenever possible.

MLPA studies for BRCA1 *and* BRCA2

The MLPA assay was used for cases with *BRCA1* and *BRCA2* negative results by amplicon sequencing. The DNA samples were investigated by "SALSA MLPA probemix P002-D1 *BRCA1*-v04 and *BRCA2*-CHEK2-v01 (MRC-Holland, Holland)" which contains probes specific for *BRCA1* and *BRCA2* genes and control regions.

Next-Generation Sequencing (NGS) of the breast cancer related genes panel

The content of the gene panel was constructed according to the literature [32]. DNA quality and concentration measurements were performed by Qubit (Thermofisher, USA) for the preparation of DNA samples to test kit (Oncorisk, Celemics, South Korea). After the adjustment of proper DNA amount, library preparation was performed. Steps of the targeted NGS test kit included, in an orderly fashion: DNA fragmentation, DNA end repair, A-tailing, purification, adapter ligation, indexing, probe hybridization, targeted library selection by streptavidin beads, amplification of targeted library according to the recommendations of the producer. The library run was achieved by NGS platforms (MiniSeq and NextSeq500, Illumina, USA). The data were analyzed by software Genomize Seq (V.16.7) (Genomize, Turkey). Additionally, the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), Varsome web tool (https://varsome.com/), Franklin web tool (https://franklin.genoox.com - Franklin by Genoox) was also used. The coding regions (± 10 base pair intronic, 5' and 3' UTR are excluded) of "*APC (ENST00000257430), ATM (ENST00000278616), BARD1 (ENST00000260947), BLM (ENST00000355112), BMPR1A (ENST00000372037), BRCA1 (ENST00000357654), BRCA2 (ENST00000380152), BRIP1 (ENST00000259008), CDH1 (ENST00000261769), CDK4 (ENST00000257904), CDKN2A (ENST00000361570), CHEK2 (ENST00000382580), EPCAM (ENST00000263735), MLH1 (ENST00000231790), MRE11A (ENST00000323929), MSH2 (ENST00000233146), MSH6 (ENST00000234420), MUTYH (ENST00000450313), NBN (ENST00000265433), PALB2 (ENST00000261584), PRSS1 (ENST00000486171), PTEN (ENST00000371953), RAD50 (ENST00000265335), RAD51C (ENST00000337432), RAD51D (ENST00000345365), SLX4 (ENST00000294008), SMAD4 (ENST00000342988), STK11 (ENST00000326873), TP53 (ENST00000269305), VHL (ENST00000256474)*" genes were investigated. The mean read depth was more than 95X. For the pathogenicity scoring and classification of genetic variants, the ACMG recommendations were used [33,34]. Chromosome positions of variants were reported according to "hg19/GRCh37 Human Reference Genome"

Statistics

Statistical analysis in this study was performed using SPSS Version 22. Dichotomous variables were tested with either Pearson's chi-square statistics or Fisher's exact test. Continuous variables were tested with Manne-Whitney U test. *P*-value of 0.05 or less was considered significant.

Results

BRCA1/2 *Sequencing and MLPA Results*

The age at admission was 47 (median, range 24–90) and age of diagnosis was 44 (median, range 22–87). The number patients with *BRCA1/2* amplicon sequencing and oncorisk gene panel were 76 (15.4%) and 333 (67.3%), respectively. Both tests were applied to 86 cases (17.4%) in a stepwise manner. In total, *BRAC1/BRCA2* genes were sequenced in 495 cases.

Table 2
BRCA1/2 mutations.

BRCA1 gene	BRCA2 gene
c.66dupA (exon 2)	c.67+1G>A (intron 2) *2
c.135-2A>G (intron 3)	Exon 3 deletion
c.181T>G (exon 4)	c.632-4_632-3delAC (intron 7)
c.671-1G>C (intron 9)	c.632-3_632-2del (intron 7)*2
c.788dupG (exon 10)	c.657_658delTG (exon 8)
c.844_850dupTCATTAC (exon 10)	c.672_674delTAC (exon 8) n.
c.1504_1508del (exon 10)	c.810delA (exon 10)*4 n.
c.2611_2612delCC (exon 10)	c.1853delC (exon 10)
c.2800C>T (exon 10)	c.2761_2762insT (exon 11)
c.2869C>T (exon 10)	c.2866_2867delAA (exon 11) n.
c.3569_3570delCT (exon 10)	c.3186_3189delTCAG (exon 11)
c.3700_3704delGTAA (exon 10)	c.4521delG (exon 11) n.
c.4065_4068delTCAA (exon 10)	c.5066_5067insA (exon 11)
c.4356delA (exon 12)	c.5718_5719delCT (exon 11)
c.5095C>T (exon 17)	c.5916delA (exon 11)
c.5102_5103delTG (exon 17)	c.5969delA (exon 11)
c.5152+5G>A (intron 17)	c.6468_6469del (exon 11)
Exon 18 and exon 19 deletion	c.8827C>T (exon 22)
c.5266dupC (exon 19)*5	c.9027delT (exon 23)
Exon 20 deletion*2	

* Number of patients, *n. Novel.*

Four point four per cent of the cases (*n*=22) had pathogenic variants in the *BRCA1* gene and 4.7% (*n*=23) had *BRCA2* pathogenic variation. *BRCA1* and *BRCA2* MLPA analysis included 438 cases (438/495, 88.5%). Three cases had a deletion in *BRCA1* gene [exon 20 (*n*=2), exon 18 and 19], one case had deletion in *BRCA2* gene (exon 3). We detected deletion in 0.91% of patients. There was no duplication. The list of *BRCA1/2* gene mutations is given in **Table 2**.

Frameshift (*n*=15, 68.2%), splice site (*n*=3, 13.6%), stop codon (*n*=2, 9.1%), and missense (*n*=2, 9.1%) pathogenic variants were identified in *BRCA1* gene. There was one novel variant namely c.4356delA (exon 12).

BRCA2 gene pathogenic variants included frameshift variants (69.6%, *n*=16), splice site (21.7%, *n*=5), stop codon (4.3%, *n*=1), and *in frame* deletion (4.3%, *n*=1) variants. Four variants were novel: c.672_674delTAC (exon 8), c.810delA (exon 10), c.2866_2867delAA (exon 11), c.4521delG (exon 11), c.8827C>T (exon 22).

The triple negative histological type was found more in cases with *BRCA1/2* pathogenic variants (*p*=0.006). The Ki-67 proliferation index (*p*<0.001, Median 70 vs. 30) and histological grade of the tumors were increased (*p*=0.003, median 2 vs. 3) in patients with *BRCA1/2* pathogenic variants, as well. The age of onset was decreased in these cases (*p*=0.031, Median 40 vs. 44) and bilateral involvement was more common (*p*=0.001).

Oncorisk gene panel results

The pathogenic variants of frequently reported genes were found in 57 of 419 cases (13.6%). *BRCA1/2* genes were the most affected ones (26/419 6.2%). The other frequently affected genes were as follows: *CHEK2* (4.3% *n*=18), ATM (1% *n*=4), *PALB2* (0.5% *n*=2), *RAD50* (0.5% *n*=2). More rarely affected genes were *BRIP1, NBN, RAD51C, STK11* and *VHL* genes (0.24%, *n*=1) (**Table 3**).

After the addition of pathogenic variants of ***MLH1*** (*n*=1), ***MSH2*** (*n*=1), and ***MUTYH*** (*n*=6) genes, 15.5% of the cases (65/419) had pathogenic/likely pathogenic variants. Two cases had more than one pathogenic variant: One of them had *BRCA2* and *MUTYH* genes and the other one had *CHEK2* and *MUTYH*.

The cases with VUS were as follows: 35.6% of the cases (*n*=149) had one, 9.3% (*n*=39) had two, 2.2% (*n*=9) had three VUS, whereas

Table 3
Non-*BRCA* mutations.

Gene	cDNA-change	Predicted aminoacid change	Mutation type	Age at diagnosis	Cancer family history	Secondary cancer
ATM	c.3077+1G>T		Splice site	38	None	
ATM	c.3576G>A	p.Lys1192=	synonymous	43	Mat. aunt: leukemia?	
ATM	c.7159_7160 insAGCC	p.Phe2387Ter	nonsense	41	Cousin (pat. uncle): breast (34)	
ATM	c.8278_8279delCT	p.Gln2762AlafsTer6	frameshift	39	None	
BRIP1	novel, c.507+1G>T		splice site	49	Mat. uncle: colon. Pat. uncle: larynx (70)	
CHEK2	c.573+2T>C		splice site	40	Father: lung (65), pat. aunt: endometrium (60), pat. uncle: liver, mat. uncle: lung and his daughter: colon (50), cousin (pat. aunt): leukemia and her brother: lung (45)	
CHEK2	c.599T>C	p.Ile200Thr	missense	38	Cousin (pat. aunt): colon (40), cousin (mat. aunt): skin	
CHEK2	c.599T>C	p.Ile200Thr	missense	41	Mother: bone,pat. aunt: pancreas, pat. uncle: gastric? pancreas?, pat. uncle:lung, mat. aunt: lung, mat. uncle: lymphoma, cousin (pat. uncle): thyroid	
CHEK2	Novel,c.592delTinsCCA	p.Ser198ProfsTer7	frameshift	48/53	Sister: breast (42)	
CHEK2	c.628G>A	p.Gly210Arg	missense	31	None	
CHEK2	c.628G>A	p.Gly210Arg	missense	63	Sister: stomach (48), mother: colon (72), mat. uncle: prostat (70), cousin (mat. aunt): cancer (35)	
CHEK2	c.721+3A>T		splice site	47	None	
CHEK2	c.721+3A>T		splice site	45	Mat.aunt: breast (50), two pat. uncle: prostat (55), pat. grandmother: endometrium? (60)	
CHEK2	c.1268_1269delTC	p.Leu423HisfsTer14	frameshift	52	Mother: lymphoma, father: lung, cousin (mat. aunt): colon	
CHEK2	c.1298A>C	p.Tyr433Ser	missense	51	None	Renal cell ca.
CHEK2	c.1309G>A	p.Glu437Lys	missense	36	Father: lung (69), mat. grandfather: prostat (65)	
CHEK2	c.1361G>A	p.Trp454Ter	nonsense	42	Brother: brain (23)	
CHEK2	c.1556C>T	p.Thr519Met	missense	37	Pat. uncle: liver and prostat (50), grandmother: larynx (82), cousin (pat. uncle (liver)): liver (50), mat. granduncle larynx (80)	
CHEK2	c.1556C>T	p.Thr519Met	missense	50	Father: lung, pat. uncle: lung	
CHEK2	c.1556C>T	p.Thr519Met	missense	49	Pat. uncle: larynx (60), pat. uncle:lung	
CHEK2	c.1556C>T	p.Thr519Met	missense	36	Pat. aunt: endometrium, cousin (mat. uncle): endometrium	
CHEK2	c.1556C>T	p.Thr519Met	missense	42	None	
CHEK2	c.1556C>T	p.Thr519Met	missense	43	Mother: gastric, father: gastric, cousin (pat. uncle): breast	
MLH1	c.1007G>A	p.Gly336Asp	missense	32	Father: malign melanoma	
MSH2	c.942+3A>T		splice site	56	Mat. aunt: endometrium (68), mat. aunt: thyroid (70), cousin (mat. aunt (thyroid)):breast (45)	
NBN	c.657_661delACAAA	p.Lys219AsnfsTer16	frameshift	57	Mother: breast (52) brother: colon (34), chidren of brother (colon):polyposis	
PALB2	c.2587-1G>C		splice site	44	Pat. uncle: breast, cousin (pat. aunt): breast (45), cousin (pat. uncle): gastric	
PALB2	c.509_510delGA	p.Arg170IlefsTer14	frameshift	35	Mother: ovarian, mat. uncle: colon	
RAD50	c.1052-2A>C		splice site	41	Pat. uncle: brain	Malign melanom
RAD50	c.2157delA	p.Lys722ArgfsTer14	frameshift	61	Mother: colon, mat. uncle: colon	
RAD51C	c.179_180delCT	p.Leu61AlafsTer11	frameshift	72	Father: lung, sister: breast (40), pat. Uncle: larynx cousin (pat. aunt): basal cell carcinom (65)	
STK11	c.250A>T	p.Lys84Ter	nonsense	33	Cousin (mat. aunt): breast (45)	
VHL	c.598C>T	p.Arg200Trp	missense	39	Brother: brain mass? (34), mat. grandfather: lung (80)	Thyroid (19)

mat.: maternal; pat.: paternal.

52.8% (n=221) had no VUS. Therefore, a total of 254 VUSs were found in 197/419 (47,0%) individuals in this study.

BRCA1/2 *mutations*

In our study, *BRAC1/BRCA2* genes were sequenced in 495 cases. There were 25 pathogenic *BRCA1* variants (**Table 2**). The most frequent pathogenic *BRCA1* variant was c.5266dupC (exon 19) and it was determined in 5 cases. The variant was a known, common, pathogenic variant of Ashkenazi Jewish ancestry. One previously not reported *BRCA1* variant was identified in exon 12 (c.4356delA).

There were 24 *BRCA2* pathogenic variants. Four variants were novel and not reported: c.672_674delTAC (exon 8), c.810delA (exon 10), c.2866_2867delAA (exon 11), c.4521delG (exon 11), c.8827C>T (exon 22). The clinical findings and family histories of four, novel *BRCA2* cases were available and are shared below:

Two cases of BRCA2 c.810delA

The first case had excision of the right breast and was diagnosed with invasive ductal carcinoma at 46 years of age. She also had major depression for 20 years and was medicated by Prozac and Xanax. Her paternal grandmother was deceased at age 66 because of colon cancer. She had two healthy sons (18 and 30 years old).

The second case was diagnosed at age 40 as right, invasive breast cancer (apocrine differentiation) following excision biopsy. Her mother had endometrium cancer. Her paternal aunt had breast cancer and the daughter of her paternal uncle had stomach cancer (exitus at 38 years).

Case of BRCA2 c.2866_2867delAA

The case had the diagnosis of right-sided invasive ductal carcinoma at age 31. Right subcutaneous mastectomy was performed. Her 35-year-old sister was followed up for tumor in the breast (without biopsy) and had cervix pathology (CIN2).

Case of BRCA2 c.4521delG

The case was 34 years old and diagnosed with left breast cancer. The mastectomy revealed intraductal carcinoma with fibroadenoma, nodular sclerosing adenosis and fibrocystic alterations. The pathogenic variation was maternal. Chemotherapy and radiotherapy were given. She had no child. Her maternal aunt died at 60 years due to breast cancer and paternal aunt also died at 38 years because of breast cancer. Her pathogenic variant carrier mother was healthy (60 years).

Case of BRCA2 c.8827C>T

The female case noticed a lump on her right mammary tissue at the age of 29. Frozen section demonstrated invasive ductal carcinoma grade 3 (30 years). Her chemotherapy and follow up was done in a distinct center. Her family history included cervix carcinoma in her maternal aunt (45 years), and testis tumor in her maternal uncle (38 years). She had two healthy daughters (2 and 7 years old).

Case of BRCA2 *c.672_674delTAC*

The case presented to clinic with palpable mass of right breast when she was 30 years old. Patology report of the mass was infiltrative ductal carcinoma. Radical mastectomy (right) was performed and she recieved chemotherapy and radiotherapy. Her 85 year old mother also diagnosed with breast cancer when she was 72 year old, and no pathogenic variation detected. Conversely, she has a healthy 80 year old father who carries same pathogenic mutation.

MLPA results

BRCA1 and *BRCA2* MLPA analysis included 438 cases. Four cases had heterozygous microdeletions and none of the cases had duplication. The clinical findings and family histories of deletion 3 cases were available and are shared below:

Case of BRCA1 exon 18 and 19 deletion

The case was diagnosed as triple negative, invasive ductal carcinoma at age 44. She had one brother (42 years), one sister (24 years), one daughter (12 years), mother (68 years) and they were healthy. Her father had a diagnosis of lung cancer (68 years). The daughter of her maternal aunt had breast cancer (27 years).

Case of BRCA1 exon 20 deletion

The case was 50 years old and diagnosed as BRADS 5 by imaging at 47 years. Following right segmental mastectomy, the pathologic investigation revealed invasive ductal carcinoma. She had accompanying serous carcinoma of the right ovary and peritoneal metastasis. Three years later, after chemotherapy and radiotherapy, she had left-sided invasive ductal carcinoma. Her parents were consanguineous (2^{nd} degree). Her mother had the diagnosis of breast cancer at age 50 and endometrium cancer at 65. Her maternal grandfather died at 75 years due to skin tumor. The sister of her maternal grandmother died because of the tumor of the tongue at 60 years.

Case of BRCA2 exon 3 deletion

The case was admitted to hospital because of axillary lymphadenopathy. The biopsy showed ductal carcinoma *in situ* (solid, apocrine, intermediate type). Her mother died at 50 years due to breast cancer. Her maternal aunt was 50 years old and had breast cancer. The maternal uncle died at age 43 years due to pancreas cancer.

In our study group, the triple negative ratio was increased in cases with *BRCA1/2* pathogenic variants. In these cases, the histologic grade of the tumors was increased, the age of onset was decreased, and bilateral involvement was more common.

Non-BRCA *mutations*

The most frequent non-*BRCA* pathogenic variants included *CHEK2, ATM, PALB2, RAD50* gene variants. The pathogenic variants of *BRIP1, NBN, RAD51C, STK11,* and *VHL* genes were also identified (**Table 3**). Additionally, cases with *MLH1, MSH2,* and monoallelic *MUTYH* gene variants were also present which are involved in colorectal cancer pathogenesis. Two cases had more than one pathogenic variant: one of them had *BRCA2* and *MUTYH* genes and the other one had *CHEK2* and *MUTYH*(8 monoallelic pathogenic MUTYH variant carriers). Their clinical findings and family histories are shared below:

The case with BRCA2 and MUTYH pathogenic variation

The case was 53 years old. She had left mastectomy at 43 years. She was admitted due to recurrence at right side. Her history included diabetes and hypertension. Her brother and 2 sisters were healthy (aged 40–55 years). Her father had smoking history and died because of lung cancer at age 56. Her paternal uncle died at 70 years (lung cancer). Her mother was healthy (89 years). Her maternal aunt died at 85 years due to breast cancer, which was diagnosed at 70 years.

The case with CHEK2 and MUTYH pathogenic variation

The patient was 49 years old. She was diagnosed with invasive ductal carcinoma (2^{nd} degree) by tru-cut biopsy and right mastectomy. She had 3 sisters (45–53 years) and 1 brother (48 years). One of the sisters had the history of "total abdominal hysterectomy and bilateral salpingoophorectomy" but the reason is unknown; the sister had 3 healthy daughters and one healthy son.

Discussion

The frequency of breast cancer is more than 50/100,000 in our country and there were 22,500 new cases in year 2018 [1]. Majority of these cases were diagnosed in advanced stages, which shows the importance of early detection of breast cancer. Indeed, disease-causing mutations were identified in 17.8% (45 BRCA1/2 + 4 deletions of BRCA1/2 + 39 other genes)/495) of the study cohort. If they were screened before breast cancer diagnosis, clinical utility of the NGS test would be higher.

The median age at diagnosis was 44 years, which was less than the previous TFBDS (Turkish Federation of Breast Diseases Societies) report [1]. The localization of the breast cancer was bilateral in 6.8% (n=29) of the cases. The histopathology revealed invasive

ductal carcinoma in 74.8% (n=303) of the cases, which is comparable with the literature. The triple negative breast cancer cases constituted the 12% (n=44). Lymph node metastasis was positive in 45.4% (n=108). Distant metastasis was observed in 84.9% (n=186) **(Table 1)**.

The screening strategies, recommendations for follow-up and prophylaxis differ according to the mutant gene [35,36].

The proportion of "*BRCA1/2* associated hereditary breast and ovarian cancer" attributed to pathogenic variants in the gene was 66% for *BRCA1* and 34% for *BRCA2*. The pathogenic variants of these genes were identified by sequencing in more than 80% of the cases and MLPA analysis for deletions/duplications of the *BRCA1* and *BRCA2* genes determined in about 10% [37,38,39,40].

When the previous studies conducted in our country are reviewed, it seems difficult to make a generalization due to serious methodological differences **(Table 4)**. Methods used in studies published before 2018 [11,12,13,14,15,16,17,18,19,20,21,27] mainly focused on heteroduplex scanning followed by confirmation by Sanger sequencing [11,12,13,15,16,20,21,27]. Additionally, protein truncation test [11,13,14], denaturing gradient gel electrophoresis [14,17], MLPA [18,19], western blot [20] and restriction enzyme digestion-based methods [14] were used as well. Searching for selected exons in the *BRCA1* and *BRCA2* genes does not make it possible to see the whole picture. On the other hand, even when all coding exons and exon-intron junctions of *BRCA1* and *BRCA2* genes were examined with indirect mutation detection techniques, fewer sensitive results were obtained compared with direct sequencing.

In 2018 and later, the studies become primarily based on NGS. In addition to NGS, Sanger confirmation [22,25,26,28], bioinformatic copy number variation (CNV) analysis [13,29] and MLPA [22,25,26,29] was added in some publications. In another study, the relationship between *BRCA* variants and telomere lengths was investigated by quantitative PCR [28]. The rates of pathogenic variants in the *BRCA1* and *BRCA2* genes in non-NGS studies have been reported to vary between 4.5% and 20%. In NGS studies, positivity rates vary between 8.6% and 41.6%. In this study, the detection rate of pathogenic variants in *BRCA1* and *BRCA2* was 9.1% (45/495) when large genomic rearrangements were excluded. As expected, NGS yields higher positivity rates. Also as expected, positivity rates between 4.5% and 10.5% were reported in studies in which selected exons were evaluated [12,13,14,15], whereas in studies where all exons were evaluated [11,16,17,20,21,22,24,25,26,27,28,29,30] it was 5% to 41.6%. Therefore, investigating all coding regions of BRCA1/2 with direct sequencing should be the method of choice.

Patient selection criteria and pathogenicity evaluation algorithm of the obtained genetic data are two major factors that can affect the rate of positive results. When considering patient selection criteria, all studies from our country, including this study, are based on hereditary/familial cases in line with National Comprehensive Cancer Network (NCCN) guidelines. Choosing patients with at least one first degree affected relative not only improves the diagnostic rate but also modifies the risk in patients with pathogenic results as shown in CARRIERS study [41]. Only Bişgin et al. examined 129 index breast cancer cases regardless of family history [23]. They found a higher-than-expected pathogenic *BRCA1/2* variant rate of 14% [23]. However, three important limitations of their study exist. First, the parameters that may affect the results, such as the average age at diagnosis or triple negativity status were not provided, second the pathogenicity evaluation of detected variants was made only by in silico analysis. Lastly, it should be considered that ascertainment bias may alter the results conducted on cases referred to medical genetic department. In studies originating from Turkey, a population-screening study or an unselected screen of all breast cancer cases has not been reported yet. An important shortcoming in some published studies is the lack of information about the interpersonal kinship status. In only 7 of the 21 publications (12,18,21,22,26,27, this study), it was reported how many families the cases came from. Carrying the same haplotypes in investigated individuals may cause high false positivity, as well as high false negativity. Although when this information is not given specifically, the general consideration is that the evaluated cases are not related but the researchers' questioning and reporting of this situation will provide a more objective evaluation of the results.

The interpretation algorithm of the testing data is the second major factor that directly affects the results. In particular, the pathogenicity of variants detected in studies published before the 2015 guidelines of American College of Medical Genetics and Genomics (ACMG) are expressed as mutation or polymorphism [33]. Since this terminology and interpretation criteria are incompatible with today's classification algorithms, the positivity rates in some publications are open to discussion. For example, when all the studies in our country are considered, the study of Eyuboğlu et al. stands out with a variant detection rate of 41.6% as an extreme value [28]. Since the relationship between *BRCA* variants and telomere length was investigated in this publication, no definitive judgment was made about the clinical pathogenicity of detected variants. According to NCBI database 7 variants (12/113 cases – 10.6%) were reported as pathogenic [28]. It was also stated that according to SIFT predictions extra 8 variants or according to PROVEAN predictions extra 5 variants were estimated as damaging [28]. This example alone can emphasize how easily pathogenicity assessment can dramatically change positivity rates. In only four (18,19,22, this study) out of 21 studies, it was clearly stated that the variant classification was made according to ACMG 2015 criteria. In these publications (18,19,22, this study), mutation positivity rates for *BRCA1* and *BRCA2* genes were found to be 8.6%, 17.8%, 8.9% and 9.9% (49/495), respectively. These pathogenicity rates are very close to each other. Considering that some of the reported variants of uncertain significance will shift to the pathogenic class in the future, we think that the *BRCA1/2* pathogenic variation rate in hereditary/familial breast cancer cases in the Turkish population is approximately 10%. The reason for the relatively higher rate found in the study of Demir et al [25]. may be because of geographic differences, since that study included cases specific to the Thracian region. In particular, the *BRCA1* c.5266dupC mutation, which is especially common in Ashkenazi Jews, is the most common mutation detected in a large number of (5.4%) cases [25]. It was interpreted as a general hotspot mutation, but it may be a more common haplotype in the Thracian region of Turkey. Other studies also reported the c.5266dupC variant as a common variant, but the frequencies are somewhat lower [13,16,26]. It is the most common (2.2%) variant in an international publication including cases originating from Turkey [42]. In our serie, it is also the most common variant detected in 1% (5/495) of the cases.

Large genomic rearrangements (LGR) take a special place among other pathogenic variant types mainly because they cannot be detected by sequencing methods. MLPA is the choice of method to uncover this type of mutation. Recent advancements in bioinformatic data analysis are suggested as a new solution for delineating these mutations. In the study of Yazıcı et al., amplicon-based NGS analysis was studied in 741 cases and in these cases, CNV analysis was performed from bioinformatic data and later confirmed with MLPA [22]. Concordance or discordance ratio between bioinformatic CNV analysis and MLPA results was not reported [22]. Akçay et al. reported that del/dup estimation could be made with bioinformatics CNV analysis only for the *BRCA1* gene in more than 90% of their NGS cases [29]. The results were 100% concordant with MLPA in all cases where estimation could be made [29]. However, it has been reported that bioinformatics algorithms are insufficient for other genes and many false positive and false negative results are encountered [29]. For this reason, MLPA should still be used for screening and diagnostic purposes, not only for confirmation. Dele-

Table 4
Summary of studies determined BRCA1/2 mutations in hereditary and/or early-onset breast cancer patients from Turkey.

Year	Total Numberof Individuals	Total Number of Unrelated Families in Tested Individuals	Female / Male Count	Average age at testing	Family history (At least one affected first or second degree relative)	Early onset (≤45 years old with or without family history)	Triple Negative (%)	Cancer status in tested individuals	Experiment Method	Mutation scanning of BRCA1 gene	Mutation scanning of BRCA2 gene	Notes***	Mutation frequency	Reference
1999	Not provided (At least 17)	15	Not provided (At least 3 families had male BC individuals)	Not provided	12/15 (80%)	Age not provided in all patients (At least 12 families had early onset cancer individuals)	Not provided	BC, OC, healthy relatives	Protein Truncation Test for *BRCA1* Exon 11 and *BRCA2* Exon 10+11. Heteroduplex screening for remaining exons followed by Sanger sequencing.	All coding exons	All coding exons	-	BRCA1: 2/15 (13,3%) BRCA2: 1/15 (6,7%)	Balci et al. (1999)
2000	50	Not provided	40-10	40,7	13/50 (26%)	36/50 (72%)	Not provided	BC, BC+OC	Heteroduplex screening followed by Sanger sequencing and second confirmation in cloned plasmid sequencing.	Exon 2, 5, 10, 11, 13, 20, 24	Exon 11	-	BRCA1: 2/50 (4%) BRCA2: 2/50 (4%)	Ozdag et al. (2000)
2000	105	Not provided	Not provided	Not provided	53/105 (50,4%)	Age not provided in all patients (At least 52 patients had an onset ≤50 years)	Not provided	BC, OC, BC+OC	Protein Truncation Test for *BRCA1* Exon 11 and *BRCA2* Exon 10+11 in all patients. Sanger sequencing of remaining *BRCA1* exons in selected 24 patients. Heteroduplex screening for *BRCA1* Exon 2,14,20 in the remaining patients.	Exon 2, 11, 14, 20. (All exons in 24 patients)	Exon 10, 11	-	BRCA1: 9/105 (8,6%) BRCA2: 2/105 (1,9%)	Yazici et al. (2000)
2003	83	Not provided	73-10	46,4	Not provided in all patients (At least 25/83 patients had family history)	Age not provided in all patients (At least 18 patients had an onset ≤40 years)	Not provided	BC, OC, BC+OC	PCR+Restriction enzyme digestion analysis for four mutations (*BRCA1*; 185delAG, Tyr978X, 5382insC and *BRCA2*; 6174delT). Protein truncation test for *BRCA1* Exon 11. Denaturing gradient gel electrophoresis (DGGE) screening for *BRCA2* and confirmation with Sanger sequencing.	Three spesific mutations and Exon 2, 11	All coding exons	-	BRCA1: 2/83 (2,4%) BRCA2: 4/83 (4,8%)	Manguoglu et al. (2003)

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Table 4 (*continued*)

Year	Total Numberof Individuals	Total Number of Unrelated Families in Tested Individuals	Female / Male Count	Average age at testing	Family history (At least one affected first or second degree relative)	Early onset (≤45 years old with or without family history)	Triple Negative (%)	Cancer status in tested individuals	Experiment Method	Mutation scanning of BRCA1 gene	Mutation scanning of BRCA2 gene	Notes***	Mutation frequency	Reference
2005	Not provided	22	Not provided	Not provided	22/22 (100%)	Age not provided in all patients (At least 13 families had early onset cancer individuals ≤40 years)	Not provided	BC, OC, healthy relatives	Heteroduplex screening for 9 BRCA1 mutations (185delAG, 1294del40, 3452del4, 3875del4, 4184del4, 2312del5, 2800delAA, 3166ins5, 3600del11) and 4 BRCA2 mutations (3034/6del4, 5447del7, 5946/50delCT, 6503delTT)	Nine spesific mutations	Four spesific mutations	TP53, MLH1, NF1 screening in selected families	BRCA1: 1/22 (4,5%) BRCA2: 0/22 (0%)	Guran et al. (2005)
2006	87	38	87-0	44,6	87/87 (100%)	41/87 (47,1%)	Not provided	BC, OC, BC+OC, healthy relatives	Heteroduplex screening followed by Sanger sequencing in positive samples	All coding exons	All coding exons	-	BRCA1: 1/38 (2,6%) BRCA2: 3/38 (7,9%)	Egeli et al. (2006)
2010	156 (+50 controls)	Not provided	153-3	Not provided (Average age at diagnosis is 44,9 in 156 patients)	50/156 (32,1%)	Age not provided in all patients (At least 58 patients had an onset ≤40 years)	Not provided	BC, OC, BC+OC, Prostate Cancer, healthy controls	DGGE or denaturing high performance liquid chromatography screening for BRCA1 and BRCA2 genes and confirmation with Sanger sequencing in positive samples.	All coding exons	All coding exons	-	BRCA1: 3/156 (1,9%) BRCA2: 4/156 (2,6%) +26 unclassified variants (BRCA1: 13, BRCA2: 13)	Manguoglu et al. (2010)
2011	50	Not provided	47-3	Not provided	16/50 (32%)	Age not provided in all patients (At least 28 patients had an onset ≤40 years)	Not provided	BC, OC	MLPA analysis for BRCA1 / BRCA2 and a spesific probe for CHEK2 c.1100delC mutation	del&dup analysis in all exons	del&dup analysis in all exons	Possibly same cohort of the group's previous research [Manguoglu et al. (2010)] since it is stated that all participating patients were tested for BRCA1/ BRCA2 point mutations previously.	BRCA1: 0/50 (0%) BRCA2: 0/50 (0%)	Manguoglu E et al. (2011)

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Table 4 (*continued*)

Year	Total Numberof Individuals	Total Number of Unrelated Families in Tested Individuals	Female / Male Count	Average age at testing	Family history (At least one affected first or second degree relative)	Early onset (≤45 years old with or without family history)	Triple Negative (%)	Cancer status in tested individuals	Experiment Method	Mutation scanning of BRCA1 gene	Mutation scanning of BRCA2 gene	Notes***	Mutation frequency	Reference
2011	211	Not provided	211-0	50,7	46/208 (21,6%)	Age not provided	56/211 (26,5%)	BC	MLPA analysis for BRCA1 (208 patients) and BRCA2 (185 patients)	del&dup analysis in all exons	del&dup analysis in all exons	-	BRCA1: 4/208 (1,9%) BRCA2: 0/185 (0%)	Aydin F et al. (2011)
2014	117	Not provided	117-0	42,5	56/117 (47,9%)	90/117 (76,9% of the patients had an onset ≤50 years)	33/117 (28,2%)	BC	Heteroduplex screening followed by Sanger sequencing in positive samples. In silico analyses and Western blotting to support pathogenicity evaluation.	All coding exons	All coding exons	-	BRCA1: 16/117 (13,7%) BRCA2: 0/117 (0%) +25 unclassified variants (BRCA1: 9, BRCA2: 16)	Cecener et al. (2014)
2016	223 (+60 controls)	Not provided	223-0	42,6	186/223 (83,4%)	223/223 (100% of the patients had an onset ≤50 years)	42/223 (18,4%)	BC, healthy controls	Heteroduplex screening followed by Sanger sequencing of *PALB2* gene in positive samples. In silico analyses to support pathogenicity evaluation.	All coding exons	All coding exons	BRCA1 and BRCA2 negative patients were selected from group's previous research cohorts [Egeli et al. (2006) and Cecener et al. (2014)].	PALB2: 9/223 (4,0%) +11 unclassified variants	Cecener et al. (2016)
2018	1809 (+125 controls)	Not provided	1769-40	Not provided (Average age at diagnosis is 41,9 in 1809 patients)	Not provided in all patients (All 25/25 del&dup carriers had family history)	Age not provided in all patients (At least 17/25 of del&dup carriers had an onset ≤45 years)	272/1473 (18,5%)	BC, OC, BC+OC, BC or OC with other types of cancer, healthy controls	Sanger sequencing for *BRCA1* and *BRCA2* in 1785 patients. Also NGS analysis for both genes in 741 patients. MLPA and CNV analysis in NGS data to search for deletions and duplication in 1262 patients. Confirmation with different test/probe sets.	All coding exons + del&dup analysis	All coding exons + del&dup analysis	Mutation identities were not disclosed in this study apart from del&dup's!	Overall mutation rate: 293/1785 (17%) *BRCA1/2* difference were not disclosed!	Yazıcı H. et al (2018)

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Table 4 (*continued*)

Year	Total Numberof Individuals	Total Number of Unrelated Families in Tested Individuals	Female / Male Count	Average age at testing	Family history (At least one affected first or second degree relative)	Early onset (≤45 years old with or without family history)	Triple Negative (%)	Cancer status in tested individuals	Experiment Method	Mutation scanning of BRCA1 gene	Mutation scanning of BRCA2 gene	Notes***	Mutation frequency	Reference
2019	129	Not provided	129-0	Not provided	This study selected patients without regard of family history!	Age not provided	Not provided	BC	Amplicon based NGS analysis for *BRCA1* and *BRCA2*	All coding exons	All coding exons	-	BRCA1: 7/129 (5,4%) BRCA2: 11/129 (8,5%)	Bisgin A et al. (2019)
2019	99	Not provided	99-0	45,6	29/99 (29,3%)	Age not provided in all patients (At least 41 patients had an onset ≤40 years)	33/99 (33,3%)	BC, BC+OC	NGS analysis for *BRCA1* and *BRCA2* (Method details were not provided)	All coding exons	All coding exons	-	BRCA1: 11/99 (11,1%) BRCA2: 8/99 (8,1%)	Geredeli C et al. (2019)
2019	1419	Not provided	Not provided	Not provided	Not provided	Not provided	Not provided	Not provided	Amplicon based NGS analysis for *BRCA1* and *BRCA2*. Sanger validation for homopolymer regions, low quality variants, insertions and/or deletions, splice site alterations and novel variants MLPA analysis for both genes in NGS negative patients.	All coding exons + del&dup analysis	All coding exons + del&dup analysis	A total of 64 VUS were reported in 91/1419 (6,4%) individuals in this paper. Large del&dup's identities were not disclosed in this study!	BRCA1: 58/1419 (4,1%) BRCA2: 64/1419 (4,5%) +17/1419 patients with pathogenic del&dup variants (1,2%)	Bahsi T et al. (2020)
2020	493	493	485-8	46,5	214/493 (43,4%)	213/493 (43,2%)	26/493 (5,3%)	BC, OC, BC+OC, other types of cancer, healthy relatives	Amplicon and Target Capture based NGS analysis for *BRCA1* and *BRCA2* combined with MLPA analysis to search for deletions and duplications. Confirmation with Sanger sequencing in positive samples.	All coding exons + del&dup analysis	All coding exons + del&dup analysis	-	BRCA1: 58/493 (11,8%) BRCA2: 30/493 (6,1%)	Demir S et al. (2020)

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Table 4 (*continued*)

Year	Total Numberof Individuals	Total Number of Unrelated Families in Tested Individuals	Female / Male Count	Average age at testing	Family history (At least one affected first or second degree relative)	Early onset (≤45 years old with or without family history)	Triple Negative (%)	Cancer status in tested individuals	Experiment Method	Mutation scanning of BRCA1 gene	Mutation scanning of BRCA2 gene	Notes***	Mutation frequency	Reference
2020	113	113	113-0	Not provided	50/113 (44,2%)	Age not provided in all patients (At least 60 patients had an onset ≤50 years)	Not provided	BC, BC+OC, healthy relatives	Amplicon based NGS analysis for *BRCA1* and *BRCA2*. Sanger confirmation in positive samples. Telomere length measurement with quantitative polymerase chain reaction.	All coding exons	All coding exons		BRCA1: 18/113 (15,9%) BRCA2: 30/113 (26,5%) 1 patient has variants in both genes	Eyuboglu İP et al. (2020)
2020	603	Not provided	596-7	Not provided (Average age at diagnosis is 44 in 603 patients)	Not provided in all patients (At least 24/30 of mutation carriers had family history)	Age not provided in all patients (At least 213 patients had an onset ≤40 years)	77/603 (12,8%)	BC	Heteroduplex screening followed by Sanger sequencing in positive samples.	All coding exons	All coding exons	-	BRCA1: 15/603 (2,5%) BRCA2: 15/603 (2,5%)	Cecener G et al. (2020)
2021	302	Not provided	297-5	44,2	108/302 (35,8%)	Age not provided in all patients (At least 134 patients had an onset ≤40 years)	107/302 (35,4%)	BC	NGS analysis for *BRCA1* and *BRCA2* (Method details were not provided)	All coding exons	All coding exons	Mutation identities were not disclosed in this study!	BRCA1: 41/302 (13,6%) BRCA2: 37/302 (12,3%)	Atcı MM et al. (2021)

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Table 4 (*continued*)

Year	Total Numberof Individuals	Total Number of Unrelated Families in Tested Individuals	Female / Male Count	Average age at testing	Family history (At least one affected first or second degree relative)	Early onset (≤45 years old with or without family history)	Triple Negative (%)	Cancer status in tested individuals	Experiment Method	Mutation scanning of BRCA1 gene	Mutation scanning of BRCA2 gene	Notes***	Mutation frequency	Reference
2021	917 (+490 controls)	1407	720-12 (BC cases)	Not provided	189/732 (25,8%)	Age not provided in all patients (At least 211 patients had an onset ≤40 years)	68/732 (9,3%)	BC, BC+OC, CRC, BC+CRC, healthy controls	Target capture based NGS analysis for APC, ATM, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, MLH1, MSH2, MSH6, PMS2, MUTYH, NBN, PALB2, PTEN, RAD51C, RAD51D, STK11, TP53 genes in all patients. BARD1, MRE11, ABRAXAS1, XRCC2, RAD50 and PIK3CA analysis in a subset of patients. MLPA analysis of BRCA1, BRCA2 in 60% of BC patients. Additional MLPA testing for MLH1, MSH2, MSH6, EPCAM, PMS2, APC, CDH1, STK11 in a subset of patients when NGS CNV analysis point to a possible variation.	All coding exons + del&dup analysis (in a subset of patients)	All coding exons + del&dup analysis (in a subset of patients)	A total of 404 VUS were reported in 487/1407 (34,6%) individuals in this paper. Pathogenic results in colorectal carcinoma patients are not shown here.	BRCA1: 44/732 (6,0%) BRCA2: 21/732 (2,9%) CHEK2: 11/732 (1,5%) PALB2: 9/732 (1,2%) ATM: 6/732 (0,8%) TP53: 3/732 (0,4%) BRIP1, PMS2, RAD51C: 2/732 (0,3%) CDH1, MLH1, MRE11, MSH6, MUTYH, NBN, RAD50, STK11, XRCC2: 1/732 (0,1%)	Akcay IM et al. (2021)

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Table 4 (*continued*)

Year	Total Numberof Individuals	Total Number of Unrelated Families in Tested Individuals	Female / Male Count	Average age at testing	Family history (At least one affected first or second degree relative)	Early onset (≤45 years old with or without family history)	Triple Negative (%)	Cancer status in tested individuals	Experiment Method	Mutation scanning of BRCA1 gene	Mutation scanning of BRCA2 gene	Notes***	Mutation frequency	Reference
2021	495	495	493-2	47	139/286 (48,6%) Family history not available / known or doubtfully negative in the remaining 209 patients	274/495 (55,4%)	44/367 (12,0%)	BC, BC+ secondary malignancies in sporadic individuals (OC, CRC, endometrium cancer, papillary thyroid, skin cancer, ewing sarcoma, urothelial cancer, renal cell cancer)	Amplicon based NGS analysis for BRCA1 and BRCA2 genes in 162 individuals. Target capture based NGS analysis for APC, ATM, BARD1, BLM, BMPR1A, BRCA1, BRCA2, BRIP1, CDH1, CDK4, CDKN2A, CHEK2, EPCAM, MLH1, MRE11A, MSH2, MSH6, MUTYH, NBN, PALB2, PRSS1, PTEN, RAD50, RAD51C, RAD51D, SLX4, SMAD4, STK11, TP53 and VHL genes in 419 individuals. MLPA analysis of BRCA1, BRCA2 in 438 individuals.	All coding exons + del&dup analysis	All coding exons + del&dup analysis	A total of 254 VUS were found in 197/419 (47,0%) individuals in our study. ***Variant classification according to the current ACMG guidelines were not performed in most of the papers. Therefore positive rates are subject to debate.	BRCA1: 25/495 (5,1%) BRCA2: 24/495 (4,8%) CHEK2: 18/419 (4,3%) MUTYH: 6/419 (1,4%) ATM: 4/419 (1,0%) PALB2, RAD50: 2/419 (0,5%) BRIP1, MLH1, MSH2, NBN, RAD51C, STK11, VHL: 1/419 (0,2%)	This study (All data available upon request)

tions and duplications were investigated with the MLPA method in a total of seven studies (15,18,19,22,29,30,this study). Manguoglu et al. did not detect any LGR in 50 cases that are wild type in prior *BRCA1/2* sequencing [18]. Aydın et al [19]. detected LGR in the BRCA1 gene in 1.9% of cases, Yazıcı et al [22]. in 2% of cases, Demir et al [25]. in 0.2% of cases, and Akçay et al [29]. in 2.6% of cases. The striking point common to all of these studies is that although they consist of large case series, no LGR originating from the *BRCA2* gene has ever been reported. In the publication of Bahsi et al., del/dup was detected in 1.2% of cases, but mutation localizations were not reported [26]. To our knowledge we report the first LGR in *BRCA2* gene from Turkey which is a deletion of exon 3 in this study. In total we found large genomic rearrangements in 4/495 (0.8%) patients. Therefore, it is reasonable to assume that LGRs of *BRCA2* gene are exceedingly rare in Turkey and LGRs of *BRCA1* gene can be seen in around 2% of hereditary/familial breast cancer cases. Compared with the abovementioned 10% pathogenic results from sequencing studies, MLPA should be performed as a second step in such patients. Deletions involving *BRCA1* exon 18 (17-18 deletion or 18-19 deletion) are the most frequent of large genomic rearrangements and reported from individuals of northern Black Sea region in Turkey [19,22,29].

Advances in the understanding of pathophysiologic relationships of non-*BRCA1/2* genes in hereditary/familial breast cancers give rise to the need to expand the number of tested genes in clinical care. The results of a recent population-based study from the United Kingdom involving more than 60,000 patients has shown significantly higher risk for individuals with protein truncating variants in 9 genes (*ATM, BARD1, BRCA1, BRCA2, CHEK2, PALB2, RAD51C, RAD51D* and *TP53*) for breast cancer [43]. Also, missense variants in *ATM, BRCA1, BRCA2, CHEK2* and *TP53* are also associated with clinically significant increased lifetime risk [43]. Another large population-based study from United States involving more than 60,000 patients has also shown similar results with increased risk of breast cancer if the patient carries pathogenic variants of *ATM, BARD1, BRCA1, BRCA2, CDH1, CHEK2, NF1, PALB2, RAD51C* and *RAD51D* [41]. Our panel here includes all these genes except *NF1* and furthermore several known genes with distinct cancer syndromes involving breast cancer such as *STK11* and *PTEN*. From the pre-NGS studies of Turkey, only one study investigated the *PALB2* gene [21]. This is partly because of the relationship between non-*BRCA* genes and breast cancer has not been clearly defined in the world literature at that time and partly due to the difficulties and cost of applying genetic testing before NGS. However, more interestingly, a cancer susceptibility multigene panel including 25 genes was applied in only one study in the post-NGS period [29]. That study included colorectal cancer cases as well as breast cancer cases. Specific to breast cancer cases, pathogenic variant outcomes were reported in 6% of cases in other susceptibility genes (plus 8.9% from *BRCA1* and *BRCA2* which is discussed above). VUS was also detected in 34.6% of all cases. In our study, a panel-containing 30 genes was applied, and the non-*BRCA1/2* pathogenic variant was detected in 9.3% (39/419) of the cases. When the *MLH1, MSH2, MUTYH* variants that may not be associated with breast cancer pathogenesis are excluded from these variants (8 variants), the rate decreases to 7.4% (plus 9.9% from *BRCA1* and *BRCA2*). Additionally, various numbers of VUS variants were detected in 47% of cases. As expected, as the number of genes being studied increases, the detected pathogenic variant ratio and uncertain variant ratio increase in parallel. In the study of Tsaoussis et al., which reflects the results of two multigene panels (25 and 33 genes), a high pathogenic variant rate of 22.1% was reported (15.8% in a subset of 158 cases originating from Turkey, including *BRCA1* and *BRCA2* mutations) even though patient selection criteria were not strict since this study reflects patients investigated in a private diagnostic laboratory [42]. Additionally, variants classified as VUS were detected in 34.8% of cases. In other words, even in narrow NGS panels containing 25, 26, 30, or 33 genes, the need for genetic counseling and follow-up arises in more than 50% of cases. When the three aforementioned studies are evaluated together, it is obvious that the search for medium/high risk group genes that predispose to breast cancer increases the genetic diagnostic rate considerably.

With these results it is our belief that NGS panels involving *BRCA1, BRCA2,* and other moderate/high risk breast cancer genes should be the first step of evaluation in hereditary/familial breast cancer patients and complemented with second step MLPA analysis of *BRCA* genes in patients with negative results. On the other hand, this strategy creates inconclusive results in 30–50% of patients who are aligned with the literature. Total number of variants of uncertain significance can only be reduced with more genetic testing/research and implementation of genetic knowledge with clinical data. Variant by variant research are needed because in biology there are always exceptions. For example, in Breast Cancer Association Consortium's research, missense variants that were interpreted as pathogenic according to ACMG criteria increased breast cancer risk, whereas those that were not classified as pathogenic / likely pathogenic did not cause a statistically significant clinical risk [43]. This finding shows that not every missense variant is equal in terms of phenotypic outcome, and how efficient the standard criteria are in distinguishing said variants. Another study by Richardson et al., demonstrated the impact of validated functional assays in variant classification. When functional assays were used 86% of uncertain variants were reclassified either as likely pathogenic or likely benign [44]. Therefore, functional research should be planned in patients with inconclusive results. They must be informed by genetic counselors so that they fully understand their biological conditions. It is also of paramount importance that these cases be followed by a multidisciplinary cancer team involving specialists of medical genetics, oncology, radiology, pathology, and other related physicians. Over time, testing for medium/low risk genes will increase through the demonstration of new pathophysiological effects and the development of polygenic risk scores so wide gene panels will be applied. Therefore, the need for genetic counseling will grow evermore. Clinical utility should be top priority in devising these testing algorithms and a more prospective randomized clinical research is needed in that matter.

As a summary, the pathogenic variants were found in 17.8% of the cases and the pathogenic *BRCA1/2* genes were the most affected ones (9.9%). 4 novel, pathogenic variants of *BRCA1* and *BRCA2* are identified. Four of the cases have deletion of *BRCA1/2* genes in MLPA analysis.

Conclusion

Cost effectiveness of gene panel approach is demonstrated by our study. So, instead of stepwise approach, we suggest that the breast cancer cases with an indication for genetic etiology testing should be investigated directly using oncorisk gene panels. The distribution pathogenic variants among our population was described.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Elcin Bora: Conceptualization, Resources, Writing – original draft, Writing – review & editing. **Ahmet Okay Caglayan:** Conceptualization, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Altug Koc:** Investigation, Data curation, Writing – review & editing. **Tufan Cankaya:** Resources, Writing – review & editing. **Hande Ozkalayci:**

Data curation, Writing – review & editing. **Mehmet Kocabey:** Data curation, Writing – review & editing. **Demet Kemer:** Data curation, Writing – review & editing. **Suleyman Aksoy:** Resources, Writing – review & editing. **Zumre Arican Alicikus:** Resources, Writing – review & editing. **Isil Basara Akin:** Resources, Writing – review & editing. **Merih Guray Durak:** Resources, Writing – review & editing. **Duygu Gurel:** Resources, Writing – review & editing. **Tugba Yavuzsen:** Resources, Writing – review & editing. **Ali Sevinc:** Resources, Writing – review & editing. **Isil Somali:** Resources, Writing – review & editing. **Ilknur Gorken:** Resources, Writing – review & editing. **Pinar Balci:** Resources, Writing – review & editing. **Aziz Karaoglu:** Resources, Writing – review & editing. **Serdar Saydam:** Resources, Writing – review & editing. **Ayfer Ulgenalp:** Conceptualization, Resources, Investigation, Writing – review & editing, Supervision.

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