Egyptian Journal of Medical Human Genetics

RESEARCH Open Access

Turkish population-based screening for first identified changes of *BRCA1* and *BRCA2* genes in breast and/or ovarian cancer patients

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Abstract

Introduction It is known that *BRCA1* and *BRCA2* genes' mutation carriers are predisposed to breast and ovarian cancers and other organ cancers such as prostate, colon and cervix. In the previous study performed at X University, all coding exons of both genes were screened by denaturing gradient gel electrophoresis (DGGE). In addition to various nonsense, missense mutations, polymorphisms and intronic region changes, seven novel missense mutations, including H513L, H816P and S1517Y in BRCA1 and S326R, G258P, E2903K and N2742S in BRCA2, had been identified.

Methods To determine whether these unclassified variants are pathogenic, DNA samples of 150 healthy individuals without a known cancer history in the family were screened in this study for these seven novel missense mutations. These DNA samples were recruited from archives of previous polymorphism studies. PCR performed DNA amplifications, and denaturing high-performance liquid chromatography (DHPLC) techniques did mutation screenings.

Results Peak patterns suggestive of a change in DNA fragments were considered for sequencing analyses. Analyses revealed that none of the 150 DNA samples had any change in the seven screened fragments. As a result, it is assumed that these seven mutations might be novel pathogenic mutations described in the Turkish population.

Conclusion In conclusion, these carriers must be informed about the mutation and given appropriate genetic counseling by their physicians. In addition, genetic testing must be offered to high-risk individuals (men/women) in the family so that it would be possible for other family members to have genetic counseling and contribute to disease prevention. On the other hand, these findings would contribute to current literature with novel results and shed light on future research.

Keywords *BRCA1*, *BRCA2*, Mutation, DHPLC

Introduction

Breast cancer (BC) is the most common type observed in women, and its incidence is increasing drastically [1]. Similarly, it is the most frequently diagnosed type of cancer among Turkish women and accounts for 25% of all cancer types [2]. BC has remained Turkey's second most common cause of death for many years [3, 4]. Genetic predisposition is one factor that increases the risk of developing breast or ovarian cancer. Many genes are responsible for developing breast cancer [5]. *BRCA1* and *BRCA2* are the genes most frequently responsible for the genetic susceptibility to these malignancies. Having *BRCA1* and *BRCA2* gene mutations increases the risk of

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encountering breast or ovarian cancers in the early stages of life. Among carriers of these mutations, the lifetime risks of developing breast cancer and ovarian cancer are between 60–85% and 10–40%, respectively [8].

The *BRCA* genes are essential for protecting the chromosome structure and provide the stability of the genome, like a "caretaker" [6]. The *BRCA1* is located at chromosome 17q21, and *BRCA2* is located at chromosome 13q12. Both the protein products of these genes bind to some essential cellular proteins [7]. In addition to tumor suppressor functions in cell proliferation, BRCA1 and BRCA2 proteins are closely related to other essential checkpoint proteins in the cell cycle involved in DNA damage repair, transcription regulation and DNA recombination. Therefore, mutations of *BRCA1* and *BRCA2* genes cause dysfunction of tumor suppressor proteins and mediate the inactivation of the genome protector proteins. As a result, these changes lead to cancer formation [8, 9]. More than one hundred mutations causing different DNA sequence changes have been identified in *BRCA1* and *BRCA2* genes [10]. However, mutation scanning studies in breast or ovarian cancer patients from the Turkish population have not shown any constructor mutations considered specific to the population [11].

In this study, the genetic alterations were investigated in *BRCA1* and *BRCA2*, which were described for the first time in a previous study by X et al. These findings were new mutations observed for the first time, but their biological importance was obscure. Therefore, the present study aimed to determine whether these changes are polymorphisms or novel mutations in the Turkish population. For this purpose, 150 healthy individuals without any family cancer story were scanned with denaturing high-performance liquid chromatography (DHLPC) analysis.

Material and methods

Samples

This study used randomly selected DNA samples from the X University Faculty of Medicine, Department of X archive. The samples in this archive were used as control groups in some previous studies and were obtained from healthy individuals without a familial cancer history. (A total of 150 DNAs that were obtained from 150 healthy women individuals with no family history of cancer in X University, Faculty of Medicine, Department of X, used as a control in previous projects, stored at −20 °C were included in the study after obtaining the informed consent forms.) Samples were derived from blood and stored in proper conditions. Samples with insufficient DNA content were replaced with others randomly selected from the archive. Thus, 150 samples were arranged to be included in the study.

DNA isolation and PCR analysis

The genomic DNAs were isolated by changing the standard DNA isolation method in a previous study [11]. Then, the DNA samples were taken to the spectrophotometric measurement for quantity and purity determination (NanoDrop 1000). As mentioned, samples with too little DNA quantity to be studied were replaced with other DNA samples in the archive.

The PCR was carried out with a PCR System 9700 (Gene Amp®) thermal cycler device. For different exons of these genes, proper PCR programs were designed. For example, the exons for the BRCA1 gene were exon 16 and 05 and 07 areas of exon 11. For the BRCA2 gene, exon 9 A area was of exon 10, exon 21 and exon 18. The primers used in the PCR are presented in Table 1.

Agarose gel electrophoresis

The samples were controlled in gel electrophoresis to confirm the achievement of the amplification. The PCR procedure was repeated with new dilution rates for the non-amplified samples. The 1% agarose gel was placed in an electrophoresis tank containing Tris–Borate–EDTA. Then, the PCR product and 50-bp marker were loaded onto wells using the buffer and run for 20 min at 120 V. Ultimately, the samples were examined with UV transilluminator syngene (Ingenius), and images were taken.

DHPLC analysis

The DHPLC analysis was carried out using the WAVE Maker system (Transgenomic Inc., San Jose, CA) to

Table 1 Primer sequences used in PCR analysis

Name of primer	Sequence of primer (5′ to 3′)	Gene
1105F	AGGAGCATT TGTTACTGAT	BRCA1
1105R	AGACTTCCTCCTCAGCCTATT	
1107F	CTAAGTGTTCAAATACCAGT	
1107R	ATTTCTATGCTTGTTTCCCG	
16F	AATTCTTAACAGAGACCAGAAC	
16R	AAAACTCTTTCCAGAATGTTGT	
9F	CAGATAACTGAAATCACCAAAAGTG	BRCA2
9R	ACAACAACAAAAAAACCTGTAGTTC	
10AF	TATAAAATATTAATGTGCTTCTGTT	
10AR	AAAGGGCTTCTGATTTGCTAC	
18BF	TGTTTCTGACATAATTTCATTGAGC	
18BR	AAACTTTAACTGTCTGAAGAATATGC	
21F	GGGTGTTTTATGCTTGGTTCT	
21R	CATTTCAACATATTCCTTCCTG	

determine the genetic variations of the coding areas 1105, 1107 and 16 in the *BRCA1* gene and 9, 10A, 21, 18B coding areas in the *BRCA2* gene. For this procedure, 10 µl of PCR product from individuals included in our study were hybridized with the same amount obtained from healthy individuals' DNAs. Subsequently, denaturation stages were carried out with the PCR system (Mycycler). Then, the denatured and hybridized amplicons were loaded onto the device and studied under proper working conditions. After the first DHPLC analysis, the DHPLC analysis of amplicons showing different peaks was repeated.

DNA sequence analysis

The DNA sequence analysis included samples showing different peaks in repeated DHLPC analysis. First, the amplicons were cleaned with the PCR product purification kit (Roche) for this process. Then, these cleaned amplicons were used as the pattern for DNA sequence analysis. The results obtained from the process were evaluated using the device's software and an ABI 310 system. DNA sequence analysis was performed using the US National Center for Biotechnology Information nucleotide database (*BRCA1* GenBank accession no. L78833.1; *BRCA2* GenBank accession no. AY436640.1).

Results

PCR and agarose gel electrophoresis

The isolated DNA samples were taken to spectrophotometric measurements for quantity and purity determination. Samples containing sufficient DNA were included in the study, and the samples with too little DNA quantity to be studied were replaced with other DNA samples in the archive.

In the *BRCA1* gene; for the H513L change, the 05 areas of exon 11, for the H816P change, the 07 regions of exon 11 and the S1577Y change, the areas of exon 16 were amplified with the PCR technique. In the *BRCA2* gene, for the G258P, S326R, N2742S and E2903K changes, exon 9, the A area of exon 10, the B area of exon 18 and exon 21 were amplified with PCR, respectively. These samples amplified in PCR were checked by agarose gel electrophoresis. An example of the gel image of these exonic regions is given in Fig. 1.

DHPLC assay results

Products of amplicons after amplification obtained from DNAs of 150 healthy individuals were studied with DHPLC. Among all the samples, three examples from standard DHPLC charts of *BRCA1* and *BRCA2* genes are randomly selected in Figs. 2 and 3. In the *BRCA1* gene, five samples for 05 areas of exon 11 at 55.7 °C, eight for exon 16 at 55.8 °C and 34 at 59.3 °C showed these different peak patterns. In the *BRCA2* gene, for exon 9, 2 samples at 55 °C and 15 samples at 57 °C, for A area of exon 10, 15 samples at 53.7 °C, for B area of exon 18, 10 samples at 55 °C and 20 samples at 55.5 °C, and for exon 21, 5 samples at 55.5 °C and 47 samples at 59.5 °C showed abnormal peaks. All samples presenting different peaks were analyzed with DHPLC again under the same working conditions. After repeated analysis, most of these other peaks disappeared; their previous results were considered false negative. However, the *BRCA2* gene for exon 9 in five samples at 57 °C working conditions and the area of exon 10 in five samples at 54.7 °C operational conditions continued to show different peak patterns in the repeated analysis. Then, these samples were taken for DNA sequence analysis for further investigation.

DNA sequence analysis

As a result of repeated DHPLC analysis, ten samples were included in the sequence analysis. No changes were detected in the relevant regions. The results of two

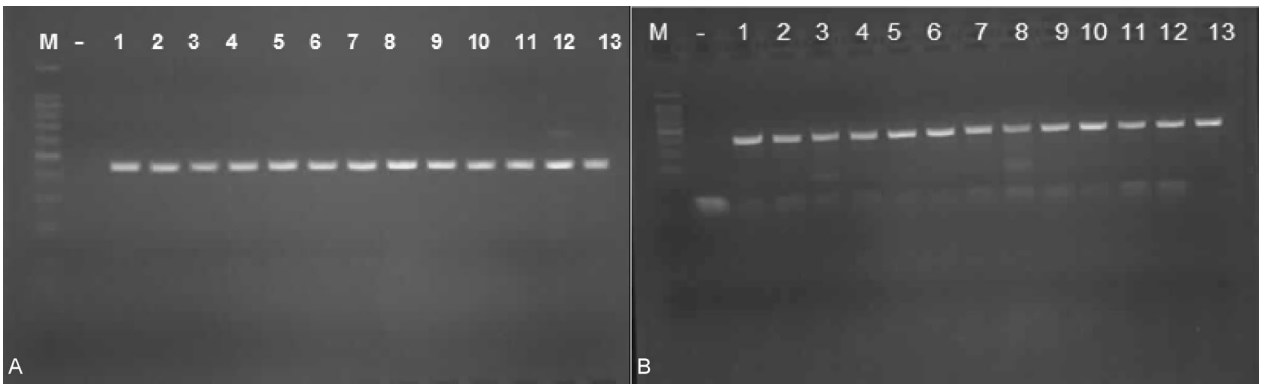

Fig. 1 Agarose gel image of the amplicons. **A** Exon 16 on *BRCA1* gene from 1 to 13 individual. **B** A area of exon 10 on *BRCA2* gene from 1 to 13 individual

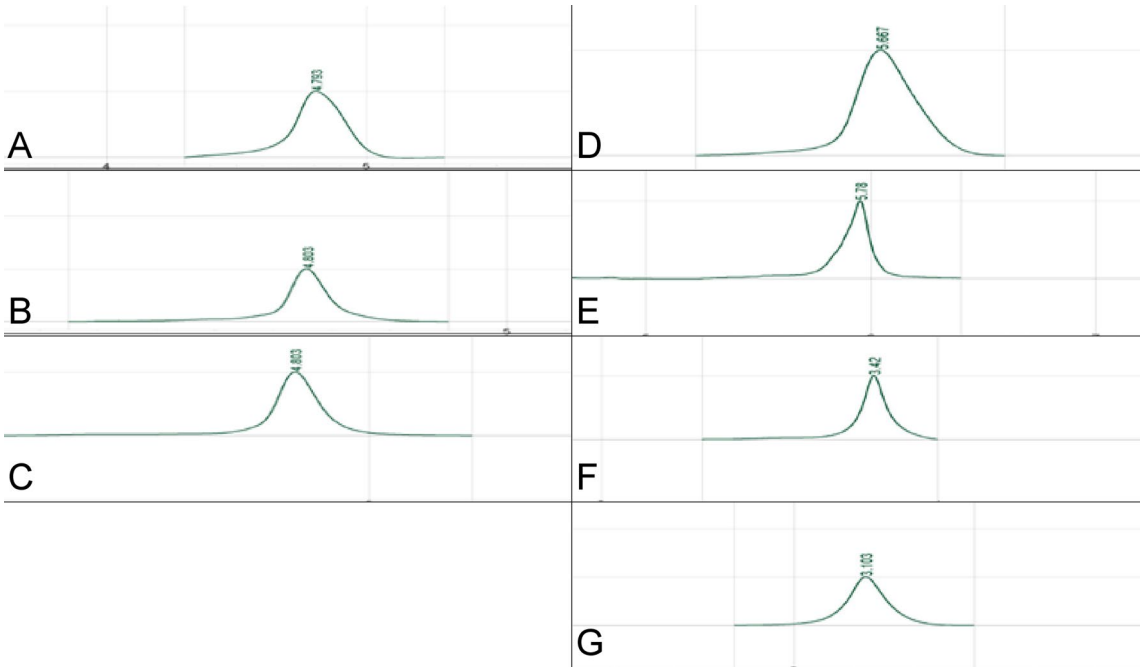

Fig. 2 DHPLC charts of *BRCA1* and *BRCA2* genes were randomly selected among all control samples. **A**–**C** Three examples from normal DHPLC charts of the *BRCA1* gene. **D**–**G** Four examples from normal DHPLC charts of the *BRCA2* gene

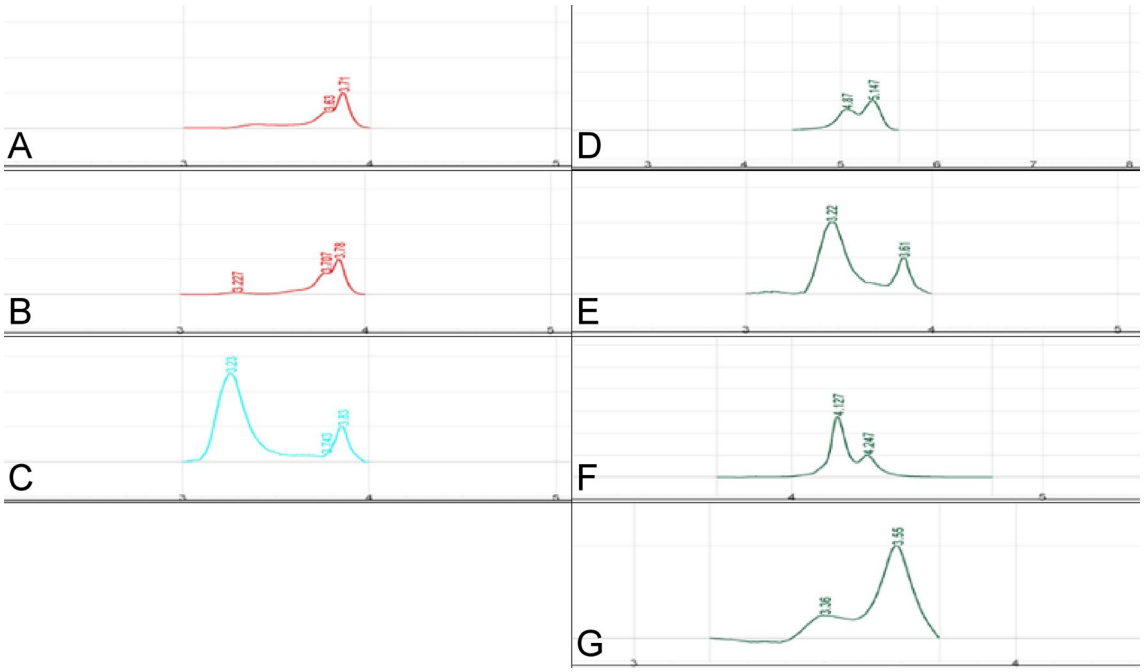

Fig. 3 DHPLC charts of *BRCA1* and *BRCA2* genes were randomly selected among all control samples. **A**–**C** Three examples from normal DHPLC charts of the *BRCA1* gene. **D**–**G** Four examples from normal DHPLC charts of the *BRCA2* gene

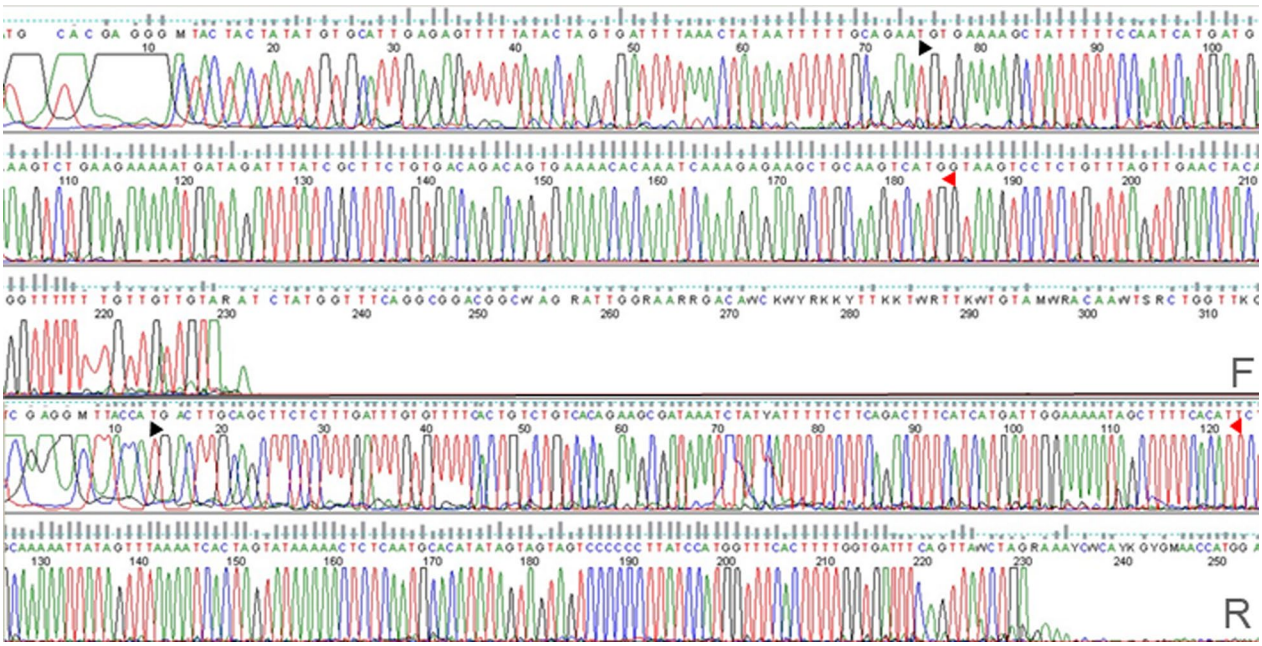

Fig. 4 Images of DNA sequence analysis from exon 9 of the *BRCA2* gene

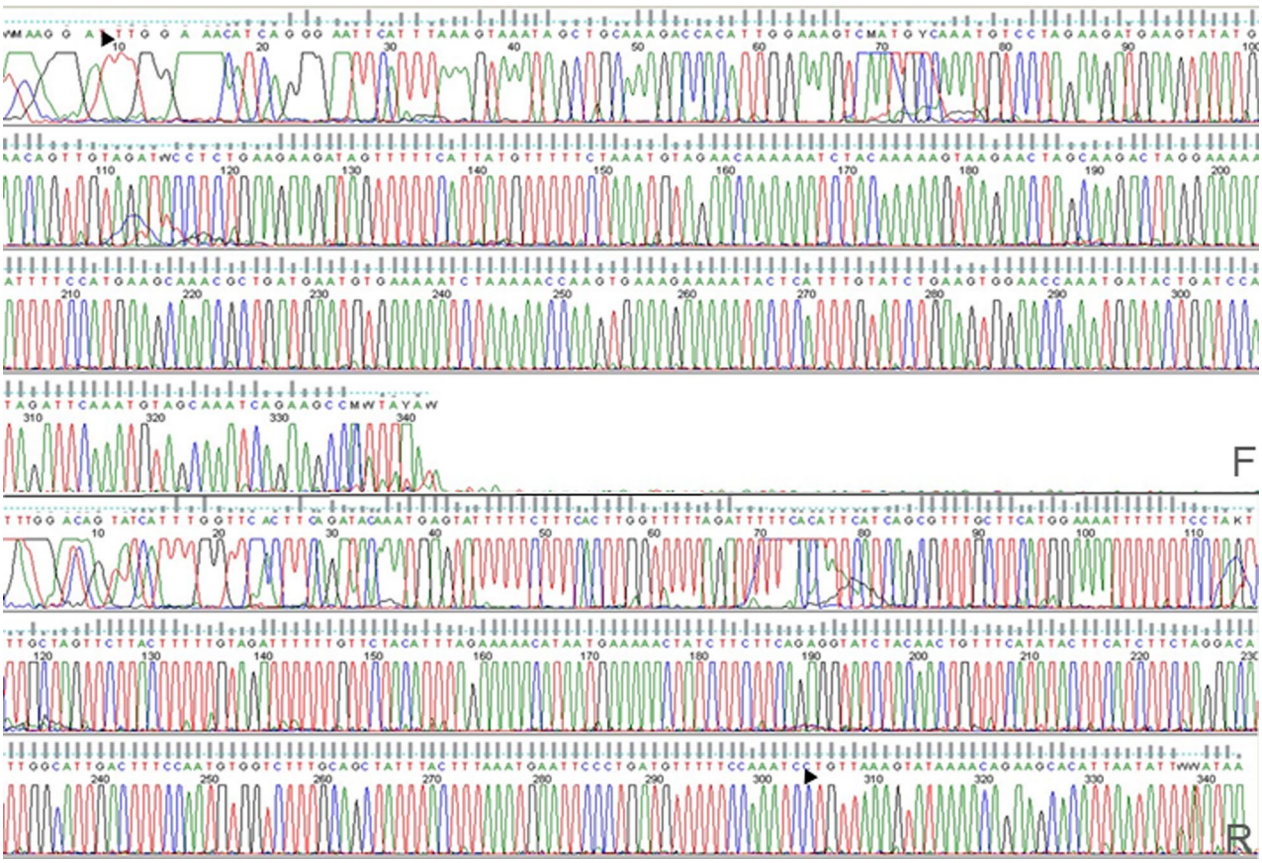

Fig. 5 Images of DNA sequence analysis from exon 10 of the *BRCA2* gene

randomly selected sequence analyses are presented in Figs. 4 and 5.

Discussion

Today, the importance of cancer is becoming more significant. Despite all new treatment approaches, death from cancer is still the second cause of all deaths in developed countries [12, 13]. Among women, breast cancer (BC) is the most fundamental reason for cancer-related deaths [14]. It has been known that women with BC cases in their families have a higher risk of BC development. Furthermore, it has been shown that genetic predisposition is an essential factor for breast, ovarian, colon and some other types of cancer [15]. While ovarian cancer has a lower prevalence compared to breast cancer, its lethality surpasses it by threefold. Projections suggest a significant increase in the mortality rate of ovarian cancer by 2040. This heightened mortality is attributed to the asymptomatic and indolent growth of tumors, delayed symptom onset and the absence of effective screening methods leading to diagnoses at advanced stages. Consequently, ovarian cancer has earned the moniker "silent killer" [16]. Our knowledge about the etiology of familial ovarian cancer has increased as we defined the mutations of tumor suppressor genes on *BRCA1* and *BRCA2* in hereditary breast/ovarian cancer patients and by determining the DNA wrong coupling repair genes such as MSH2 and MLH1 in Lynch syndrome. It is reported that the mutations in *BRCA1* and *BRCA2* genes are responsible for 90% of familial ovarian cancer cases, and individuals carrying these mutations possess 60–70% more risk for ovarian cancer throughout their life span [11].

Changes in the *BRCA1/BRCA2* in 350 Swedish breast–ovarian cancer patients and a control group consisting of 70 individuals with no familial or separate breast–ovarian cancer were examined in a study with DHPLC due to its effectiveness and speediness. Eventually, 80 previously defined mutations (51 in *BRCA1*, 29 in *BRCA2*), 61 clinically significant unknown changes (36 in *BRCA1*, 25 *BRCA2*) and 23 new mutations (2 meaningless, 12 wrong meaningful, three frameshifts, six intronic changes) were observed. Because two of the newly detected changes were more than 1% in the control group, these changes were evaluated as polymorphism [17].

DHPLC investigated mutations in the *BRCA1* and *BRCA2* genes in a study conducted in Korea with 1020 BC patients and 167 control subjects without a familial history of breast cancer. Again, we see that the DHPLC method was used in this study because the individual number was high, facilitating the procedure. Consequently, 78 mutations were observed. These changes were 14 deletions, 38 wrong meaningful and 26 polymorphisms. Three of the 14 deletions were reported as new mutations in the *BRCA*1, and three were reported as new mutations in the *BRCA2*. Two of the newly detected changes were found to be more than 1% in the control group, and therefore, they were evaluated as polymorphism [18].

In the previous study that was conducted in our department, all exons of both genes were scanned with protein truncation test (PTT) and denaturing gradient gel electrophoresis (DGGE) methods in the DNA of 75 patients, including 26 familial breast and/or ovarian cancer patients, six bilateral BC patients, three breast and ovarian cancer patients, 32 early-stage BC patients, five early-stage ovarian cancer patients and three male BC patients. The missense mutations, polymorphisms and intronic region changes of these genes, whose biological significance was unknown entirely, and first-detected modifications were found. These seven detected changes were H513L, H816P and S1577Y changes in the *BRCA1* gene and S326R, G258P, E2903K and N2742S changes in the *BRCA2* gene [11].

Despite having this knowledge about these changes, more studies are necessary with healthy individuals to understand whether these changes are polymorphisms specific to the Turkish population or pathological mutations. DNA samples from 150 healthy women individuals with a clear familial cancer history were studied in the present study to determine this. When the threshold value between mutation and polymorphism is taken as 1%, the DNA sequence alterations seen in 1% or more than 1% of the population are referred to as polymorphism; on the other hand, when they are detected in less than %1 of the population, they are called a mutation. According to this information, in this study conducted with a control group of 150 individuals, the H513L, H816P and S1577Y changes were not detected in the *BRCA1* gene, and S326R, G258P, E2903K and N2742S changes in the *BRCA2* gene. Hence, their incidence is less than 1%, and they have not been mentioned in the literature before. As a result, it is possible to argue that these are the new mutations observed for the first time.

A study was conducted in Turkey on *BRCA1* and *BRCA2* genes with only some exons in 53 familial BC and 52 early-stage breast cancers in 105 Turkish women. In this study, the exons 2, 11, 14 and 20 of the *BRCA1* and exon 11 of the *BRCA2* gene were scanned with PTT (protein truncation test) or the heteroduplex analysis. In the *BRCA1* gene, two 5382 insC mutations were detected—one in breast cancer and one in ovarian cancer patients. Also, in the *BRCA1* gene 1623delTTAAA, 2139delC, 3819delGTAAA, 247delT, 4508delC, IVS-14+1delG mutations and in *BRCA2* 5295insC, 6656delC mutations were spotted in each of the different individuals [19]. In another study, exons 2, 5, 11, 13, 20 and 24 of the BRCA1

gene and exon 11 of the *BRCA2* gene were scanned in 6 hereditary, seven familial, 27 early-stage and ten males with high-risk breast or ovarian cancer in a total of 50 Turkish individuals. In 23 individuals, in addition to previously defined changes, five different polymorphisms, 3034delAAAC and 6880insG mutations in the *BRCA2* gene of 2 individuals and other patients 1201insA and 2080A--> G mutations in the *BRCA1* gene were spotted [20].

Studies that focus on all exons of *BRCA1* and *BRCA2* are insufficient. In one study, all exons of *BRCA1* and *BRCA2* genes of 15 individuals with high-risk group breast and/or ovarian cancer were scanned with PTT or CSGE (Conformation Sensitive Gel Electrophoresis), and 3414delTCAG mutation of *BRCA2,* 5622C--> T and 5382insC mutations of *BRCA1* were spotted [21]. In another study on 15 familial and 87 non-familial ovarian cancer patients, mutation scanning of these genes was performed using the PTT method. The results showed mutations in 17 individuals [22]. Another study with 87 breast and/or ovarian cancer patients was carried out with DNA sequence analysis; and 5382insC mutation and a new polymorphism (3663C--> A) in the BRCA 1 gene, two new mutations (9329insC and 9934insG), a new intronic polymorphism 7069 + 41(TTTT--> AAAG) and an early reported polymorphism (1093A--> C) were found [23].

In this study, DHPLC was used due to its effectiveness. First, samples representing different peaks were analyzed again using the same experimental conditions as the present study. After this second analysis, for the samples still representing other peaks, the PCR, denaturation and hybridization processes were repeated and worked with the DHPLC device again. At the same time, the working conditions of the device were optimized by cooling the room temperature. The device may face a problem while studying the samples. The DHPLC device is a sensitive tool. When the temperature of the working environment is high, the stability of the device might deteriorate, and different peaks can be obtained. Another reason for different peaks can be the incorrect mixing of the test samples and reference samples with different amounts. Different peaks might also be observed if the PCR protocol is not optimized well.

Recent studies have detected that de novo mutations of *BRCA1* and *BRCA2* are increasing; therefore, correctly defining the molecular scanning strategies is becoming more critical. Necessary studies must be carried out on the other members of mutation-detected individuals or families, physicians must be informed and provided with the results of these studies, and an entirely correct genetic consultation must be served by defining all risks.

Because of the location of our country and the lack of funding mutations as in other hereditary diseases, it is crucial for early diagnosis and treatment to screen for changes in these genes in breast, ovarian and prostate cancer risk groups with the DHPLC method, which is a reliable method, and to provide counseling after the risks are determined. Furthermore, when it is considered that new mutations related to these genes will continue to be found for the first time in our population, which is a heterogeneous society, we think it would be helpful to include control groups in future studies.

Abbreviations

BC	Breast cancer
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
CSGE	Conformation sensitive gel electrophoresis
DGGE	Denaturing gradient gel electrophoresis
DHPLC	Denaturing high-performance liquid chromatography
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
PCR	Polymerase chain reaction
PTT	Protein truncation test
UV	Ultraviolet

Acknowledgements
We would like to thank Prof. Dr. Sibel Karaüzüm for DNA samples.

Author contributions
TSS, AEM and GL conceived, designed research and obtained financing for the project. TSS and AEM conducted experiments. TSS and MS wrote the manuscript. GL supervised the experimental work. TSS, AEM and MS performed a literature review. All authors read and approved the manuscript.

Funding
This study was supported by Akdeniz University Scientific Research Projects Management Unit (Project no: 2007.02.0122.015).

Availability of data and materials
The principal data generated and/or analyzed during the current study are included in manuscript.

Declarations

Ethics approval and consent to participate
The study is approved by Akdeniz University Ethical Committee. This study used randomly selected DNA samples from the Akdeniz University Faculty of Medicine, Department of Medical Biology and Genetics archive. The samples in this archive were used as control groups in some previous studies and were obtained from healthy individuals without a familial cancer history. (A total of 150 DNAs that were obtained from 150 healthy individuals with no family history of cancer in Akdeniz University, Faculty of Medicine, Department of Medical Biology and Genetics, used as a control in previous projects, stored at − 20 °C were included in the study after obtaining the informed consent forms.) Written and signed informed consent was obtained from all persons included in the study. The authors assert that all procedures contributing to this work comply with the ethical standards with the Helsinki Declaration.

Consent for publication
Written informed consent to publish was obtained from the parents who participated in the study.

Competing interests
The authors declare that they have no competing interests.

Received: 15 June 2023 Accepted: 1 April 2024
Published online: 09 April 2024

Publisher's Note

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