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A case–control study of *BRCA1* founder mutations 185delAG and 5382insC in a cohort of Egyptian ovarian cancer patients using pyrosequencing technique

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Abstract

Background: Ovarian cancer (OC) is considered a leading cause of death among women with gynecological malignancies. OC, like breast cancer, shows a familial predisposition to germline mutations in genes *BRCA1* or *BRCA2*, which have proved to play important roles in the incidence and progression of cancers. In Arab countries there are limited data concerning *BRCA1* or *BRCA2* founder mutations associated with familial ovarian cancer (FOC). Therefore, the aim of this pilot study was to assess two common founder mutations of *BRCA1* (185delAG and 5382insC) in a cohort of Egyptian patients with FOC. The study included fifty female patients with FOC and twenty healthy controls. Clinical, laboratory, and pathological findings were assessed as well as response to therapy. Genetic testing for *BRCA1* (185delAG and 5382insC) mutations was performed on peripheral blood samples using a short-fragment sequencer (pyrosequencer).

Results: The *BRCA1* 185delAG mutation was not observed in either the FOC patients or the controls. However, the carrier frequency of heterozygous *BRCA1* 5382insC mutation was 8%. All the FOC patients with a *BRCA1* 5382insC mutation had a positive family history of cancer ($p = 0.009$). All carriers of the *BRCA1* 5382insC mutation showed a preliminary good response to chemotherapy. The majority of carrier patients were diagnosed at an advanced stage of the disease with high-grade tumors and distant metastasis (75% of cases).

Conclusion: The frequency of the *BRCA1* 5382insC mutation in FOC patients was 8%. The strong association between the mutation and the positive family history suggests that a wider screening for *BRCA1* founder mutations would be valuable in predicting high-risk individuals.

Keywords: Familial ovarian cancer, *BRCA1* gene, *BRCA2* gene, Founder mutation, Pyrosequencing

Background

Among the most common gynecological malignancies, ovarian cancer (OC) ranks third after cervical cancer and uterine cancer. OC has a poor prognosis and a fatal outcome in those affected. The main reason for the high

mortality rate in OC is due to the fact that in the early stages, most patients are asymptomatic or have non-specific symptoms. The silent spread of OC in conjunction with the lack of satisfactory screening tests mean that advanced, widespread disease often occurs before diagnosis [1, 2].

The exact cause of familial ovarian cancer (FOC) is not well understood and may be attributed to genetic factors, environmental or lifestyle factors, or it may occur by chance [3, 4]. It has been found that 8–15% of OC patients have germline mutations of the *BRCA* genes

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(BRCA1 and BRCA2) and this is considered as one of the underlying causes of FOC [5, 6].

BRCA 1 and 2 are tumor suppressor genes that play an important role in DNA repair and the maintenance of chromosomal stability through homologous recombination (HR). Any change in the nucleotide sequence of either BRCA 1 or BRCA 2 genes results in loss of heterozygosity, genome instability, and an increased risk of malignancy, usually as OC or breast cancer (BC) [7–9].

There are three common germline founder mutations implicated in FOC. Two founder mutations are found in the BRCA1 gene (185delAG and 5382insC), and one founder mutation in the BRCA2 gene (BRCA2 6174delT) [8].

Both BRCA1 185delAG and BRCA1 5382insC founder mutations are frameshift mutations that result in truncated non-functioning proteins. In the case of the BRCA1 185delA mutation, there is a deletion of adenine and guanine at position 185 of exon 2 of the BRCA1 gene [10]. With the BRCA1 5382insC mutation, there is an insertion of a cytosine nucleotide at position 5382 of exon 20.

The BRCA1 5382insC mutation originated in northern areas of Russia and spread to Ashkenazi Jews and other populations in Eastern Europe [11, 12]. Previous studies investigated the frequency of those mutations using different techniques. BRCA1 185delAG was studied using duplex/multiplex-PCR [13], while the 5382insC mutation was detected by ARMS-based PCR [14]. Both studies were performed in Eastern India [13, 14].

However, there are a lack of reports regarding the carrier frequency of BRCA1 founder mutations in the Middle East and Africa. A recent study done by Ashour and Ezzat Shafik [15] on 104 epithelial OC patients of different ancestries (61.54% of patients were of Arabic origin) revealed 21 pathogenic variants in 22 patients of Arabic and Asian origins. This study was done through sequencing the translational exons of BRCA 1 and 2 and the immediately adjacent introns.

The increased use of germline genetic testing in patients with OC or BC has had a significant effect on cancer care [16] because the presence of BRCA1 or BRCA2 mutations can alter the management of OC patients to involve, for example, targeted therapy with Poly (ADP-ribose) polymerase (PARP) inhibitors [17].

The traditional Sanger sequencing is relatively expensive compared to the short-fragment sequencer (pyrosequencer) [18]. The use of the cost-effective method of pyrosequencing in detecting point mutations has a great significance in determining mutation carriers, whether in patients or at-risk relatives. Consequently, the purpose of our work was to detect two well-known BRCA1 founder mutations: 185delAG and 5382insC in Egyptian females with FOC using a pyrosequencer.

Methods

Patients

Seventy females were enrolled in the present study. Fifty FOC patients were recruited from the inpatient and outpatient clinics of the oncology department during the period from June 2017 to June 2018. Twenty healthy, age-matched females with a negative family history were enrolled as controls. All patients signed a written, informed consent, and the study was approved by the local ethics committee. The study protocol was in agreement with the Declaration of Helsinki guidelines 1975, as revised in 2000.

Females who participated in the study were selected by having one or more of the following inclusion criteria:

1. A first-degree relative (mother, sister, or daughter) or second-degree relative who has ovarian, colorectal, or breast cancer.
2. A personal history of breast cancer before age 40 years.
3. A personal history of breast cancer diagnosed before age 50 years, and/or one or more relatives (first or second degree) who have breast cancer or ovarian cancer or both.

Any patient diagnosed with OC only and without personal or reported family history was excluded. Data were collected for age, menstruation, marital status, parity, and family history of breast, ovarian, and colorectal cancer. A full clinical examination was done.

Radiological investigations including ultrasonography examination and computed tomography (CT) of the abdomen and pelvis were done at the time of diagnosis in conjunction with routine laboratory investigations. Tumor markers as carbohydrate antigens (CA 125, CA 19.9, and CEA) and histopathological examination after definitive surgical procedure, tumor stage, and metastasis were extracted from the medical records of each patient.

Mutation analysis

DNA extraction

For mutation detection, DNA extraction was done using a QIAamp Blood Mini Kit (catalog no. 51104). DNA concentration and quality were assessed by a NanoDrop spectrophotometer (NanoDrop ND-2000, ThermoScientific, USA).

PCR amplification of target regions

PCR amplification of target regions was done using a Pyromark PCR kit (catalog number: 978703, Qiagen). The sequence of oligonucleotide primers flanking the mutation loci were used as described: [10] BRCA1 185delAG forward: 5'-AAGTTGTCATTTTATAAACCTT-3',

reverse: 5'-TGTCTTTTCTTCCCTAGTATGT-3'/biotin, sequencing primer 5'-TGACTTACCAGATGGGA-3'/BRCA1 5382insC forward: 5'-AAAGCGAGCAAGAGAATCCC-3', reverse 5'-TGGGGTGAGATTTTGTCAAC-3'/biotin, sequencing primer: 5'-CGAGCAAGAGAAATCCC-3'

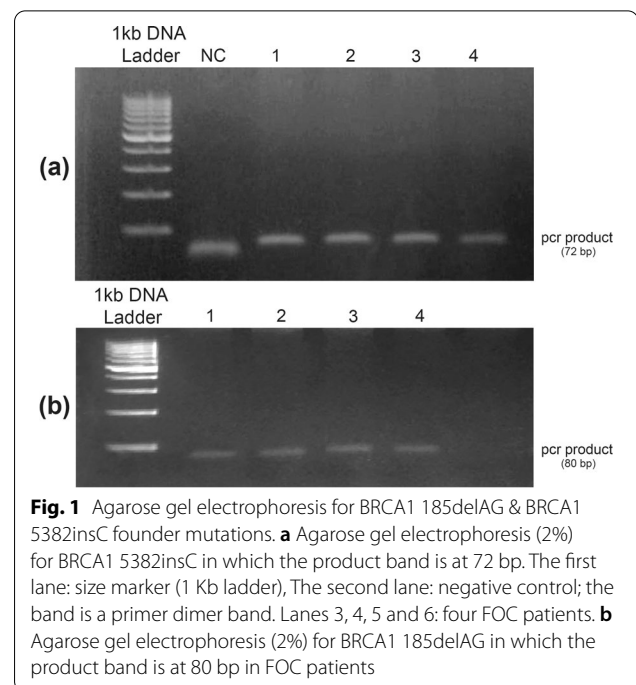
The PCR reaction mix was as follows: 12.5 µl of PyroMark PCR Master Mix (2X), 1 µl of each forward and reverse primer (10 µmol), 2.5 µl Coral Load Concentrate (10X), 5.5 µl RNase-free water and 2.5 µl Template DNA. The PyroMark PCR Master Mix contains HotStarTaq DNA Polymerase and optimized PyroMark PCR Buffer (includes 3 mM MgCl₂ and dNTPs). PCR was performed using Thermo Scientific Arktik Thermal Cycler PCR Machine (Arktik thermocycler, ThermoScientific, USA). The thermal cycling conditions started with an initial activation for 15 min at 95 °C then for BRCA1 185delAG: 50 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and extension for 30 s at 72 °C. For BRCA1 5382insC the amplification was done for 45 cycles with annealing temperature at 55 °C for 30 s. The final extension step for both mutations was for 10 min at 72 °C.

Pyrosequencing

Before running the pyrosequencer, it is necessary to confirm the presence of a single clear PCR product over the gel. Therefore, PCR amplicons were checked over 2% agarose gel electrophoresis; a band sized 72 bp was detected in BRCA1 5382insC, while in BRCA1 185delAG, the product band size was 80 bp (Fig. 1). Pyrosequencing was then performed using Qiagen Pyromark Q24 GOLD kit (Catalog No. 970802), Samples were loaded on a PyroMark Q24 following the manufacturer's instructions.

Briefly, pyrosequencing is based on hybridization of the sequencing primer to a single-stranded PCR-amplified DNA template. The template is incubated with enzymes and substrates that are supplied in the pyrosequencing kit. During sequencing, the four nucleotides [adenine (A), thymine (T), cytosine (C), guanine (G)] are added sequentially. If a nucleotide is complementary to a base in the template strand, it will be incorporated into a DNA strand by a polymerase enzyme. Each incorporation event is accompanied by release of pyrophosphate (PPi). ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate. This drives the conversion of luciferin to oxyluciferin by luciferase enzyme, generating a visible light in amounts proportional to the amount of ATP. Light is detected using charged coupled devices (CCDs) and seen as a peak.

Internal controls were obtained by negative insertion of nucleotide dispensations. Analysis of the results was done by PyroMark Q24 software in the form of a pyrogram for each sample, where peak heights are proportional to the



nucleotides' numbers that are incorporated with each dispensation. For BRCA1 185delAG, the intensities of C and T peaks were approximately half of those in the wild-type internal controls, indicating a deletion of CT in one allele. While for the BRCA1 5382insC mutation, incorporation of an extra C was present.

Data were fed to the computer and analyzed using a Graphic PAD Prism software package version 6.0. Qualitative data were described using numbers and percentages. Quantitative data were described by the mean and standard deviation. Chi-square test, Fisher's exact test, or Monte Carlo correction were used to compare between the carrier and non-carrier groups. Student t test was used for normally distributed quantitative variables and Mann Whitney test for abnormally distributed quantitative variables. P-values less than 0.05 were considered statistically significant.

Results

The mean age of FOC patients included in the study was 50.08 ± 14.56 years. Comparing FOC patients and controls revealed no significant difference in age (Additional file 1: Table S1). Pyrosequencing of the DNA samples of FOC patients and controls revealed the absence of either heterozygous or homozygous BRCA1 185delA mutations (Fig. 2).

On the other hand, as Fig. 2 shows, heterozygous BRCA1 5382insC mutation was found in 4 out of 50 FOC patients with a carrier frequency of 8% (95% CI 2.2–19.2).

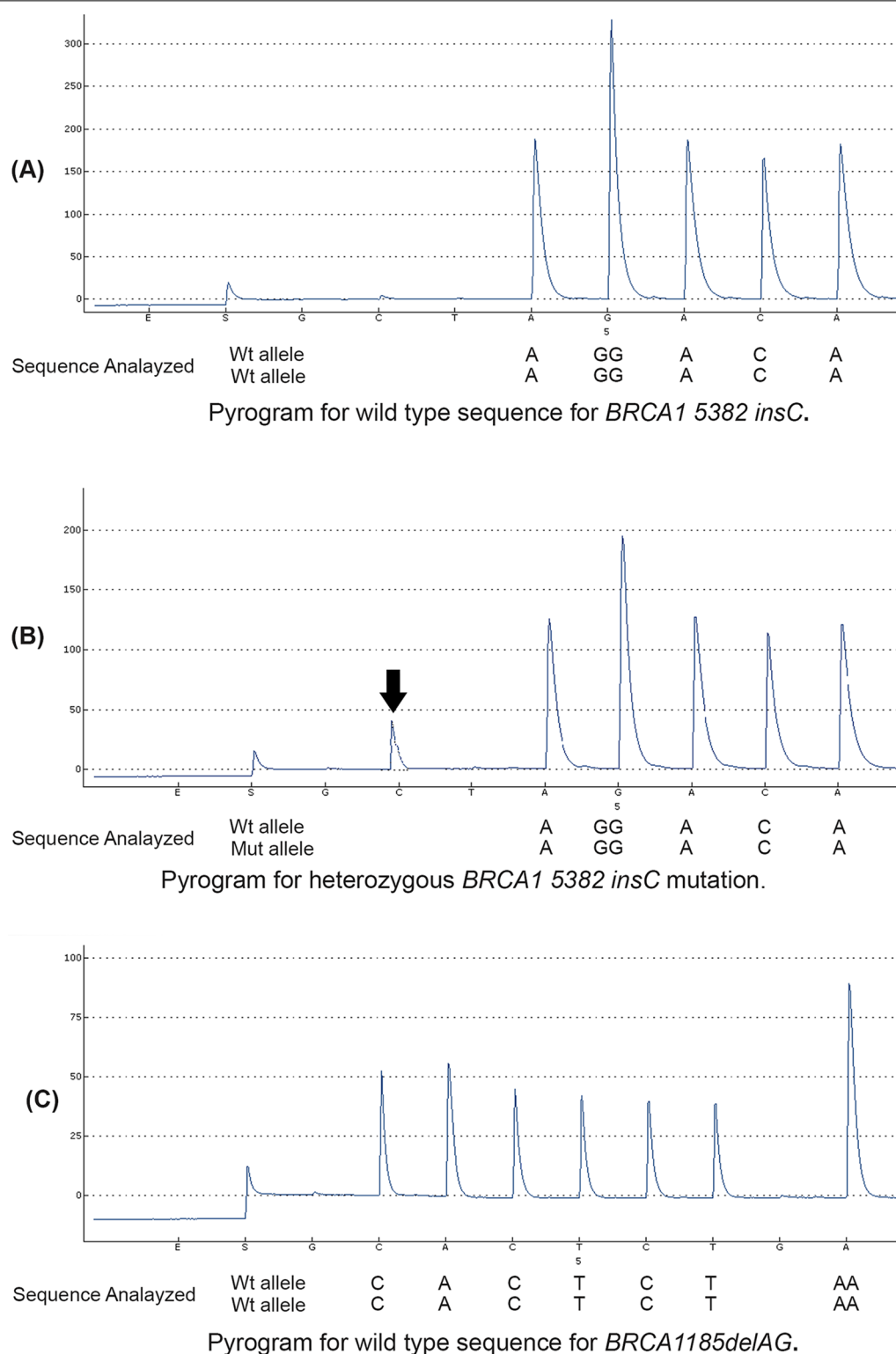


Fig. 2 Representative pyrogram for *BRCA1 185delAG* and *BRCA1 5382 insC* founder mutation. **A, B** Pyrogram for wild-type sequence for *BRCA1 5382 insC*. **B** Pyrogram for heterozygous *BRCA1 5382 insC* mutation in a carrier FOC patient (case number 1). At position 2 there is an insertion of extra C. Negative internal controls were obtained by negative nucleotide dispensation of G unit at position 1 and T unit at position 3. **C** At the wild-type sequence for *BRCA1 185delAG*, the generated peak heights for nucleotide dispensations at positions 2, 3, 4, 5, 6, and 7 (CACTCT in the reverse strand) are approximately equivalent and represent incorporation of a single nucleotide in both alleles (2 light units), but the signal intensity of A at position 9 is two times greater (4 light units) and reflects the incorporation of two as in tandem in both alleles

However, BRCA1 5382insC mutation was not detected among the controls. Demographic and clinical characteristics of BRCA1 5382insC mutation in carrier patients are summarized in Table 1.

According to the presence of heterozygous BRCA1 5382insC, the FOC patients were further divided into two subgroups: carriers and non-carriers. The mean age of carriers was 48.25 years, and for non-carriers it was 56.50 years. Histopathological examination showed papillary serous invasive carcinoma in most of the FOC patients in the present study (44%): 45% of non-carriers and 25% of carriers (Additional file 1: Table S2). For the most part, OC was diagnosed in advanced stages; 60.9% of non-carriers and 75% of carriers were stage III–IV. High-grade tumors (G 3) were manifested in both groups. The difference between carriers and non-carriers regarding cancer stage, grade, or metastasis was not significant ($p=0.421$, $p=1.000$, and $p=0.14$ respectively) (Additional file 1: Tables S3 and S4). In addition, Additional file 1: Table S5 shows that the difference between carriers and non-carriers concerning the three measured tumor markers (CA125, CA19.9, and CEA) was not significant ($p=0.174$, $p=0.508$, and $p=0.844$ respectively).

As Table 2 shows, there was no significant difference among the FOC subgroups regarding marital status, parity, and menopause. However, all carriers (100%) had at least one first-degree relative affected by cancer (OC, BC, or colon cancer) with a significant difference between both carriers and non-carriers ($p=0.009$). A pedigree of the carriers' families is demonstrated in Fig. 3.

Table 2 Comparison between BRCA1 5382insC carriers and non-carriers

Characteristic	FOC patients				FEp
	Non-carrier (n = 46)		Carrier (n = 4)		
	No	%	No	%	
<i>Marital status</i>					
Not married	4	8.7	0	0.0	1.000
Married	42	91.3	4	100.0	
<i>State of menopause</i>					
Premenopausal	13	28.3	1	25.0	1.000
Menopausal	33	71.7	3	75.0	
<i>Parity</i>					
Nullipara	5	10.9	1	25.0	0.563
Unipara	3	6.5	0	0.0	
Multipara	38	82.6	3	75.0	
<i>Affected family members</i>					
One	35	76.1	1	25.0	0.009*
Two	4	8.7	2	50.0	
Three	0	0.0	1	25.0	
<i>Response to chemotherapy</i>					
Good	20	43.5	4	100.0	0.046*
No response	26	56.5	0	0.0	
<i>Survival</i>					
Died	1	2.2	0	0.0	1.000
Survived	45	97.8	4	100.0	

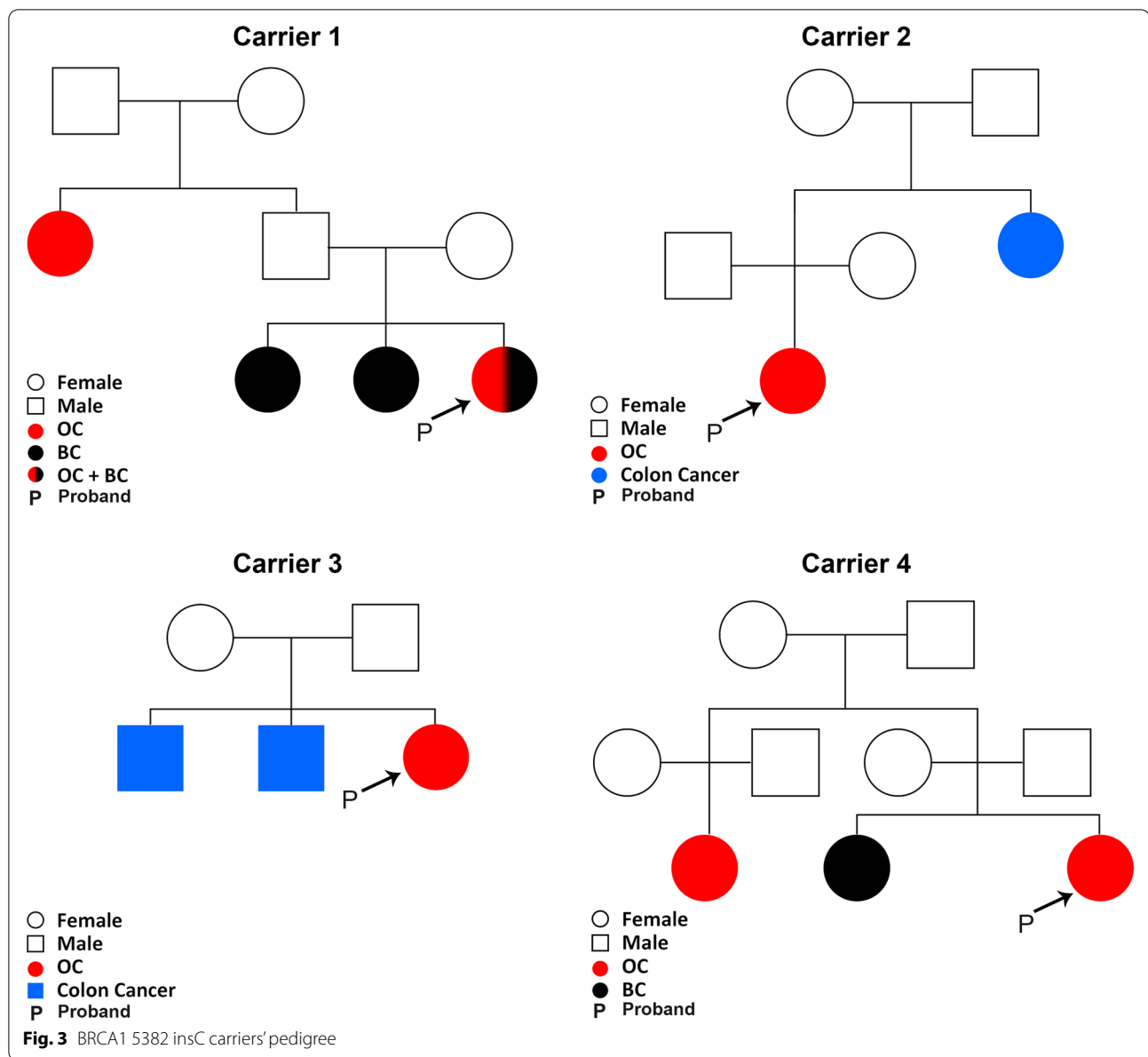
FE, Fisher's exact test

*Statistically significant at $p \leq 0.05$

Table 1 Demographic and clinicopathological characteristics of BRCA1 5382insC carrier patients

	Carrier 1	Carrier 2	Carrier 3	Carrier 4
Age	43	52	48	50
Age of first presentation	40	50	45	48
Menopausal status	Premenopausal	Menopausal	Menopausal	Menopausal
Parity status	Five	Four	Zero	Three
Pathology	Metastatic undifferentiated carcinoma	Invasive papillary mucinous cystadenocarcinoma	Invasive papillary serous adenocarcinoma	Endometrioid carcinoma
Stage	IV	IV	IV	I
Grade	3	3	3	1
Family history	Personal history of neglected breast lobular carcinoma, aunt (OC), 2 sisters (BC)	Her aunt (Colon cancer)	2 brothers had colon cancer	Sister(BC), cousin (OC)
Bone metastasis	Positive	Free	Free	Free
Visceral metastasis	Positive(liver)	Positive(liver)	Positive(liver)	Free
Survivability	Still alive	Still alive	Still alive	Still alive
Response to chemotherapy	Respond	Respond	Respond	Respond

BC, breast cancer; OC, ovarian cancer; MAC, metastatic undifferentiated carcinoma; PSCC, papillary serous adenocarcinoma; PMCAC, papillary mucinous cystadenocarcinoma; EC, endometrioid carcinoma



With regard to the response to platinum-taxane chemotherapy among 46 non-mutation/non-carrier patients, twenty had a preliminary good response to chemotherapy, while all mutation/carrier patients showed a good response (Table 2).

Discussion

OC is a worldwide gynecological malignancy. Although it is less common than BC, its importance comes from the occult nature of its spread leading to late diagnosis and poor prognosis. The 5-year survival rate is estimated to be approximately 30% in advanced stages [19].

The current study aimed to detect two common BRCA1 founder mutations (185delAG and 5382insC) to assess the carrier frequency in a cohort of Egyptian females with FOC using the pyrosequencing technique.

Heterozygous BRCA1 5382insC mutation was found in 4 out of 50 FOC patients enrolled in the current study with a carrier frequency of 8%. This was within the previously reported range of 5–15% [20]. Synoweic et al. [21] reported BRCA1 5382insC mutation in 9.6% of Polish FOC patients. Additionally, Moslehi et al. [22] found a frequency of 6.7% in Ashkenazi Jewish patients. Some discrepancy in the frequency of BRCA1 5382insC

mutation carriers has been found among different populations, and it seems to be affected by ethnicity, study design, and methods of detection.

On the other hand, we did not find either heterozygous or homozygous BRCA1 185delAG, mutations among the FOC patients. Again, the frequency of the BRCA1 185delAG mutation is variable among different populations; a low carrier frequency of 1.1% was reported in Morocco [23], while a higher carrier frequency of 16.4% was found in India [24].

The main significant finding regarding the BRCA1 5382insC mutation in our study was the strong association with the history of cancer in at least one first-degree relative, particularly breast, ovarian, or colon cancer (100% in carriers vs. 84.8% in non-carriers; $p=0.009$). This issue could be explained by the fact that germline mutations of BRCA1 pass through an autosomal dominant pattern during familial aggregation [22, 25, 26]. The strong association between a family history of OC and/or BC in first-degree relatives and BRCA1 founder mutation highlights the importance of screening for BRCA mutations in high-risk families in order to provide proper genetic counseling and prophylactic management such as salpingo-oophorectomy, which may reduce the risk of developing OC by 90% [27, 28].

Also in the present study, the 4 BRCA1 5382insC mutation carriers showed a preliminary good response to chemotherapy. Many studies showed BRCA1 mutation carriers had a better response to chemotherapy and prognosis than non-carriers [29–31], while several studies found no significant difference between carriers and non-carriers regarding the chemotherapeutic response [25]. BRCA mutations enhance the DNA damaging effect of chemotherapeutic agents such as platinum, causing an increase in platinum sensitivity, which makes it the cornerstone of the initial regimen of treatment in such cases. This can be explained by it impairing the DNA repair mechanism of mutated cells, so they remain susceptible to damage by cytotoxic drugs [29]. However, platinum resistance may occur in some BRCA1 mutation carriers and results in poor prognosis and/or disease recurrence. In those patients, a shift to alternative drugs such as PARP inhibitors might be valuable [32, 33].

Intracellular PARP1 and PARP 2 enzymes play a crucial role in DNA repair mechanisms. Inhibition of such enzymes by PARP inhibitors leads to the accumulation of single-stranded DNA breaks and impaired DNA repair mechanisms with subsequent cytotoxicity and enhanced apoptotic effect [34].

The age of onset and tumor histopathology stage were also compared between carrier and non-carrier patients. It was observed that BRCA1 5382insC mutation was

associated with advanced-stage, high-grade OC tumors and evident metastasis in 75% of enrolled patients. However, the small sample size rendered this association insignificant.

Overall, despite good facilities for diagnosis and management, there are no adequate data on the prevalence of BRCA1 germline mutations in Arab women with FOC; this is in addition to insufficient research on the underlying genetic mechanisms of OC and its familial aggregation. Based on the literature review, we can claim that the current study is the first in Egypt to apply screening for BRCA1 founder mutations in FOC patients using the pyrosequencing technique. Pyrosequencing is characterized by its high accuracy to pick up single nucleotide changes in short DNA fragments. Furthermore, uncomplicated automation in comparison with other methods, along with its reasonable cost, renders it more accessible for predicting disease outcome and high-risk individuals [35, 36].

Conclusion

The frequency of BRCA1 5382insC mutation in a cohort of Egyptian FOC patients was 8%. There was a strong association between family history of HBOC-related tumors and BRCA1 mutations. Moreover, all BRCA1 5382insC mutation carriers showed a preliminary good response to chemotherapy. Therefore, screening for BRCA1 5382insC mutation is valuable for the prevention of OC and for offering appropriate genetic counseling to high-risk families. A large-scale follow-up study with an expanded screening panel of BRCA mutations including those that have been recently recognized in Arabic patients [15] is highly recommended to assess the underlying molecular mechanisms.

Abbreviations

AST: Aspartate aminotransferase; BC: Breast cancer; BRCA1: Breast cancer1 gene; BRCA2: Breast cancer 2 gene; FOC: Familial ovarian cancer; HBOC: Hereditary breast ovarian cancer syndrome; OC: Ovarian cancer; PARP: Poly ADP ribose polymerase inhibitors.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43042-022-00226-8>.

Additional file 1: Comparison between BRCA1 5382insC carriers and non-carriers according to different laboratory data.

Acknowledgements

We are thankful to all patients who participated in this study. We also thank the Medical Research Center (MRC), Faculty of Medicine, Alexandria University for providing the pyrosequencing services.

Authors' contributions

MMR conceived and designed the study. NME, LME, EME, and MHS did the practical work, statistical analysis of the data, and writing the manuscript. All authors read and approved the final manuscript.

Funding

No funding was received for this study.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations**Ethics approval and consent to participate**

All patients signed a written informed consent, and the study was approved by the local ethics committee of Faculty of Medicine, Alexandria University, Egypt. The study protocol was in agreement with the Declaration of Helsinki guidelines 1975, as revised in 2000. The reference number is not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Received: 14 July 2021 Accepted: 21 November 2021

Published online: 26 January 2022

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