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Identification of *CHEK2* Germline Mutations in *BRCA1/2*- and *PALB2*-Negative Breast and Ovarian Cancer Patients

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CHEK2 gene · Early-onset breast cancer · c.1103A>G · Variant of uncertain significance

Abstract
Introduction: The *CHEK2* gene is known to be an important signal transducer involved in DNA repair, apoptosis, or cell cycle arrest in response to DNA damage. The mutations in this gene have been associated with a wide range of cancers, both sporadic and hereditary. Germline *CHEK2* mutations are linked to an increased risk of breast cancer. Therefore, the aim of this study was to identify the prevalence of *CHEK2* variants in *BRCA1/2*- and *PALB2*-negative early-onset patients with breast cancer and/or ovarian cancer in a Turkish population for the first time. ***Methods:*** The study included 95 patients with *BRCA1/2*- and *PALB2*-negative early-onset breast cancer and/or ovarian cancer and also 60 unaffected women. All the intron/exon boundaries and coding exons of *CHEK2* were subjected to mutational analysis by heteroduplex analysis and DNA sequencing. ***Results:*** A total of 16 *CHEK2* variants were found in breast cancer patients within the Turkish population. *CHEK2* c.1100delC mutation most frequently studied in the *CHEK2* gene was not detected in our study. The prevalence of variants of uncertain significance in *CHEK2* was found to be 7.3% ($n = 7$) in *BRCA1/2* and *PALB2* mutation-negative Turkish patients with early-onset breast and/or ovarian cancer. ***Conclusion:*** The present study may shed light on alternative variations that could be significant for understanding the prevalence and clinical suitability of the *CHEK2* gene.

Introduction

Breast cancer is a global health problem and one of the most prevalent types of malignancy among females in the world [1, 2]. Although it has a high prevalence, only 5–10% of all cases are due to the inheritance of high-penetrance cancer susceptibility genes. Germline mutations in two major tumor suppressor genes, *BRCA1* and *BRCA2*, which are associated with both hereditary breast and ovarian cancer, cause genetic susceptibility [2]. Many studies have reported evidence that germline mutations in other susceptibility genes, such as *PTEN*, *STK11*, *CDH1*, *ATM*, *TP53*, *PALB2*, and *CHEK2*, also lead to breast cancer risk [3–5]. Genetic mutation of Checkpoint kinase 2 (*CHEK2*) functions as a multiorgan cancer susceptibility gene contributing to the development of nu-

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merous cancers, including colorectal, prostate, thyroid, kidney, ovarian, and breast. Downregulation of *CHEK2* protein expression has also been observed in these types of cancer [6, 7]. *CHEK2* is a serine-threonine kinase activated by the *ATM* protein in double-strand breaks in DNA and plays an important role in DNA repair. Activated *CHEK2* is a tumor suppressor gene that inhibits the cell cycle by phosphorylating critical cell cycle proteins, including p53, Cdc25C, Cdc25A, and BRCA1, and blocks carcinogenesis and cell transformation by promoting the activation of DNA repair [7–9]. Germline mutations in *CHEK2* – more specifically, the c.1100delC mutation carriers – have a significantly increased risk of bilateral and contralateral breast cancer. *CHEK2* c.1100delC is known as a pathogenic moderate-risk mutation because of the estimated two-fold increased risk of breast cancer in women with *CHEK2* c.1100delC [7]. Although it is observed in 5% of the non-*BRCA1/BRCA2* breast cancer families, *CHEK2* c.1100delC is present in only 1% of the general population [10]. The distribution of the *CHEK2* c.1100delC allele shows wide geographical variation, and its frequency differs in different populations. In Northern Europe, this allele is observed to be more prevalent, while in Southern Europe it is rare and sometimes not observed at all [11, 12]. There is no known study investigating all coding regions of the *CHEK2* gene in Turkish breast and/or ovarian cancer patients. In this study, we sequenced the coding regions of the *CHEK2* gene in 95 Turkish patients with *BRCA1/2*- and *PALB2*-negative breast/ovarian cancer and in unaffected controls for the first time. In this way, we performed a comprehensive review of *CHEK2* germline mutations and evaluated the spectrum, prevalence, and clinical suitability of *CHEK2* germline mutations in this cohort.

Table 1. Summary of demographic, clinical, and pathological characteristics of breast cancer patients

Mean age, years	42.7
Range	24–50
Family history, *n* (%)	
Positive	69 (72.6)
Negative	26 (27.3)
Invasive tumor type, *n* (%)	
Invasive ductal carcinoma	72 (75.7)
Other	23 (24.2)
Histological grade, *n* (%)	
I	14 (14.7)
II	31 (32.6)
III	23 (24.2)
IV	3 (3.1)
Localization, *n* (%)	
Left	32 (33.6)
Right	45 (47.4)
Bilateral	7 (7.4)
Tumor size, *n* (%)	
≤2 cm	50 (52.6)
>2 cm	45 (47.4)
Estrogen receptor, *n* (%)	
Positive	57 (60.0)
Negative	38 (40.0)
Progesterone receptor, *n* (%)	
Positive	48 (50.5)
Negative	47 (49.5)
c-erbB-2 expression, *n* (%)	
Positive	23 (24.2)
Negative	55 (57.8)
Triple negative patients	17 (18.0)
In situ component, *n* (%)	
<25%	32 (33.6)
>25%	29 (30.5)
Ki67, *n* (%)	
≥20%	36 (37.9)
<20%	63 (62.1)
Metastasis, *n* (%)	
Positive	27 (28.4)
Negative	68 (671.6)

Materials and Methods

Patient Selection

The study involved 95 women with breast or ovarian cancer who were diagnosed before 50 years of age and known to not carry mutations in the *BRCA1*, *BRCA2*, and *PALB2* genes. Our control group consisted of 60 people without breast cancer and without a family history of breast cancer until the time of the study period, which continued through 2017. *BRCA1*, *BRCA2*, and *PALB2* mutations for each case and controls had been previously identified by Sanger sequencing [5, 13, 14]. The blood samples of the study were collected from the General Surgery Department of the Medical Faculty at Bursa Uludag University in Bursa, Turkey, and clinical/genetic data in the study participants were analyzed at the Medical Biology Department of the University. The demographic features of the clinical data of the study cases are provided in Table 1. This study was approved by the Local Ethics Committee (2015-4/5) of Uludag University and conforms to the ethical standards of the Declaration of Helsinki. All participants were informed and gave their written informed consent.

Mutation Analysis of the CHEK2 Gene

Polymerase Chain Reaction Analysis

Genomic DNA was isolated from peripheral blood using standard kit procedures (Omega Bio-Tek, USA). The quantity and quality of DNA samples were determined by UV absorbance using a NanoDrop spectrophotometer (Beckman Coulter, ABD). The entire coding sequence of the *CHEK2* gene (OMIM: 604373; Transcript: ENST00000404276.5) and the flanking intron boundaries were analyzed by polymerase chain reaction (PCR). A 25-μL reaction mixture was used for PCR, and this volume contained 0.05 mM of each deoxyribonucleoside triphosphate (dNTP-Genetbio

Table 2. *CHEK2* PCR condition: primer sequences, annealing temperature, and gene fragment size (ENST00000404276.5)

Exons	Product size, bp	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Annealing temperature, °C
1	430	CTCACCTTTGTTGTTGGACAC	CCACCTGGTAATACAACTTTCTG	65
2.Oca	586	TCTGATTGCCTTCTTAGGCT	CCATATTCTGTAAGGACAGGAC	65
4	397	TCAGTGATCGCCTCTTGTGA	GGGTCTTACTGTAGCCCTGG	58
5	353	GTCCTCTGCAAACAGGGACAA	CTAAGCAGGGGGTTATTCCTGAG	58
6	453	AGTAGAGCTGGGTTTGGAACT	AGCTAGGCATGTGTGTGAATG	56
7	239	TCCCTCTGGGCAGATGTTCTA	GGATGAGAAAGGCAAGCCTACAT	60
8	364	TCCAACTGGGACATCTGATTGTG	CCACGGTCCCTCGATTTCTG	65
9	331	CTGTGAGATGTGTGTGTTGGTAAC	TCTGGATAAGAGCAGTATCACCTG	65
10	288	TACGTGTCTTCTTGGACTGGC	CTCCTACCAGTCTGTGCAGC	60
11	325	CTGAATGCCACTGAGAATGCCA	CCAGTGCCTCTCAAATGGTGT	60
12	254	CTGCTCTTTCTGAATGGTGGC	CCTCTGTCTCATGTCTCTCAGG	60
13	363	ATGTGGATGTGAGTCAGCCAG	ATCAGCTCCTTAAGCCCAGACTA	65
14	386	ATGCCCCCACTTTACTGGAA	GCCATTCAACTAAAGAACCGAT	65

G-9000, Korea), 10 pmol of each primer, 1 unit of HS Prime Taq DNA Polymerase (Genetbio G-7000, Korea), and 100 ng of genomic DNA. Primer sequences and PCR annealing temperatures are shown in Table 2. The cycling profile included an initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 55–68°C (temperature set according to primer base composition) for 30 s, and elongation at 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were visualized under UV transilluminator after the electrophoresis process on 2% agarose gel stained with ethidium bromide.

Heteroduplex Analysis

The amplified products were assessed with heteroduplex analysis (HDA). For the analysis, 20 μL of each PCR product was heat-denatured at 95°C for 6 min, then cooled at 50°C for 10 min, 37°C for 15 min, and 20°C for 30 min, respectively. Next, 3 μL of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added, and then the mixture was loaded into the gel. Four microliters of each processed PCR product were electrophoresed on 1× MDE gel (BMA Rocland, ME, USA). These products were run at 900 V for 17–21 h at room temperature in 0.5× TBE buffer solution (89 mM tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.0). The gels were stained using a nonradioactive silver staining method. After the process, the results were photographed and visualized with an imaging analyzer (Vilber Laurmat, Marne La Valle, France). Samples showing one or two bands separated from wild-type bands were identified as HDA-positive. All samples containing the mutation were subjected to the HDA analysis procedure three times to prevent contamination ($n = 3$).

Sanger Sequencing Analysis

HDA-positive PCR products were purified using an E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, USA) to carry out a Sanger sequencing analysis, and sequencing reactions were performed using a PCR product sequencing kit (DTCS, Quick Start Mix-M010812, USA) in accordance with the protocol. As a final step, the samples were analyzed on a Beckman Coulter Automated Sequencer using a CEQ-8000 Automated DNA Sequencing System (Beckman Coulter, Inc., Fullerton, CA, USA). To confirm whether these mutations potentially affected the structure and splicing ability of *CHEK2*, the results of the sequencing analysis were evaluated using the following web-based programs: the Ensemble Genome Browser (http://www.ensembl.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/all.php), the Leiden Open Variation Database (LOVD) (http://www.lovd.nl/3.0/home), and the Human Genome Variation Society Database (http://www.hgvs.org/dblist/glsdb.html).

In silico Analysis

Briefly, amino acid substitutions were identified with PROVEAN (http://provean.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), and Align Grantham Variation with Grantham Deviation (Align-GVGD) (http://agvgd.iarc.fr/) tools to determine the potential consequences of missense variants on protein function. The prior probability of pathogenicity was determined for each variant of uncertain significance (VUS) according to the Align-GVGD grades (C0, C15, C25, C35, C45, C55, and C65), C65 (most likely deleterious) to C0 (most likely neutral), and the class ≥C15 (probably damaging) was considered the threshold for deleterious variants in Align-GVGD (Table 3). The mutation taster (http://www.mutationtaster.org/) and MUpro (https://www.ics.uci.edu/~baldig/mutation.html) programs were used to evaluate protein stability. The 3D structural changes in the C-terminal region in *CHEK2* were visualized in SWISS-MODEL. The transcript number of the *CHEK2* gene is ENST00000404276.5.

Statistical Analysis

SPSS software package, version 20.0 (SPSS Inc., USA), was used to analyze the outcomes statistically. To have multiple comparisons, the identified variants were carried out by correlation test and χ^2 test (both tests are two-sided). The result ($p < 0.05$) was statistically significant.

Table 3. Results of in silico analysis for *CHEK2* variants

P	Protein change	SIFT	Align GVGDa	Polyphen	LOVD	Ensembl	Clinical significance
c.463T>C	p.Ser155Pro	Tolerated (0.06)	C0	Possibly damaging (0.515)	–	–	–
c.1067C>T	p.Ser356Leu	Deleterious (0.01)	C15	Possibly damaging (0.832)	–	+	Uncertain significance
c.1103A>G	p.Asp368Gly	Deleterious (0.00)	C65	Probably damaging (1)	–	+	Uncertain significance
c.1169A>G	p.Tyr390Cys	Deleterious (0.00)	C65	Probably damaging (0.977)	–	+	Likely pathogenic; uncertain significance
c.1176G>T	p.Ala392=	NA	NA	NA	–	+	Likely benign
c.1193C>G	p.Ser398Cys	Deleterious (0.006)	C15	Probably damaging (0.956)	–	–	–
c.1314C>T	p.Asp438=	NA	NA	NA	–	–	–
c.1333T>C	p.Tyr445His	Deleterious (0.00)	C25	Probably damaging (0.965)	–	+	Uncertain significance
c.1348G>A	p.Glu450Lys	Tolerated (0.553)	C0	Benign (0.003)	–	+	Uncertain significance
c.1363G>A	p.Val455Ile	Tolerated (0.232)	C25	Benign (0.048)	–	–	–
c.1420C>T	p.Arg474Cys	Deleterious (0.00)	C65	Probably damaging (1)	–	+	Uncertain significance
c.1561C>T	p.Arg521Trp	Deleterious (0.00)	C65	Probably damaging (0.998)	–	+	Uncertain significance
c.1566C>T	p.Pro522=	NA	NA	NA	–	+	Likely benign; uncertain significance
c.1573G>A	p.Gly525Arg	Tolerated (0.15)	C0	Benign (0.021)	–	+	Uncertain significance
c.1608A>G	p.Pro536=	NA	NA	NA	–	+	Likely benign
c.18C>T	_	NA	NA	NA	–	+	Benign; likely benign; uncertain significance

[a] On Align GVGD, C65 represents highest and C0 lowest genetic risk. Predictions were derived from the human *CHEK2* alignment available on the website at the Zebrafish depth.

Table 4. *CHEK2* sequence variants identified in 95 Turkish breast/ovarian cancer patients

Localization	rs Number	Variant type	Nucleotide change	Amino acid change	Percentage of carriers ($n = 95$)	Control group ($n = 60$)
E3	rs1175278074	Missense	c.463T>C	p.Ser155Pro	1	–
E9	rs121908703	Missense	c.1067C>T	p.Ser356Leu	1	1
	rs1555913929	Missense	c.1103A>G	p.Asp368Gly	1	–
E10	rs200928781	Missense	c.1169A>G	p.Tyr390Cys	3	1
	rs142692907	Synonymous	c.1176G>T	p.Ala392=	2	–
	_	Missense	c.1193C>G*	p.Ser398Cys	1	–
	_	Synonymous	c.1314C>T*	p.Asp438=	1	–
E11	rs587778194	Missense	c.1333T>C	p.Tyr445His	3	1
	rs1555913429	Missense Missense	c.1348G>A	p.Glu450Lys	3	–
	_	Missense	c.1363G>A*	p.Val455Ile	3	–
E12	rs540635787	Missense	c.1420C>T	p.Arg474Cys	1	1
E14	rs533475838	Synonymous	c.1561C>T	p.Arg521Trp	1	–
	rs202104749	Missense	c.1566C>T	p.Pro522=	3	1
	rs780512032	Synonymous	c.1573G>A	p.Gly525Arg	1	–
	rs17886242	3′-UTR variant	c.1608A>G	p.Pro536=	3	1
	rs17884403		c.18C>T	_	3	2

* These variants have not been previously reported.

Results

Patients' Characteristics

A total of 91 Turkish patients with early-onset breast cancer and 4 breast and/or ovarian cancer patients were enrolled in our study. The age range for breast cancer patients was 24–50 years. General characteristics of the patients, categorized as tumor type, histological grade, tumor location and size, in situ components, Ki67 level, metastasis, and invasion, are summarized in Table 1. In

Table 5. Clinical features of patients according to the identified *CHEK2* variants

Nucleotide change	Age at diagnosis, years	Grade	First-degree family history	Second-degree family history	Third-degree family history	ER	PR	HER2	Localization of cancer
c.463T>C	49	2	+	+	–	–	–	+	Breast
c.1067C>T	29	3	–	+	+	+	+	–	Breast
c.1103A>G	41	3	+	–	+	un	un	un	Ovarian
c.1169A>G	41	3	+	–	+	un	un	un	Ovarian
	32	3	+	+	+	un	un	un	Breast
	35	3	–	+	–	–	–	–	Breast
c.1176G>T	32	3	+	+	+	un	un	un	Breast
	35	3	–	+	–	–	–	–	Breast
c.1193C>G	32	3	+	+	+	un	un	un	Breast
c.1314C>T	39	2	–	–	–	+	+	–	Breast
c.1333T>C	54	un	+	–	–	+	+	–	Breast
	38	2	–	–	–	+	+	–	Breast
	41	2	–	–	–	+	+	–	Breast
c.1348G>A	54	un	+	–	–	+	+	–	Breast
	41	2	–	–	–	+	+	–	Breast
	38	2	–	–	–	+	+	–	Breast
c.1363G>A	54	un	+	–	–	+	+	–	Breast
	38	2	–	–	–	+	+	–	Breast
	41	2	–	–	–	+	+	–	Breast
c.1420C>T	23	1	–	–	–	+	–	–	Breast
c.1561C>T	28	2	+	–	–	+	+	–	Breast
c.1566C>T	28	2	+	–	–	+	+	–	Breast
	50	2	–	–	–	+	+	+	Breast
	46	2	–	–	+	+	+	+	Breast
c.1573G>A	50	2	–	–	–	+	+	+	Breast
c.1608A>G	28	2	+	–	–	+	+	–	Breast
	46	2	–	–	+	+	+	+	Breast
	50	2	–	–	–	+	+	+	Breast
c.18C>T	46	2	–	–	+	+	+	+	Breast
	28	2	+	–	–	+	+	–	Breast
	50	2	–	–	–	+	+	+	Breast

un, unknown.

addition to this, Table 1 also shows the identified variants with tumor characteristics (*ER, PR, HER2* status, Ki67 score, etc.) and the family history of the patients. There was no statistical difference between groups ($p > 0.05$) due to the limited number of variants. Early-onset disease was shown in most patients, and 35 (36.8%) of the patients were diagnosed under the age of 40 years. Overall, 69 patients had a family history of breast cancer, and bilateral breast cancer was reported in only 5 patients. Of the 14 patients with *CHEK2* mutations, 9 (64.2%) had a family history of breast cancer, 3 (21.4%) had a family history of other types of cancer, and 2 patients had breast cancer and no family history of cancer. *CHEK2* mutation carriers had family members with various cancers, including leukemia, breast, lung, colon, gastric, pancreatic, prostate, and laryngeal cancers.

The mean age of ovarian cancer patients ($n = 4$) was 35.2 ± 2.3 years. All patients were in early stage III. All

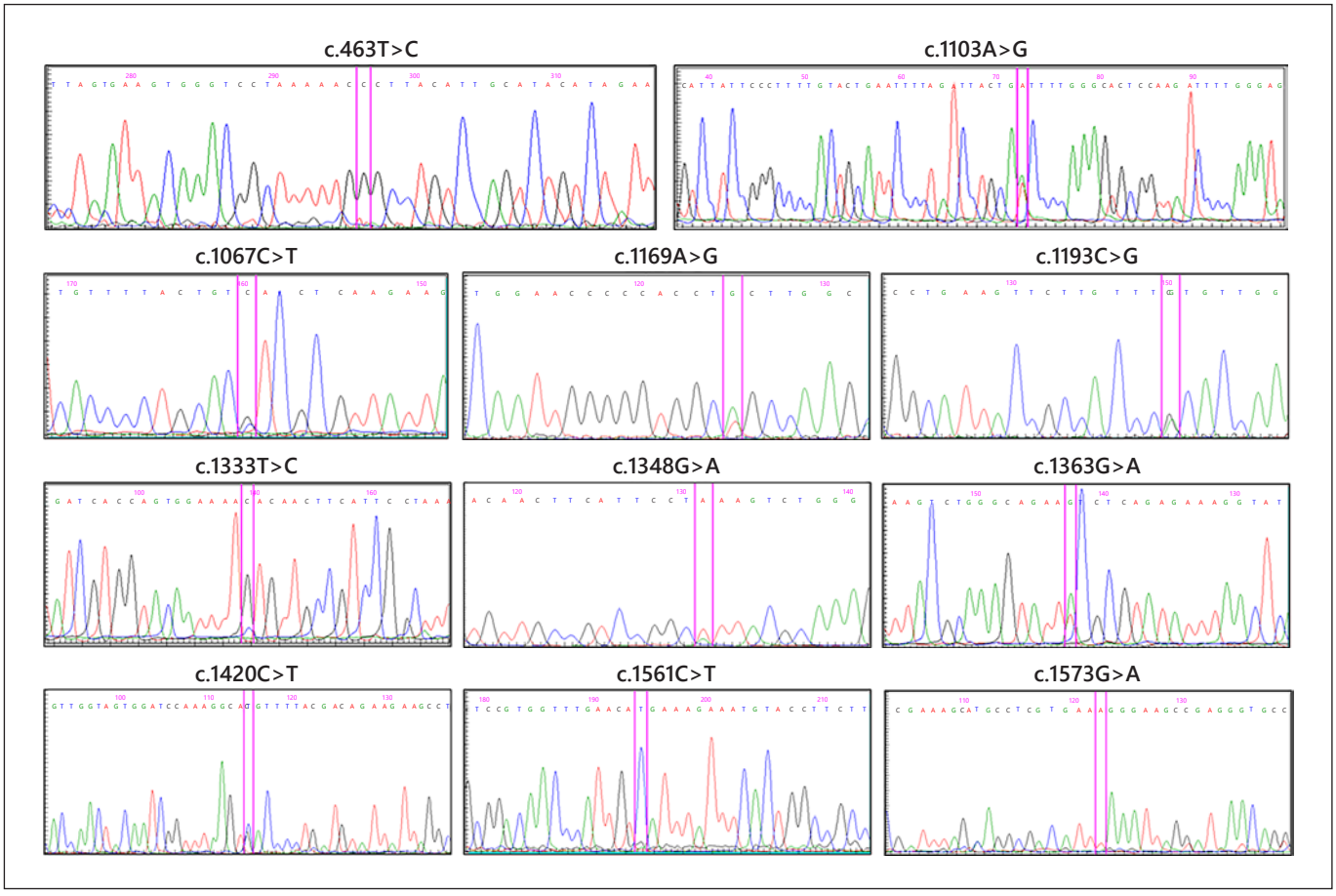

Fig. 1. A schematic diagram of identified missense variants in *CHEK2* gene by DNA sequence analysis.

patients had a family history. The mean tumor size was 5.6 ± 7.6 cm.

Mutation Screening Results

To evaluate the contribution of *CHEK2* mutations to early-onset breast and/or ovarian cancer, we sequenced the coding sequence of the gene, including intron-exon boundaries. In the case population, we identified a total of 16 different *CHEK2* sequence variants in 95 *BRCA1/2*- and *PALB2*-negative women and 60 controls, as is shown inTable 4, and clinical features of patients according to the identified *CHEK2* variants are presented in Table 5. Additionally, Figure 1 shows the sequence images of the c.463T>C, c.1067C>T, c.1169A>G, c.1193C>G, c.1333T>C, c.1348G>A, c.1363G>A, c.1420C>T, c.1561C>T, c.1573G>A, and c.1103A>G missense variants. The two novel missense variants that were detected in the study group, c.1193C>G and c.1363G>A, have not been previously reported in the Ensembl genome databases. The family history of the probands identified to carry these variants in *CHEK2* is shown in the pedigrees of Figure 2. In addition, four synonymous variants, c.1176G>T, c.1314C>T, c.1566C>T, and c.1608A>G, and one 3′-UTR variant, c.18C>T, were identified in this study. Of the eight variants, c.463T>C, c.1067C>T, c.1103A>G, c.1169A>G, c.1193C>G, c.1333T>C, c.1420C>T, and c.1561C>T were predicted to be possibly damaging or probably damaging; they were linked to disease by the PolyPhen2 and SIFT analysis programs, although the c.1348G>A, c.1363G>A, and c.1573G>A variants were presumed to be benign by PolyPhen2 and presumed to be tolerated by SIFT (Table 3). Of the variants, c.1348G>A and c.1573G>A were presumed to be likely linked to disease, less likely (C0) to be linked to disease, and benign by SIFT, Align-GVGD, and PolyPhen2, respectively. Additionally, c.463T>C was graded as C0 by Align-GVGD and was presumed to be tolerated by SIFT, yet this missense variant was categorized to be possibly

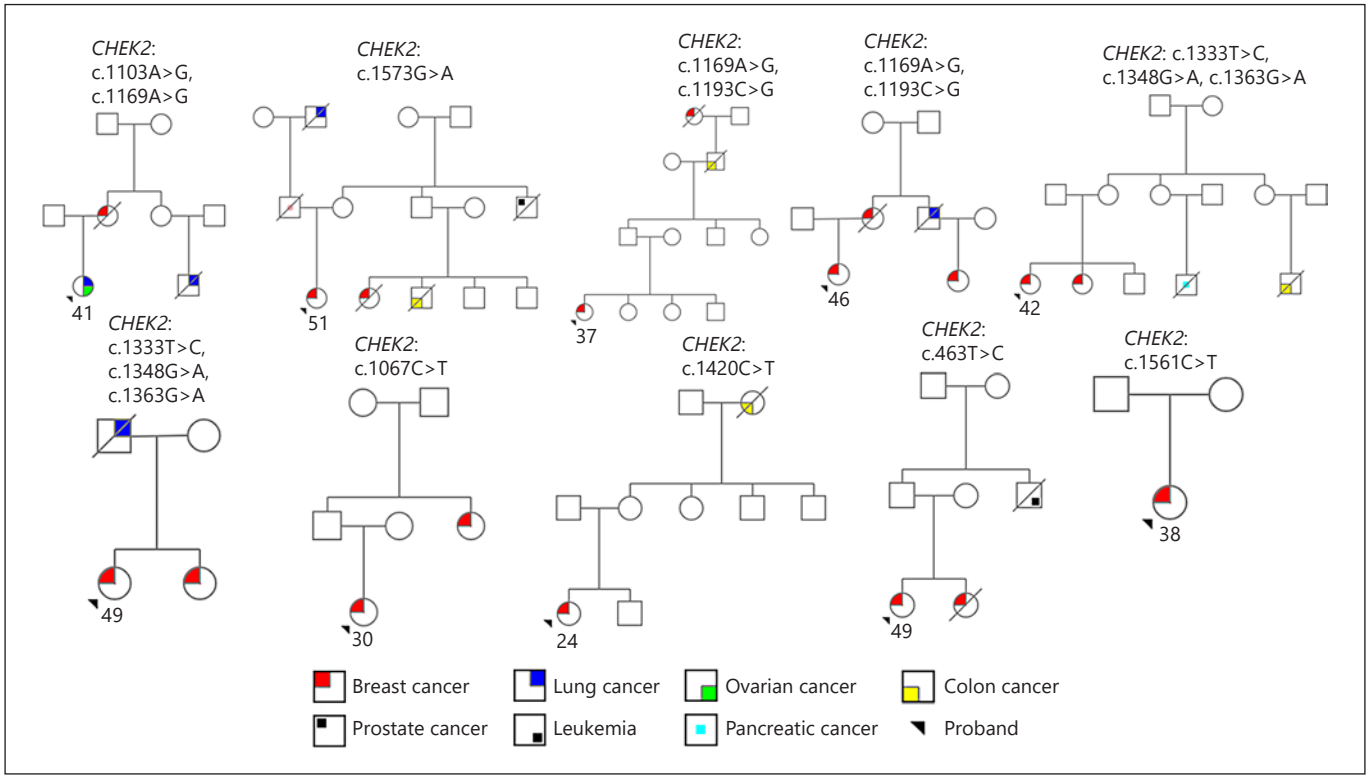

Fig. 2. Family pedigrees of probands found to carry the c.463T>C, c.1067C>T, c.1103A>G, c.1169A>G, c.1193C>G, c.1333T>C, c.1348G>A, c.1363G>A, c.1420C>T, c.1561C>T, and c.1573G>A missense variants. Circles indicate females, squares males. Filled symbols indicate affected with cancer.

damaging by PolyPhen2. The variants c.1067C>T, c.1193C>G, and c.1333T>C were graded as C15–25 by Align-GVGD and were predicted to affect protein function by SIFT and to be possibly or probably damaging by PolyPhen2. However, c.1363G>A was graded as C25 and presumed to be linked to disease by Align-GVGD and assessed as benign by PolyPhen2 and SIFT. Furthermore, c.1103A>G, c.1169A>G, c.1420C>T, and c.1561C>T were predicted to be most likely deleterious (C65) and probably damaging by SIFT, Align-GVGD, and PolyPhen2, respectively. Moreover, the four novel missense variants of *CHEK2* lead to decreased protein stability, and each variant was categorized as "disease causing." They caused changes in the amino acid sequence and splice site and were predicted to affect protein function (Table 6). The SWISS-MODEL that was used for tertiary structure prediction showed a marked variation in the structure of c.1193C>G, and c.1363G>A missense changes (Fig. 3). Briefly, 16 different variants were detected in Turkish breast and/or ovarian cancer patients, and 7 variants were detected in the 8/60 unaffected controls. However, we found no significant difference in the frequency of missense variants between the cases and controls.

Clinical Features of the Patients

In this study, we analyzed the *CHEK2* gene of 95 patients in total and detected that the majority of patients (38.9%) have ER+/PR+/HER2– tumor types. The prevalence of the c.1067C>T, c.1314C>T, c.1333T>C, c.1348G>A, c.1363G>A, c.1561C>T, c.1566C>T, c.1608A>G, and c.18C>T variants was 14.25% among the patients. In addition, the c.463T>C variant was observed in one patient with the ER–/PR–/HER2+ tumor type. Among the patients with triple-negative breast cancer, the prevalence of the c.1169A>G and c.1176G>T *CHEK2* variants was 1.9%. Additionally, the c.1566C>T, c.1573G>A, c.1608A>G, and c.18C>T variants were detected in patients with the ER+/PR+/HER2+ tumor type (6.7%). The c.1420C>T variant was present in a patient who was diagnosed with breast cancer at the age of 23; tumor status was only positive in terms of ER expression in this patient. Furthermore, tumor grade, especially

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Table 6. The prediction of identified novel variants on protein structure and stability

	Splice sites effect	gDNA position	Score	Exon-intron border	Start (aa)	End (aa)	Feature	Prediction	Predicted both value and sign of energy change using SVM	SVM	Neural network	I-mutant
c.1103A>G	Acc marginally increased	46563	wt: 0.6365 mu: 0.6529	ttgg|GCAC	220	486	Important for its protein kinase domain. LOST	Disease causing	Delta G: –1.906 (decrease stability)	Decrease the stability of protein structure	Decrease the stability of protein structure	Stability decrease (reliability Index: 4)
							Interaction with ATP. LOST	Amino acid sequence changed		Confidence score: –1	Confidence score: –0.986	
	Acc marginally increased	46549	wt: 0.8068 mu: 0.8834	ttag|ATTA	368	368	Mutagen D–>N: autophosphorylation activity LOST	Protein features (might be) affected				
	Acc increased	46556	wt: 0.66 mu: 0.83	actg|ATTT	368	394	T-loop/activation segment. LOST	Splice site changes				
c.1193C>G	Acc marginally increased	46650	wt: 0.8599 mu: 0.8664	ctgt|TGGG	220	486	Important for its protein kinase domain. LOST	Disease causing	Delta G: –0.305 (decrease stability)	Decrease the stability of protein structure	Decrease the stability of protein structure	Stability decrease (reliability Index: 3)
			0.46							Confidence score: –0.351	Confidence score: –0.505	
							Important for its helix. LOST	Amino acid sequence changed				
	Acc gained	46646		gttt|GTGT	393	398		Protein features (might be) affected				
								Splice site changes				
c.1363G>A	Donor marginally increased	47289	wt: 0.9886 mu: 0.9899	CTCA|gaga	220	486	Important for its protein kinase domain. LOST	Disease causing	Delta G: –0.148 (decrease stability)	Decrease the stability of protein structure	Decrease the stability of protein structure	Stability decrease (reliability Index: 6)
										Confidence score: –0.317	Confidence score: –0.536	
			0.86					Amino acid sequence changed				
	Donor gained	47280		GGCA|gaaa				Protein features (might be) affected				

SVM, support vector machine.

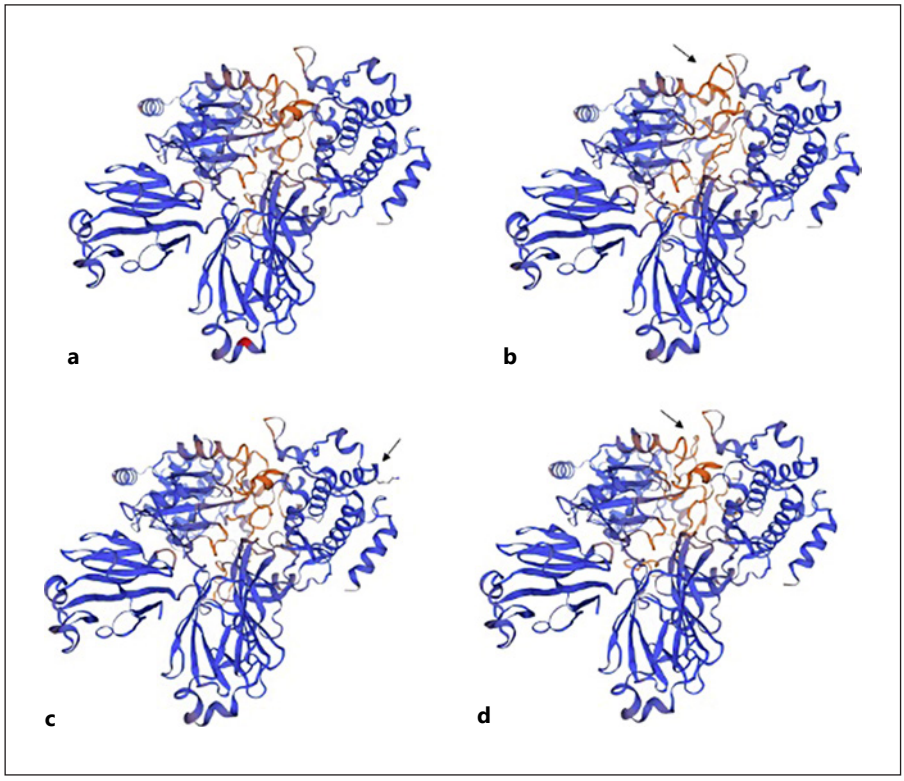

Fig. 3. The effect of c.1103A>G (**b**), c.1363G>A (**c**), and c.1193C>G (**d**) variants on protein structure by Swiss model, respectively. **a** Wild type. **b–d** Mutant protein.

Table 7. Means and medians for survival time

	Mean[a]				Median	
	estimate	std. error	95% confidence interval		estimate	std. error
			lower bound	upper bound		
Recurrence						
Negative	200.571	14.284	172.575	228.568		
Positive	114.334	11.866	91.077	137.59	108	
Overall	178.4	12.889	153.137	203.663		

[a] Estimation is limited to the longest survival time if it is censored.

grade II (32.6%), and a high level of Ki-67 expression was observed in the patients (Table 1). When the clinical features of the patient with ovarian cancer, lung cancer metastasis, and two identified missense variants were analyzed, we found that the c.1103A>G and c.1169A>G variants were detected in a young patient with tumor grade III.

Survival Analysis of the Patients

In this study, the mean age of the patients was 42.4 ± 8.8 years, and the median follow-up was 72 months for the total cohort of patients. The median overall survival rates for 10 years, 5 years, and 1 year were 70%, 92%, and 98%, respectively (95% confidence interval [CI]: 153–203 months). The recurrences were confirmed for 27 patients, and 9 patients died of breast cancer during follow-up. The recurrence rates were found to have a statistically significant negative effect on overall survival and to lead to decreased mean survival expectation by 114 months on follow-up (95% CI: 95–137 months) (Table 7). The expected disease-free survival (DFS) was 42 months (95% CI: 32–51 months), and DFS rates for 1, 3, and 5 years were 86%,

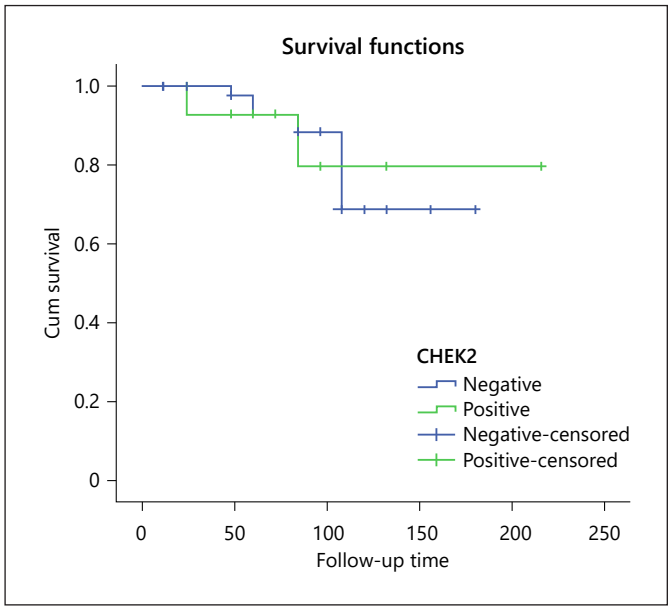

Fig. 4. Overall survival rates in breast cancer patients with *CHEK2* variants.

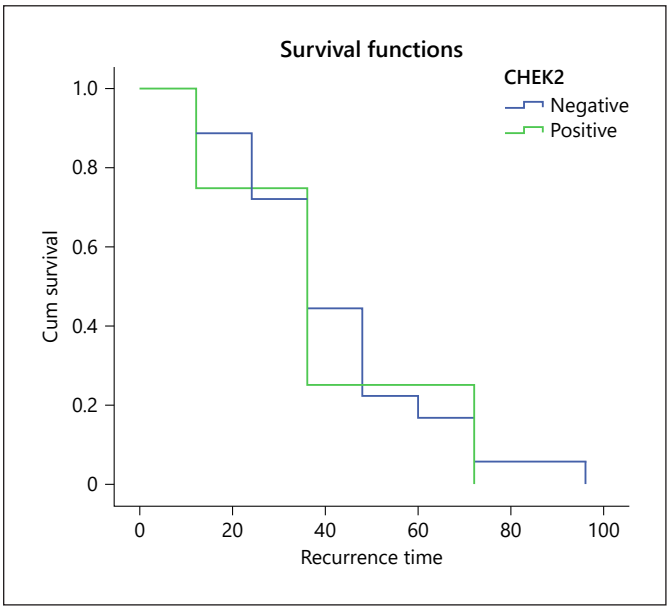

Fig. 5. Disease-free survival in relapse breast cancer patients with *CHEK2* variants.

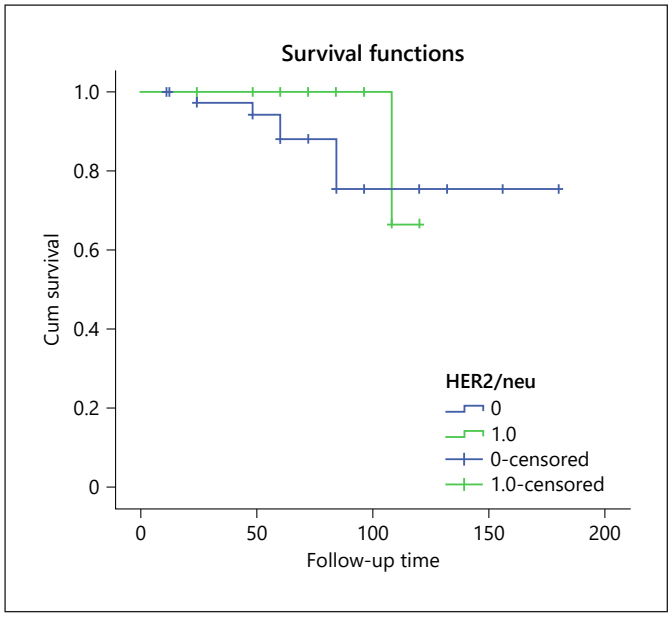

Fig. 6. Overall survival rates in HER2/neu-positive breast cancer patients with *CHEK2* variants.

40%, and 18%, respectively. Overall survival rates in 14 patients with *CHEK2* variants were examined; the presence of *CHEK2* variant led to no statistically significant difference in survival (95% CI: 145–224 months) (p = 0.82) (Fig. 4). The DFS of patients with relapse which had *CHEK2* variants is shown in Figure 5 (p = 0.84). When the effects of hormone receptor levels on overall survival were examined, it was found that only HER2/neu positivity decreased the overall survival significantly in patients with *CHEK2* variants (p = 0.019) (Fig. 6). In patients with *CHEK2* variant, the expected DFS was 39 months (range: 15–63 months), whereas it was lower in patients without *CHEK2* variant (44.2 months, 95% CI: 33–54 months). No recurrence was observed in HER2-positive patients with *CHEK2* variant; therefore, there was no statistically significant difference in survival.

Discussion

Mutations in *BRCA1* and *BRCA2* increase the risk of breast and ovarian cancer and occur in only a certain percentage of familial and/or early-onset cases. Therefore, mutation targets, such as *CHEK2, PALB2, ATM,* and *BARD*, which are known to play a role in breast cancer development, are significant in terms of breast cancer risk screening [15–17]. The rates of germline mutations in the *CHEK2* gene vary between high-risk breast and ovarian cancer families in populations of various ethnic origin, so the gene needs to be identified to determine its relevance [17]. The benefit we have provided through this research

is the establishment of the relevance of *BRCA1/2*- and *PALB2*-negative high-risk breast and/or ovarian cancer in the Turkish population by assessing the prevalence of *CHEK2* mutations. To our knowledge, this is the first study cohort ever investigated for the prevalence of *CHEK2* mutations and its breast cancer phenotype in a Turkish population. In the present study, we used HDA and sequencing analysis to screen for mutations in the coding exons of the *CHEK2* gene in 95 *BRCA1/2*- and *PALB2*-negative Turkish women with early-onset breast and/or ovarian cancer and identified a total of 11 different missense *CHEK2* sequence variants in 95 patients. Novel missense coding variants were also assessed using multiple in silico tools and evaluated based on allele frequency and gene-specific databases. We also detected four synonymous variants and one 3′-UTR variant in the *CHEK2* gene. Sixteen variants were classified as VUS, benign, likely benign, likely pathogenic, and variants of unknown clinical significance.

It is known that the breast cancer risk doubles for female *CHEK2* c.1100delC mutation carriers, and the risk for carrier women is much higher in familial breast cancer cases that arise from co-inheritance of additional genetic risk factors [18]. *CHEK2* c.1100delC carrier status confers a nearly two-fold risk of breast cancer in women of Northern and Eastern European descent, whereas the frequency was reported to be much lower in those of North American descent [18–20]. In the only study that was performed on high-risk Turkish cases, including 16 familial, 29 early-onset, 3 male breast cancer, and 2 bilateral breast/ovarian cancer cases, Manguoglu et al. [21] detected no c.1100delC variant in *CHEK2*. However, in a study focused on 2,408 Greek patients under the age of 50 years with a history of familial breast cancer, a *CHEK2* c.1100delC mutation was found in a small percentage of the cases (0.16%) [11]. In the present study, we analyzed Turkish patients and, unlike the *CHEK2* c.1100delC mutation, which is frequently identified in other populations, the c.1103A>G (p.Asp368Gly) missense variant of unknown clinical significance was identified in a 41-year-old ovarian cancer patient with a family history of breast and lung cancer. This variant had evidence of a significant impact on the protein based on in silico prediction (Table 6). The p.Asp368Gly residue is located in the kinase domain as well as the T loop of the catalytic domain (residues 220–486) of *CHEK2*. It was found that the codon exchange in c.1103A>G, a highly conserved residue located in the kinase region of *CHEK2*, significantly impaired *CHEK2* activity.

The present study showed that another significant missense variant is c.1169A>G (p.Tyr390Cys) in *CHEK2*. This variant was found in three patients (3.15%) and in one (1.6%) healthy control subject. Also, three of the c.1169A>G carriers have a family history of breast and/or ovarian cancer, and one patient was observed with the ER–/PR–/HER2– tumor type. Although classified as being of "conflicting interpretations of pathogenicity" in ClinVar, c.1169A>G (p.Tyr390Cys) has been classified as likely pathogenic based on posterior probability calculations. Indeed, the c.1169A>G variant has been reported in a different study carried out in China [17]. Functional analysis of the study suggested that the *CHEK2* c.1169A>G mutation is deleterious when evaluated by the mutant protein's inadequacy to inactivate *CDC25A* or to activate *p53* after DNA damage [22]. This study also identified a novel *CHEK2* c.1169A>G variant that is linked to increased cancer risk in high-risk Chinese breast cancer patients [22]. However, the c.1169A>G variant, which we predicted as C65 in Align-GVGD, was contradictorily classified as C0 in a study that was carried out by Desrichard et al. [2] to identify *CHEK2* mutations in French women with hereditary breast cancer. As more patients and their family members are analyzed, it may become more obvious as to whether or not the c.1169A>G variant is linked to cancer risk.

In the present study, we detected a total of 5 VUS (c.1067C>T, c.1333T>C, c.1420C>T, c.1561C>T, and c.1573G>A) in web-based programs. VUS is a variant form that has an unclear or unknown effect on protein function [23]. However, a significant proportion of genetic tests detect variants that have an undefined risk of cancer as VUS. A definite conclusion on the pathogenesis of a VUS cannot be obtained from clinical features alone. Thus, a combination of biochemical and epidemiologic data should be considered [24, 25]. However, the missense c.1067C>T, c.1333T>C, c.1420C>T, c.1561C>T, and c.1573G>A variants have been previously reported in the literature [26–29]. Furthermore, the missense variant c.1193C>G (p.Ser398Cys) was identified in this study and is present in Ensembl, but there are no population records of it. This variant was predicted to be deleterious and probably damaging by SIFT and PolyPhen, respectively. However, in 3 patients (3.15%) with ER+/PR+/HER2– tumor type, both heterozygous c.1348G>A (p.Glu450Lys) and c.1363G>A (p.Val455Ile) missense variants were identified. These variants were classified as C0 and C25 by Align-GVGD, respectively, and predicted to be benign and tolerated by PolyPhen and SIFT. Therefore, it was predicted that these variants

were less likely to affect *CHEK2* protein function by all the algorithms tested.

Previously, the c.1363G>A (p.Val455Ile) variant was reported in high-risk Finnish *BRCA1/2*-founder mutation-negative ovarian and/or breast cancer cases [30]. In our study, almost 64% of the breast cancer patients were ≤45 years of age at diagnosis, and 16.8% of them were ≤35 years of age at diagnosis; this was in line with other studies [7]. A majority of the breast cancer cases were high-grade tumors (Table 2). When the histopathological features of tumors with suspected pathogenic *CHEK2* variants were analyzed, all the tumors in our study were detected to be invasive ductal carcinomas and the majority of them were of the ER+/PR+/HER2– tumor type. *CHEK2* mutation screening detects a clinically significant breast cancer risk, and the screening should be performed for all women with a family history of breast cancer [31, 32]. A closer look at the studies by Cybulski et al. [33] shows that women with a truncating mutation in *CHEK2* and a family history of breast cancer have a lifetime risk of more than 25%. In addition, it should be considered that these women are candidates for magnetic resonance imaging screening and for tamoxifen chemoprevention.

Although 16 different variants were detected, there were some limitations to our study. This is one of the rare studies to determine the prevalence of *CHEK2* variants among breast cancer patients from Turkey. A large number of breast cancer patients and unaffected women should be screened to estimate the actual *CHEK2* mutation rate in the population, as only a small number of variants have been identified. In this present study, we observed that a *CHEK2* variation did not affect the overall survival parameters, and there was no statistically significant difference in the DFS analysis. When the effects of hormone receptor levels on overall survival were examined, it was found that only HER2/neu positivity decreased the overall survival significantly in patients with *CHEK2* variants.

Conclusion

In the present study, we analyzed the prevalence of *CHEK2* mutations in breast and/or ovarian cancer patients without *BRCA1/2* and *PALB2* mutations in Turkey and detected that the mutations were similar to other populations. *CHEK2* c.1100delC, one of the most commonly studied variants in different populations, was not detected in our study. However, c.1103A>G (p.Asp368Gly) missense variant was identified in an ovarian cancer patient. Our study indicates that a variety of deleterious *CHEK2* alleles contribute significantly to breast cancer susceptibility. The results show that *CHEK2* c.1100delC mutations have not been a genetic susceptibility factor for breast cancer in Turkish patients. The results of recent studies are not yet sufficient to change or even influence clinical practice. Although the number of patients in our study is small, our data suggest that further research into the association between the *CHEK2* mutation and clinicopathological factors should be conducted.

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Statement of Ethics

This study protocol was reviewed and approved by the Local Ethics Committee of Bursa Uludag University, approval number 2015-4/5. The ethical standards of the Declaration of Helsinki were adhered to. All participants were informed and their written consent was obtained.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

F.A., H.T.U., G.C., and M.S.G. designed the research. F.A., B.T., U.E., and E.E.E. analyzed the data, and F.A., G.G.E., and K.S. wrote the main manuscript text. All authors reviewed the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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