Wahabi Alzahabi *et al.*
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BRCA mutations: screening for germ-line founder mutations among early-onset Syrian breast cancer patients

Salma Wahabi Alzahabi[1], Maher Saifo[2] and Ghalia Abou Alchamat[1*]

Abstract

Background Breast cancer (BC) is the most common female cancers in many countries including Syria. Familial breast cancer or previous family cancer history are considered significant risk factors. Therefore, detecting the prevalence and founder mutations in the population facilitates genetic counselling, risk assessment and the development of a cost-effective screening strategy. In this study, we investigated the three germ-line founder mutations in the *BRCA1/2* genes: [NM_007294.4 (*BRCA1*):c.68_69del (p.Glu23fs), NM_007294.4 (*BRCA1*):c.5266dup (p.Gln1756fs) and NM_000059.4 (BRCA2):c.5946del (p.Ser1982fs)], to examine their incidence and frequency in early-onset breast cancer cases and determine if they are connected to familial breast cancer. One hundred early diagnosed BC females (≤ 40 years old) with no other type of cancer were recruited. Genomic DNA was isolated from peripheral blood samples, and mutations were investigated using the *Amplification-Created Restriction Site* (ACRS) method.

Results The family history of cancer was observed in 61% of the cases, of which 35% were breast cancer; however, none of the screened mutations were detected among BC patients.

Conclusions The investigated germ-line mutations were not common among Syrian female patients with early-onset BC and were not associated with familial BC. Other mutations in the *BRCA1/2* genes or other genes may have a contributing role. Future studies and the need to launch nationwide mutation screening tests for *BRCA 1/BRCA2* in the Syrian population are recommended.

Keywords Amplification-created restriction site, Breast cancer, *BRCA* gene, Familial breast cancer, Founder mutation

Background

Breast cancer (BC) is the most common and severe female cancer worldwide. Despite rigorous efforts in early detection and treatment, BC remains one of the significant causes of death claiming the lives of hundreds of thousands annually [1]. In Syria, cancer comes third among death causes and BC is the most common cancer in females forming 30% of all cancer cases [2]. The aetiology of BC was intensively studied, and mutations in candidate genes like the tumour suppressor genes *BRCA1* and *BRCA2* responsible for DNA repair and the maintenance of chromosomal stability were considered major risk factors for breast carcinogenesis [3–5]. Family history of cancer especially breast/ovarian cancer is also considered a risk factor and a critical contributor to the BC. Germ-line mutation screening is important to identify women at risk, in addition to its impact on prognosis, diagnosis and targeted therapy [6, 7]. Investigators have discovered that founder mutations are often responsible for the high prevalence of genetic disease in particular populations [8]. Specific germ-line mutations

*Correspondence:
Ghalia Abou Alchamat
dr.ghalia-aboualchamat@hotmail.com; prof.ghalia14@damascusuniversity.edu.sy
[1] Department of Biology, Faculty of Science, Damascus University, Damascus, Syria
[2] Medical Oncology, Scientific Affairs, Faculty of Medicine, Damascus University, Damascus, Syria

in the *BRCA* genes were described as founder mutations [4] and have been detected in hereditary breast cancer in many populations such as in Latin America and Korea [9–11]. Generally, most of the techniques used for mutation detection are unable to detect the numerous deleterious mutations as large deletions or insertions account for 5–15% of the total mutations. Thus, screening for founder mutations is a cost-efficient approach [12].

Mutation detection studies on BC patients are very limited in Syria. This study focused on the three germ-line founder mutations in the *BRCA1/2* genes: NM_007294.4 (*BRCA1*):c.68_69del (p.Glu23fs), NM_007294.4 (*BRCA1*):c.5266dup (p.Gln1756fs) and NM_000059.4 (*BRCA2*):c.5946del (p.Ser1982fs) to examine their incidence and frequency in early-onset breast cancer cases, and determine their association with familial breast cancer.

Methods

Study criteria

One hundred BC female patients with early diagnosis age (≤40 years old) and no other type of cancer were recruited into the study. The selection of patients was independent of any previous cancer family history. Patients were informed about the study and asked to sign a written consent before participation. Clinical data were extracted from the pathological and medical reports archived in patients' own files at al-Biruni University Hospital, where patients receive their treatments. Peripheral blood samples were collected from the patients into EDTA tubes. Samples were collected between August–November 2019 and September 2020. The study protocol was in agreement with the Declaration of Helsinki guidelines1975, as revised in 2000.

Mutation analysis

Genomic DNA extraction

Genomic DNA (gDNA) was isolated from blood samples using *Vivantis Technologies, GF-1, Blood DNA Extraction Kit, Malaysia*, according to the manufacturer's instructions. Purity and concentrations were tested by spectrophotometer (Nanodrop 2000, Thermo Scientific). DNA samples were stored at − 80°C until further analysis.

The amplification-created restriction site (ACRS) method

Mutations were screened using the *Amplification-Created Restriction Site* (ACRS) method. Briefly, the selected method enables the formation or deletion of a restriction enzyme site in a mutated or wild-type (WT) allele using a PCR primer with a mismatched nucleotide. Mutated alleles are differentiated from WT by size. The primers used to amplify the region around the *BRCA1* c.68_69del (p.Glu23fs) and *BRCA2* c.5946del (p.Ser1982fs) mutations were self-designed using AmplifX pro, and Eurofins Genomics' Oligo Analysis Tool. Primer and sequences are shown in Table 1. All PCR reactions contained: 12.5 µl of One PCR™ Mix (2X) (Gene Direx, Inc), 1 µl of each forward and reverse primers (10 µM), 80 ng of the gDNA, and the appropriate amount of nuclease-free water to reach the final volume 25 µl. Each PCR experiment included a negative control in which gDNA was replaced with the same volume of distilled water for contamination control. The PCR reactions were performed using the Eppendorf Master cycler.

HinfI restriction enzyme 2000 U (Thermo Scientific, USA) and *DraIII* restriction enzyme 1000 U (BioLabs, USA) were used to identify and cut the WT-modified allele of the *BRCA1* c.68_69del (p.Glu23fs) and *BRCA2* c.5946del (p.Ser1982fs) mutations, respectively. *BstNI* restriction enzyme 2000U (Thermo Scientific, USA) was used for the detection of the *BRCA1* c.5266dup (p.Gln1756fs) mutation by recognizing and cutting the mutant-modified allele. All digestion reactions contained: 10 µl of the PCR product, 2 µl of the enzyme buffer, 1 µl of the restriction enzyme and 18 µl of nuclease-free water. Digestion reactions were done according to manufacturer's instructions. Fragments were analysed by3% agarose gel electrophoresis using 100 bp DNA Ladder (Thermo Scientific, GeneRuler), visualized and photographed using a UV transilluminator (Olympus). Validation of *BstNI* restriction enzyme functionality, and confirmation of restriction site creation for the *BRCA1* c.5266dup

Table 1 The characterization of the primers used in the study

Mutation	Sequence*	Annealing temperature	PCR product	References
NM_007294.4(*BRCA1*): c.68_69del (p.Glu23fs)	F1- 5′ GAAGTTGTCATTTTATAAACCTTT 3′ R2- 5′ TGACTTACCAGATGGGA**G**AC 3′	55 °C	170 bp	Self-designed
NM_007294.4(*BRCA1*): c.5266dup(p.Gln1756fs)	F3- 5′ CCAAAGCGAGCAAGAGAATC**A**C 3′ R4- 5′ GACGGGAATCCAAATTACACAG 3′	62.5 °C	273 bp	[13, 14]
NM_000059.4(*BRCA2*): c.5946del (p.Ser1982fs)	F5- 5′ TTGTGGGATTTTTAGCACA**C**CAAG3′ R6- 5′ GAAGACTATGCTCAGTTCTGAT 3′	59.3 °C	497 bp	Self-designed

*Mismatched bases are underlined

(p.Gln1756fs) mutation (since no cut is expected for WT genotype), was tested on a sample of a healthy control by DNA sequencing using Big Dye Terminator chemistry (Applied Biosystems, version 3.1) following the manufacturer's instructions.

Results

One hundred early-onset breast cancer Syrian females participated in this study. Patients' ages at the time of diagnosis ranged between 20 and 40 years, with an average age of 32.5 years old.

Patients were from the various Syrian governorates, particularly Damascus and its countryside (33%), and none were of Jewish population descendants. According to our findings, 61% of the patients had a family history of cancer (61/100), and over one-third had a positive family history of breast cancer (35/100), as shown in Table 2.

Clinical data retrieved from patients' files showed that 57% of the patients had cancer in the left breast, 40% had cancer in the right breast, and only 3% had bilateral BC. Unfortunately, data regarding the histological grade and type of BC for some patients were missing from their medical files. However, out of 86 patients, 48.84% (42/86) had grade II, 51.16% (44/86) had grade III BC and none had grade I. Of 88 patients, 88.64% (78/88) had ductal carcinoma, 6.82% (6/88) had lobular carcinoma, and 4.55% (4/88) had both ductal and lobular carcinomas. Furthermore, the hormone receptors status was as follows: of 96 patients, ER status was positive in 64.58% (62/96), PR was positive in 59.38% (57/96), and HER2 was positive in 46.88% (45/96). Triple-negative BC phenotype was found in 9 patients (group1), and triple-positive was found in 18(group5). Table 3 displays detailed information about the status of the hormone receptors.

The expected sizes were achieved by amplifying the regions surrounding the three studied mutations using the specifically designed primers. Confirmation of restriction site creation near the sequence variant for the *BRCA1* c.5266dup (p.Gln1756fs) mutation was confirmed by DNA sequencing as shown in Fig. 1. None of the three studied germ-line founder mutations in the *BRCA1/2* genes were detected in BC patients (Figs. 2, 3, 4).

Table 3 The hormone receptors status in BC patients

# of Patients	ER	PR	HER2	Group
9	−	−	−	1
35	+	+	−	2
5	+	−	−	3
2	−	+	−	4
18	+	+	+	5
21	−	−	+	6
4	+	−	+	7
2	−	+	+	8
96	Total			

Discussion

Breast cancer commonly occurs as a sporadic event; however, approximately 10–15% of cases are considered to be hereditary. The possibility of developing breast cancer increases when there is a family history and cancer is diagnosed at an early age [15]. Molecular testing has become an integral part of breast cancer management globally; patients are usually offered a genetic test to determine if an inherited cancer syndrome is present [16]. Early diagnosis and treatment can be successfully achieved by the identification of patients at risk for hereditary breast cancer [6]. Furthermore, breast cancer risk is greatly increased due to certain germ-line mutations, like those in the *BRCA1* and *BRCA2* genes, reaching up to 25% [17]. Previous reports revealed that in particular populations such as the Ashkenazi Jewish, Moroccan, South Asia, and in some European countries, the *BRCA1* c.68_69del (p.Glu23fs), the *BRCA1* c.5266dup (p.Gln1756fs) and the *BRCA2* c.5946del (p.Ser1982fs) mutations are commonly found and are considered as founder mutations [13, 18–22]. In this study, the three germ-line founder mutations mentioned above were investigated for possibly present in 100 early diagnosed breast cancer patients, but none were detected. To the best of our knowledge, this study is the first to investigate

Table 2 Detailed description of the patients with positive family history of breast cancer

Positive family history of BC	Number of patients
One of the I-degree relatives (mother, father, daughter) or the II-degree relatives (sister, brother, grandma, grandfather) previously diagnosed with BC	10
Two or more relatives diagnosed with BC at the same side of family	8
One of the III-degree relatives (aunt, uncle) or the IV-degree relatives (cousins) diagnosed with BC	17
Total	35

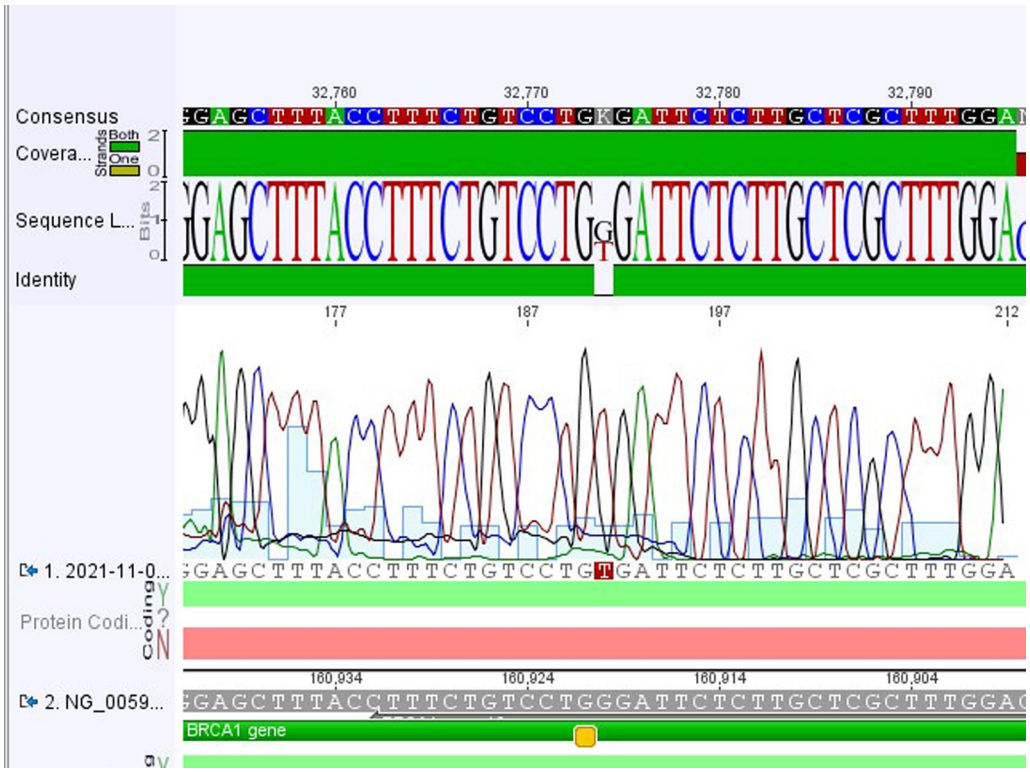

Fig. 1 Sequence alignment result of a healthy control sample against the reference *BRCA1* genome using Uber Geneious 4.8.4 bioinformatics tool. Creation of the restriction site near the sequence variant for the *BRCA1*:c.5266dup (p.Gln1756fs) mutation was successfully generated; the inserted T nucleotide was marked in red

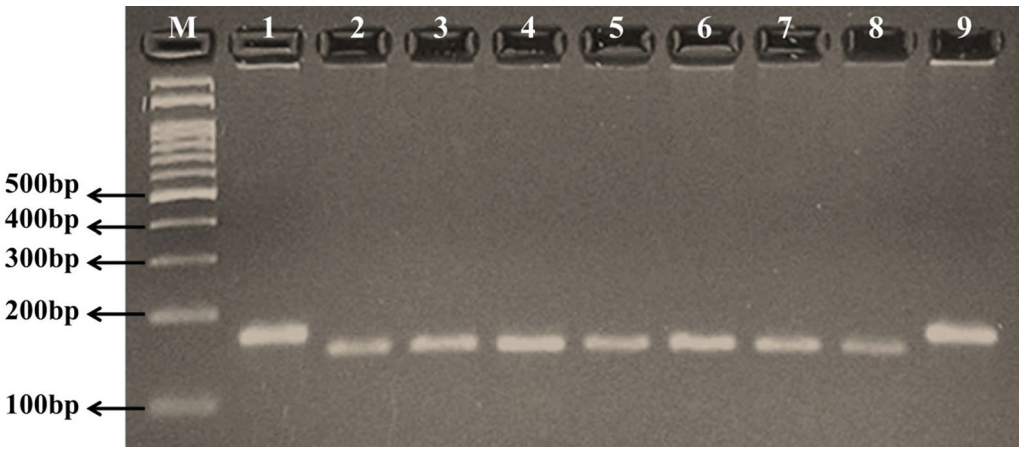

Fig. 2 Agarose gel electrophoresis (3%) of PCR and digested products for the detection of the *BRCA1*:c.68_69del (p.Glu23fs) mutation. *HinfI* restriction enzyme was used which identifies and cuts the wild-type (WT) modified allele yielding a 150 + 20 bp bands. M: 100 bp DNA Ladder, *Lane* 1&9: ACRS-PCR amplified product (170 bp), *Lanes* 2–8: one digest product (150 bp) correspond to homo WT genotype

the *BRCA1* c.5266dup (p.Gln1756fs) mutation and the second to report the status of the two mutations [*BRCA1* c.68_69del (p.Glu23fs) and *BRCA2* c.5946del (p.Ser1982fs)] among early-onset breast cancer patients in Syria. A previous study by Khalil et al. [23] conducted on 50 Syrian BC patients showed similar results. Therefore, one could conclude that the three studied mutations are not associated with familial breast cancer or have any role in the clinical presentation of early-onset breast cancer in Syrian females. Numerous studies have

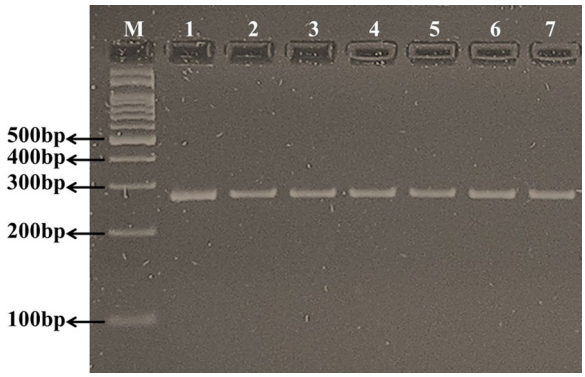

Fig. 3 Agarose gel electrophoresis (3%) of PCR and digested products for the detection of the *BRCA1*:c.5266dup (p.Gln1756fs) mutation. *BstNI* restriction enzyme was used which identifies and cuts the mutated modified allele. M: 100 bp DNA Ladder, *Lane* 1: ACRS-PCR amplified product (273 bp), *Lanes* 2–7: one undigested product (273bp) correspond to homo WT genotype. *Note that if the mutant allele was present two distinctive bands must be recognized (250 + 23 bp)*

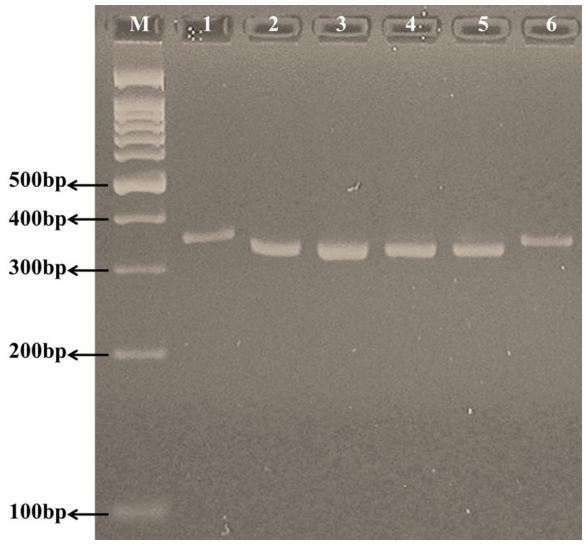

Fig. 4 Agarose gel electrophoresis (3%) of PCR and digested products for the detection of the *BRCA2*:c.5946del (p.Ser1982fs) mutation. *DraIII* restriction enzyme was used which identifies and cuts the wild-type (WT) modified allele yielding a 333 + 23 bp bands. M: 100 bp DNA Ladder, *Lane* 1&6: ACRS-PCR amplified product (356bp), *Lanes* 2–5: one digest product (333bp) correspond to homo WT genotype

examined germ-line mutations in the *BRCA1/2* genes among diverse populations, and have resulted in contradictory conclusions. For example, none of the screened mutations in our study were found in some neighbouring countries such as Jordan, where the whole coding exons and the flanking intronic regions of the *BRCA1/BRCA2* genes were screened. Also in Lebanon, where high-risk breast cancer Lebanese patients were studied, and in Saudi Arabia where Arab breast and ovarian cancer patients were studied [24–26]. However, the frequency of the screened mutations in Egypt varied among different studies [27, 28], and interestingly, the two common germ-line founder mutations in the *BRCA1* gene were also found in a cohort of familial ovarian cancer Egyptian patients [29]. Furthermore, in Tunisia, the frequency of the *BRCA1* c.5266dup (p.Gln1756fs) mutation differed also among early diagnosed patients with a positive family history of breast cancer [30, 31]. Additionally, it was suggested that beside a positive family history or early diagnose, triple-negative breast cancer genotype should be evaluated in the guidelines for genetic analysis of the *BRCA1* and *BRCA2* genes [32]. Studies found that the *BRCA1* c.5266dup (p.Gln1756fs) mutation could be considered as an effective selective criterion for genetic test among triple-negative breast cancer patients [33, 34]. Nevertheless, in our study, this mutation was not found among our patients (group 1). These results suggest that the commonly studied mutations found in some populations may not exist in our population.

Conclusion

The germ-line founder mutations screened in this study were not common, not connected to familial breast cancer or have any role in the clinical presentation of the studied Syrian early-onset breast cancer females. Different founder mutations in the *BRCA1/2* genes or in other genes may be responsible. Our findings added substantial data to the scientific literature regarding the Syrian population and emphasized the need to launch a nationwide screening test for *BRCA 1/BRCA2* in the Syrian population.

Abbreviations

ACRS	Amplification-created restriction site
BC	Breast cancer
BRCA1	Breast cancer1 gene
BRCA2	Breast cancer 2 gene
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
gDNA	Genomic DNA
PCR	Polymerase chain reaction
PR	Progesterone receptor
WT	Wild type

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Author contributions

SWZ contributed to study design, collected blood samples, performed molecular study, analysed data and results and revised the manuscript. GC was involved in study design, analysed data and results and contributed to writing

and revising the manuscript. MS was involved in revising the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All the datasets were presented in this study, and any additional data are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the ethics committee of the Damascus University in Syria. All methods were carried out in accordance with the Declaration of Helsinki. All participants were fully informed about the study, and informed consent was obtained from each participant before admitted into the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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