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. © 2022 The Author(s). Published by S. Karger AG, Basel

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-penetration 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-
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.

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 of Uludag University and conforms to the ethical standards of the Declaration of Helsinki. All participants were informed and gave their written informed consent.

| 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)              |                  |
| In situ component, n (%)        | Triple negative patients 17 (18.0) |
| Ki67, n (%)                     | ≥20% 36 (37.9)   |
| <20% 63 (62.1)                  |                  |
| Metastasis, n (%)               | Positive 27 (28.4) |
| Negative 68 (671.6)             |                  |

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...
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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.

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.ensemble.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/). 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.mutation-taster.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 χ2 test (both tests are two-sided). The result (p < 0.05) was statistically significant.

### 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             | CTCACCTTTGTGTGACAC             | CCACCTTTGTGTGACAC             | 65                       |
| 2.Oca | 586             | TCTGATTGCTGCTTACTGGCT          | CATATGGCTGCTTACTGGCT          | 65                       |
| 3     | 397             | TCAGTATCGGCGCTCTTGTGA          | GGCTCTTACGTGCTGCTGG          | 58                       |
| 4     | 397             | TGTCTCCTGCAAACAGGGAACA         | CTAACGGGAGGTTATCTGCA          | 65                       |
| 5     | 453             | AGTAGGCTGGTGGTTGGAACCT         | AGCTAGGCTGGTGGTTGGAATG        | 56                       |
| 6     | 239             | TCTCTCTGGCAGATTGTCTCTA         | GGTGAGAAAAGCGAGCTCACAT        | 60                       |
| 7     | 364             | TCACCTGGGACATCGTTGTTGGTG       | CACCGCTCTGCTATTCTCT           | 65                       |
| 8     | 331             | CGTGAGATGTTGTTGTTGTTGTTAAGC    | TCTGATGAAAGCAGATACCTG         | 65                       |
| 9     | 288             | TACGTGTTCTTCTGGAGACTG          | CCTCTACCACTGCTTCAGC          | 60                       |
| 10    | 325             | CTGATTGAGAATGTTGTTGTTGTTGTAAC  | CCAGTGGCTCTCAATGGGTG          | 60                       |
| 11    | 254             | CTGCTTCTTCTCTAATGGTG          | CCTGCTTCTCTAGTCTCTCAGG        | 60                       |
| 12    | 363             | ATGGTGGAGATGTTGACTGACCCAG     | ATCAGGTCTCTAAAAAGCCAGACTA     | 65                       |
| 13    | 386             | AGCCCTCCACCTTACTTGCAGA        | GCCATTTCAAAGAAGCCAGAT         | 65                       |
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 3. Results of in silico analysis for CHEK2 variants

| P     | Protein change | SIFT       | Align GVGDα | 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 | Possibly damaging (0.956) | – | – | – |
| c.1314C>T | p.Asp438= | NA | NA | NA | – | – | – |
| c.1333T>C | p.Tyr445His | Deleterious (0.00) | C25 | Possibly 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.Arg374Cys | Deleterious (0.00) | C65 | Possibly damaging (1) | – | + | Uncertain significance |
| c.1561C>T | p.Arg512Trp | 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.Ser398Cys | Tolerated (0.06) | C0 | Benign (0.021) | – | + | Likely benign |
| c.18C>T | _ | _ | _ | _ | – | + | Benign; likely benign; uncertain significance |

α 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 | – |
|              | rs1555913929 | Missense | c.1103A>G | p.Asp368Gly | 1 | – |
| E10          | rs200928781 | Missense | c.1169A>G | p.Tyr390Cys | 3 | – |
|              | rs142692907 | Synonymous | c.1176G>T | p.Ala392= | 2 | – |
|              | _ | Missense | c.1193C>G | p.Ser398Cys | 2 | – |
|              | _ | Synonymous | c.1314C>T | p.Asp438= | 1 | – |
| E11          | rs587778194 | Missense | c.1333T>C | p.Tyr445His | 3 | – |
|              | rs1555913429 | Missense | c.1348G>A | p.Glu450lys | 3 | – |
|              | _ | Missense | c.1363G>A | p.Val455Ile | 3 | – |
| E12          | rs540635787 | Missense | c.1420C>T | p.Arg474Cys | 3 | 1 |
| E14          | rs533475838 | Synonymous | c.1561C>T | p.Arg512Trp | 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.
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
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 in Table 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
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.1169A>G, c.1420C>T, and c.1561C>T variants was 14.25% among the patients. In addition, 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
### 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           | 46563         | wt:0.6365, mu:0.6529 | ttag|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 |
|                      |               |       |                     |           |        | Mutagen D–&gt;N; autophosphorylation activity LOST | Protein features (might be) affected |
| c.1193C>G           | 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 | Important for its helix. LOST | Amino acid sequence changed | Confidence score: –0.351 | Confidence score: –0.505 |
|                      |               |       |                     |           |        | Acc gained | Protein features (might be) affected |
|                      |               |       |                     |           |        | Splice site changes |
| c.1363G>A           | 47280         | 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) |
|                      |               |       |                     |           |        | 0.86 | Amino acid sequence changed | Protein features (might be) affected | Confidence score: –0.317 | Confidence score: –0.536 |

SVM, support vector machine.
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%,

**Table 7.** Means and medians for survival time

| Recurrence | Mean\(^a\) estimate | std. error | 95% confidence interval \(^a\) lower bound | upper bound | Median estimate | std. error |
|------------|---------------------|------------|-------------------------------------------|-------------|----------------|------------|
| Negative   | 200.571             | 14.284     | 172.575                                    | 228.568     | 108            |
| Positive   | 114.334             | 11.866     | 91.077                                     | 137.59      |
| Overall    | 178.4               | 12.889     | 153.137                                    | 203.663     |

\(^a\) Estimation is limited to the longest survival time if it is censored.
Overall survival rates in 14 patients with \textit{CHEK2} variants were examined; the presence of \textit{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 \textit{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 \textit{CHEK2} variants ($p = 0.019$) (Fig. 6). In patients with \textit{CHEK2} variant, the expected DFS was 39 months (range: 15–63 months), whereas it was lower in patients without \textit{CHEK2} variant (44.2 months, 95% CI: 33–54 months).

No recurrence was observed in HER2-positive patients with \textit{CHEK2} variant; therefore, there was no statistically significant difference in survival.

\section*{Discussion}

Mutations in \textit{BRCA1} and \textit{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 \textit{CHEK2}, \textit{PALB2}, \textit{ATM}, and \textit{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 \textit{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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