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Genetic polymorphisms and gene expression of one-carbon metabolizing enzymes and their relation to breast cancer

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Abstract

Background: Breast cancer is considered the leading cause of cancer-related death among Egyptian women (15.41%). One of the common BC risk factors is the genetic factor. One-carbon metabolism is one of the pathways reported to increase BC risk by influencing DNA synthesis and methylation. Methyl tetrahydrofolate reductase (*MTHFR*), thymidylate synthase (*TYMS) and* DNA methyl*transferase (DNMT)* enzymes are key enzymes in one-carbon metabolism directly and through influencing folate metabolism. We aimed to study the association of the gene expression level and polymorphisms of *MTHER* C677T (rs1801133), *TYMS* (rs45445694), *TYMS* 3'UTR 1494del6 and ΔDNMT3B − 149C>T with breast cancer risk in a sample of Egyptian women.

Methods: This study was conducted on one hundred female breast cancer patients. Genotyping and gene expression of the MTHFR and TYMS (1494del6, rs45445694) and DNMT3B genes were performed.

Results: There was no significant difference (OR 1.493; 95% CI 0.78–2.84; $P = 0.288$) in the frequency of the MTHFR (C677T) genotypes between breast cancer patients and control subjects and no significant difference in the frequency of the MTHFR mutant T allele. *TYMS* tandem repeats showed a significant difference (OR 2.232; CI 1.21–4.12; $P = 0.01$) in the frequency of the genotype 2R/3R among breast cancer patients and control subjects; however, the frequency of the 2R allele was not significantly different from that of the 3R allele (OR 1.461; 95% CI 0.96–2.21; $P = 0.073$). *TYMS* 3'-UTR 1494*del*6 showed a significant difference in the distribution of (+6/+6), (+6/−6) and (−6/−6) genotypes between the patient and control groups ($P \leq 0.001^*$), and its corresponding mutant allele showed P value ≤ 0.001, 95% CI = 1.64–3.76 and OR = 2.483. The expression of *MTHFR* was downregulated by 0.62-fold in all malignant tissues compared to normal adjacent tissues (0.57 ± 0.20, $P < 0.001^*$). On the other hand, no significant difference ($P = 0.143$) in the expression level of *TYMS was* found. Gene expression of *DNMT3B* was upregulated by 1.65-fold in all breast cancerous tissue samples compared with the control normal adjacent tissue with mean ± SD of 1.81 ± 0.63. ($P \leq 0.001^*$).

Conclusion: The current study showed that the 2R/3R *TYMS* allele and *DNMT3B* (− 149C>T) SNP are associated with a high risk of breast cancer and that there is a correlation between the 3'-UTR 1494*del*6 polymorphism (genotype

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− 6/− 6) and breast cancer risk. A significant reduction was found in the *MTHFR* gene expression level in BC compared with control tissues, and *the DNMT3B* (− 149C>T) SNP did affect the *DNMT3B* expression level.

Keywords: Breast cancer, MTHFR (C677T), *TYMS* 3′-UTR 1494*del*6, TYMS 5′UTR tandem repeat, *DNMT3B* (− 149C>T), Gene expression

Background

Breast cancer (BC) is described as the most frequent incident cancer and cause of death among women in Arab countries, although its incidence is lower than the global incidence. In Egypt, BC is the most diagnosed cancer and is considered the leading cause of cancer-related death among Egyptian women (15.41%). According to the American Cancer Society, the incidence rate of BC in Egypt is 46.8–59.6 per 100,000 (rates from 2011 to 2015), which is a great burden on population health [1, 2].

Breast cancer affects mainly females, and its risk increases with age. BC has many risk factors including environmental, hormonal and genetic factors, which are considered as a common risk factor for BC [3, 4]. Genome-wide association studies (GWAS) have described more than 170 single-nucleotide polymorphisms (SNPs) associated with the perceptivity to breast cancer. In addition, numerous genetic polymorphisms have been explored to explain their potential association with BC [5, 6].

Aberrantly, one-carbon metabolism is one of the pathways reported to increase BC risk by influencing DNA synthesis and methylation [7, 8]. One-carbon metabolism has two important functions in DNA synthesis and methylation. First, it is related to purine and thymidine production, which are both important for DNA synthesis. Second, it is related to its role in the supply of methyl groups through methionine metabolism [9].

Several enzymes are related to one-carbon metabolism, including (a) methyl tetrahydrofolate reductase (MTHFR), which catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which produces a methyl donor essential for the conversion of homocysteine to methionine. Methionine then allows the formation of S-adenosyl methionine (SAM), which is essential during DNA methylation [10]; (b) thymidylate synthase (TYMS), which plays an essential role in nucleotide synthesis (thymidylate) by converting 5,10-methylene tetrahydrofolate to dihydrofolate; and (c) DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from S-adenosyl methionine (SAM) to a cytosine ring usually located within the context of a CpG dinucleotide in DNA [11, 12].

These enzymes, through their role in one-carbon metabolism, also influence folate metabolism, which mediates the transfer of one carbon in many biochemical reactions and is essential in the synthesis of S-adenosyl methionine and crucial in DNA, RNA and protein methylation; thus, mutations in these enzyme genes affect DNA stability and DNA integrity and lead to mutagenesis and cancer development [12].

The *MTHFR* gene consists of 11 exons encoding a protein of 656 amino acids and is located on the short arm of chromosome 1p36.3; its length is 2.2 Kb, and it has two promoters and two isoforms (70 kDa and 77 kDa) [13, 14]. The C677T polymorphism is a point mutation missense or nonsynonymous at position 677 in the *MTHFR* gene with the substitution of cysteine to thymine nucleotide at that position. The C677T (rs1801133) polymorphism, which is located in exon 4, results in the conversion of alanine to valine at codon 222 in the *N*-terminal catalytic domain and is the most public SNP that reduces the activity of this enzyme [15, 16]. The amino acid variants that result from this polymorphism cause a reduction in enzyme activity, thus changing folate and homocysteine concentrations. The low plasma folate and high homocysteine concentrations resulted from this polymorphism influence the synthesis and DNA [17]. These changes in MTHFR enzyme activity result in tumor progression [18, 19]. Individuals with the 677TT homozygous variant have no more than 30% normal enzyme activity, and heterozygotes with the CT genotype have 65% normal enzyme activity with increased thermal instability [20]. The incidence of *MTHFR* 677C>T varies according to the different ethnicities and populations.

The thymidylate synthase (TS) gene has three known polymorphisms: The first 2 polymorphisms occur in the 5′ untranslated region (UTR) of the TS gene, while the third polymorphism is located in the 3′UTR. These three polymorphisms are the tandem repeat polymorphism in the thymidylate synthase enhancer region (TSER), the G to C single-nucleotide polymorphism (G/C SNP) and the 6-bp deletion at base pair 1494 (TS1494del6). From the tandem repeats, the triple and double repeats are the most frequent, where triple repeats result in higher TS expression [21, 22].

One of the functional SNPs that have been found in DNMTs is ΔDNMT3B − 149C>T, which is located at -149 bp from the transcription start site of the DNMT3b isoform. It was speculated that this SNP may increase the risk of many types of cancer via increasingly aberrant de

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novo methylation of CpG islands and thus transcriptional repression of some tumor suppressor genes [23, 24].

Data concerning these enzymes gene polymorphisms and their gene expression in Egyptian females with breast cancer are still insufficient.

The study aimed to investigate the association of *MTHER* C677T (rs1801133), *TYMS* (rs45445694), *TYMS* 3′UTR 1494del6 and ΔDNMT3B −149C>T polymorphisms and their gene expression levels with breast cancer risk in a sample of Egyptian women.

Methods

This study was conducted on one hundred female breast cancer patients and one hundred healthy females as controls. Breast cancer female patients were selected from patients admitted to the Clinical and Experimental Surgery Department, Medical Research Institute, University of Alexandria, during the period from September 2016 to March 2018. This study was approved by the Ethical Committee of Medical Research Institute and Ethical Committee of Faculty of Medicine (Serial number 0105660), Alexandria University, Egypt. Consent was taken from all participants to participate in the study and agreement on publication. A well-structured questionnaire was designed to cover all the following aspects: age, menopausal status, smoking habits, use of contraceptive pills and/or hormonal replacement therapies, family history of breast cancer, medical history (diabetes, hypertension, etc.) and any other medications.

Sample size was calculated statistically based on the frequencies of these genes in Mediterranean and Caucasian populations at the 80% and 5% levels of significance.

Tissue samples were collected from 10 randomly selected breast cancer patients, one from the malignant breast tissue (MT) and the other from adjacent normal breast tissue (NT) as a control group, and kept in RNA solution and stored at −80 °C till usage. Venous blood samples from the 200 participating females were collected in EDTA tubes and stored at −4 °C until subjected to DNA extraction and genotyping.

Genomic DNA was extracted from whole blood according to Sambrook et al. [25]. The integrity of the extracted DNA was assessed qualitatively by electrophoresis on a 1% agarose gel. Quantitative determination of DNA concentration and purity was performed using a NanoDrop1000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, Delaware USA).

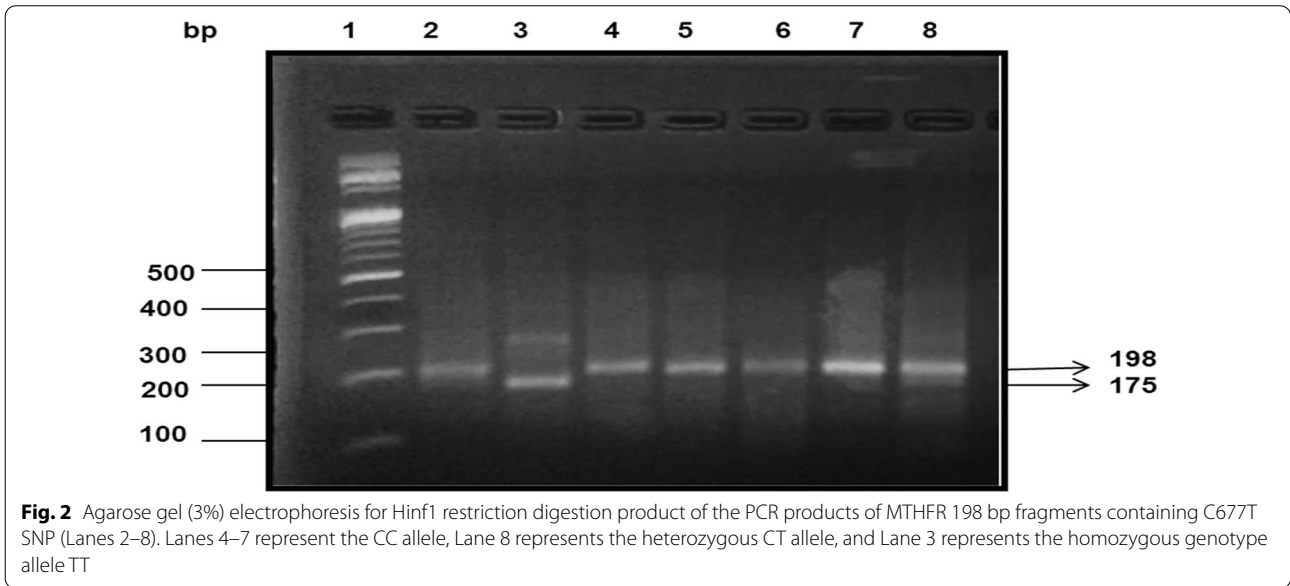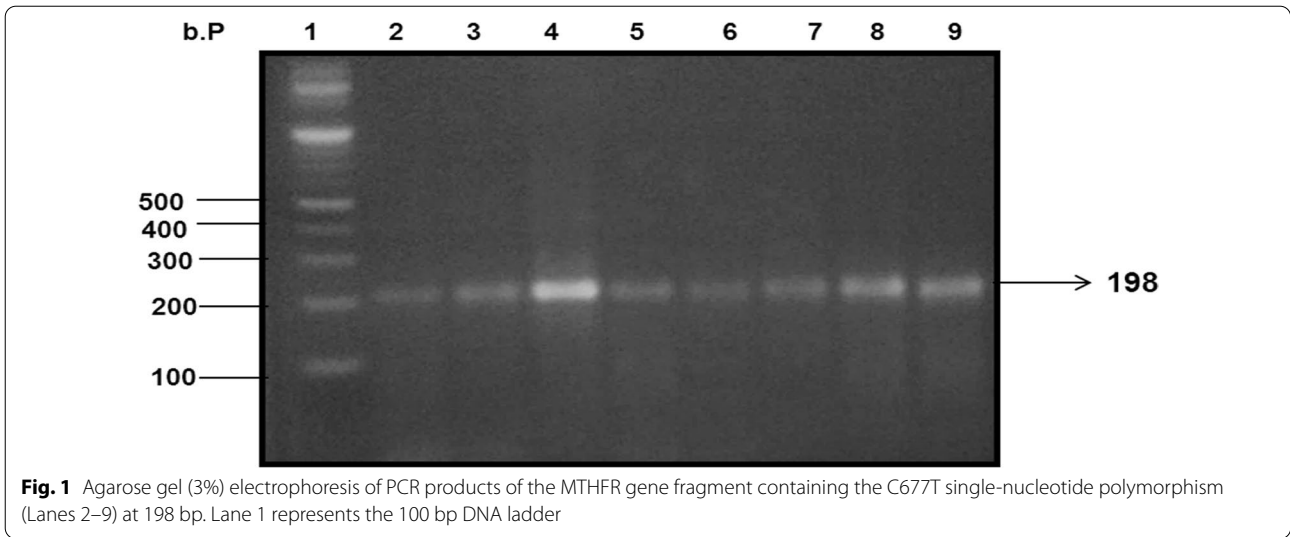### Genotyping of the studied gene polymorphisms

Genotyping conditions, including primer sequences, PCR conditions and PCR product lengths for amplification of the four studied genes (*MTHFR* and *TYMS (1494del6,* rs45445694) and *DNMT3B)*, are shown in Table 1 and Figs. 1, 2, 3, 4, 5, 6 and 7.

Table 1 Genotyping conditions of the studied genes

Studied genes	Primer sequences	PCR condition	Bands after enzyme digestion and gel electrophoresis
MTHFR (rs1801133) C677T SNP	**FW**: 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ **RV**: 5′-AGG ACG GTG CGG TGA GAG TG-3′	Initial denaturation at 95 °C for 3 min 35 cycles: Denaturation at 95 °C for 20 s Annealing at 59 °C for 20 s Extension at 72 °C for 20 s Extension at 72 °C for 5 min	CC:198 bp TT: 175 and 23 CT: 198, 175 and 23 bp
TYMS (rs45445694) 5′UTR tandem repeat	**FW**: 5′-AGG CGC GCG GAA GGG GTC CT -3′ **RV**: 5′-TCC GAG CCG GCC ACA GGC AT-3′	Initial denaturation at 95 °C for 3 min, 35 cycles: Denaturation at 95 °C for 30 s Annealing at 64 °C for 40 s Extension at 72 °C for 40 s Finally, extension at 72 °C for 5 min	(2R/2R):113 bp band (2R/3R):113 bp and 141 bp bands (3R/3R) 141 bp band
TYMS (rs151264360) of 3′ UTR [1494del6] DarI (TTAAAG) Deletion	**FW:** 5′-TCA CTG AGGGTA TCT GAC AAT GC-3′ **RV:** 5′-TGC TGT ATT CTG GTT TGG ATG C-3′	Initial denaturation: at 95 °C for 3 min 35 cycles of: Denaturation at 94 °C for 20 s Annealing at 55 °C for 20 s Extension 72 °C for 20 s Final extension for 5 min	**(+6/+6):** 240 and 160 bp bands **(−6/+6):** 394, 240 and 160 bp bands **(−6/−6):** 394 bp
DNMT3B Avr ll C<T SNP	**FW:** 5′-TGC TGT GAC AGGCAG AGC AG -3′ **RV:** 5′-GGT AGC CGG GAA CTC CAC GG-3′ (109)	Initial denaturation: at 95 °C for 3 min 35 cycles of: denaturation at 94 °C for 20 s Annealing at 55 °C for 20 s Extension at 72 °C for 20 s Final extension: at 72 °C for 5 min	Tt:207 and 173 bp Tc: 380, 207 and 173 bp Cc:380 bp

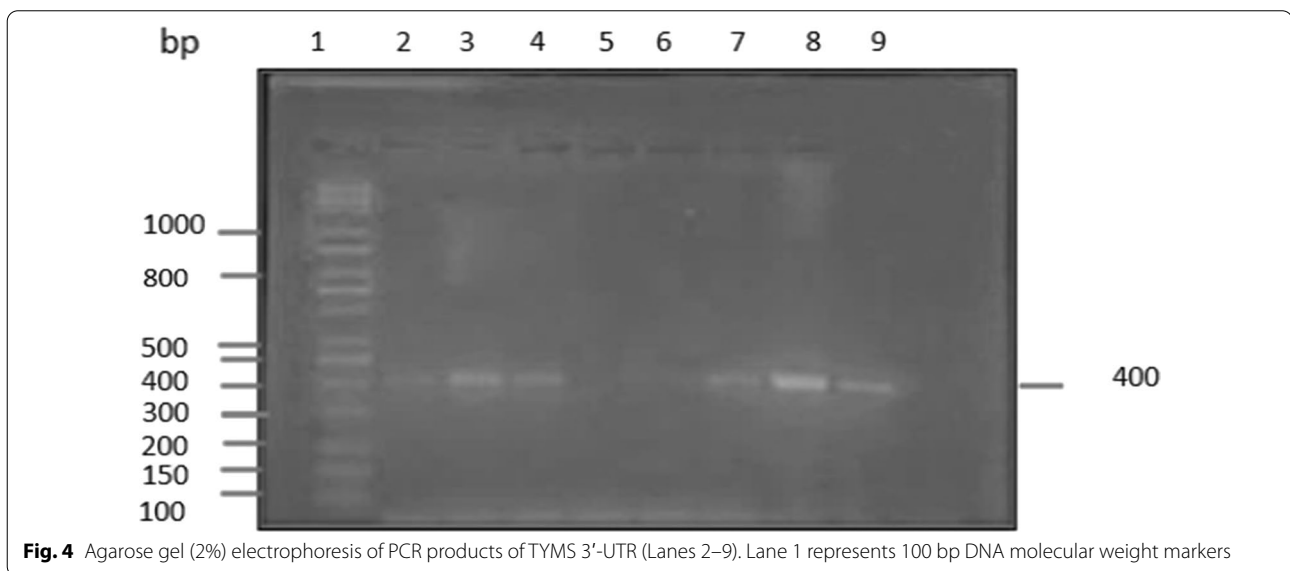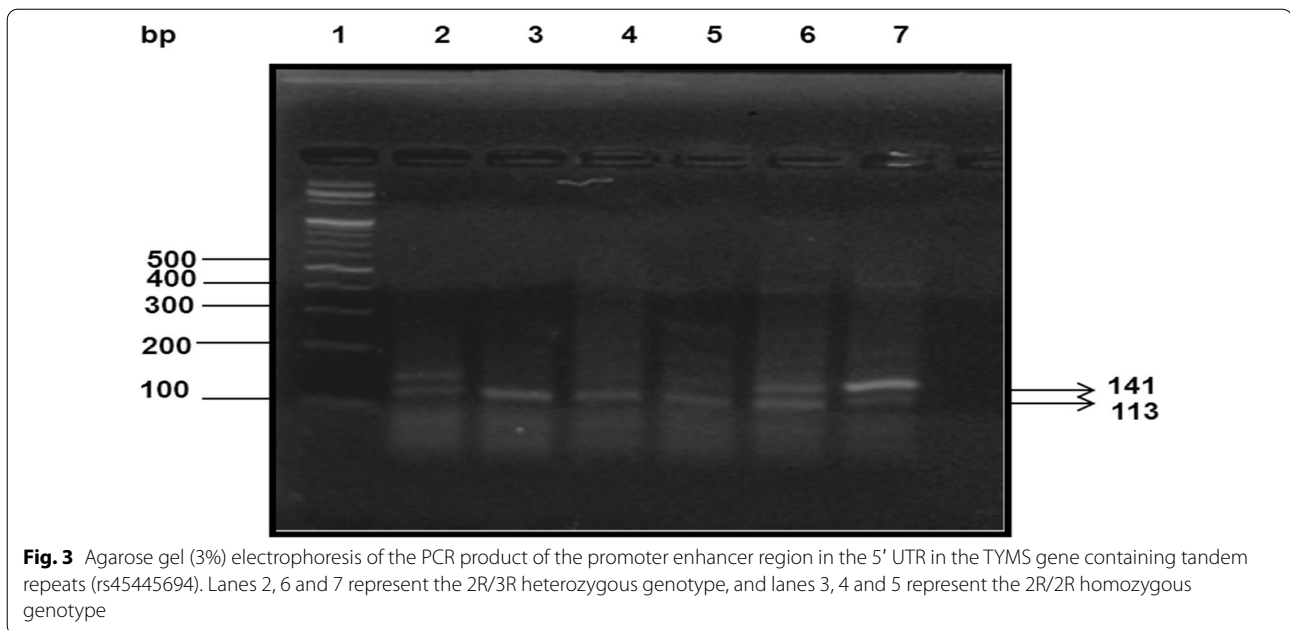The bolded region indicates homozygous genotype

FW: for word, RV: reverse

Fig. 1 Agarose gel (3%) electrophoresis of PCR products of the MTHFR gene fragment containing the C677T single-nucleotide polymorphism (Lanes 2–9) at 198 bp. Lane 1 represents the 100 bp DNA ladder

Fig. 2 Agarose gel (3%) electrophoresis for Hinf1 restriction digestion product of the PCR products of MTHFR 198 bp fragments containing C677T SNP (Lanes 2–8). Lanes 4–7 represent the CC allele, Lane 8 represents the heterozygous CT allele, and Lane 3 represents the homozygous genotype allele TT

Confirmatory sequencing of the studied genes

(a) For each of the studied genes, the PCR products of each gene were resolved on a 3% agarose gel as previously described and purified using a DNA gel extraction kit (QIAGEN, Germany) as follows: The DNA fragments were excised from the agarose gel, and three volumes of QG buffer were added to the excised agarose fragment, after which the tubes were kept at 50 °C until dissolved completely. One volume of ice-cold isopropanol was added, and the mixtures were loaded into a QIAquick spin column in 2-mL collection tubes and centrifuged at 12,000 rpm for 1 min. The flowthrough was discarded, and 0.75 mL of washing buffer PE was added to the spin column and recentrifuged again at 12,000 rpm for 1 min. The flowthrough was then discarded, and the QIAquick column was centrifuged for an additional 1 min at 12,000 rpm. Then, the QIAquick column was transferred to a clean sterilized 1.5-mL Eppendorf tube. The PCR product was eluted from the QIAquick column by adding 50 μL of EB buffer or nuclease-free
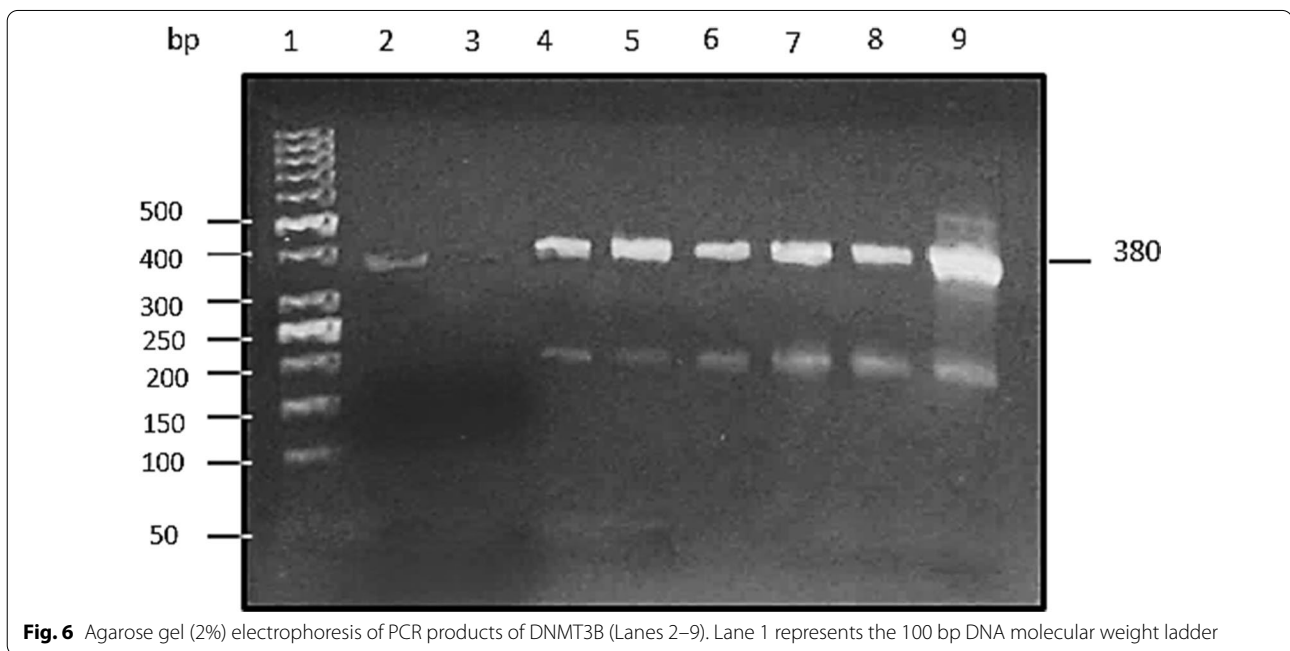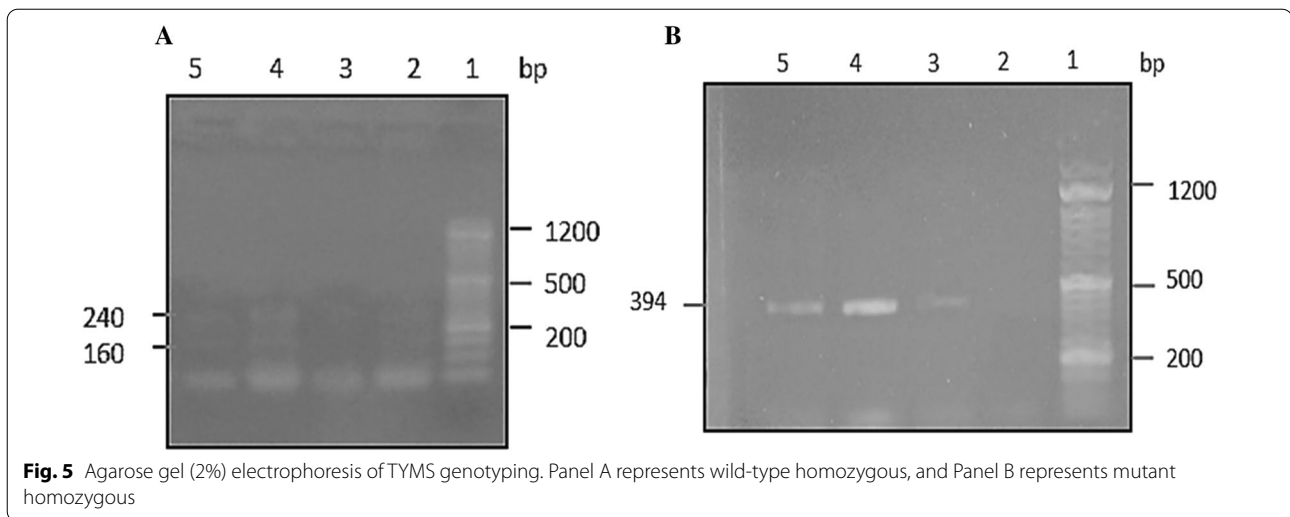
Fig. 3 Agarose gel (3%) electrophoresis of the PCR product of the promoter enhancer region in the 5' UTR in the TYMS gene containing tandem repeats (rs45445694). Lanes 2, 6 and 7 represent the 2R/3R heterozygous genotype, and lanes 3, 4 and 5 represent the 2R/2R homozygous genotype

Fig. 4 Agarose gel (2%) electrophoresis of PCR products of TYMS 3'-UTR (Lanes 2–9). Lane 1 represents 100 bp DNA molecular weight markers

water, followed by centrifugation at 12,000 rpm for 1 min. The eluted purified PCR product was checked on 3% agarose and stored at − 20 °C until further processing. Finally, the purified PCR product was subjected to sequencing using the chain termination method with an ABI 3130X DNA sequencer (Applied Biosystems, USA). DNA sequencing was performed by capillary electrophoresis (Applied Biosystems) using the Sanger method [26] (Figs. 8, 9, 10).

Determination of gene expression for MTHFR, DNMT3B and TYMS

Total RNA was extracted from all tissue samples using an RNeasy Mini Kit (QIAGEN Co., Hilden, Germany). Then, the eluted RNA was stored at − 80 °C until used for cDNA synthesis.

The purity of RNA was determined by measuring the absorbance at 260 and 280 nm (A260/A280, using a NanoDrop ND-1000 UV–Visible Spectrophotometer against TE buffer as a blank). The optical density ratio ranging from 1.8 to 2.0 was taken as an indicator for the purity

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Fig. 5 Agarose gel (2%) electrophoresis of TYMS genotyping. Panel A represents wild-type homozygous, and Panel B represents mutant homozygous

Fig. 6 Agarose gel (2%) electrophoresis of PCR products of DNMT3B (Lanes 2–9). Lane 1 represents the 100 bp DNA molecular weight ladder
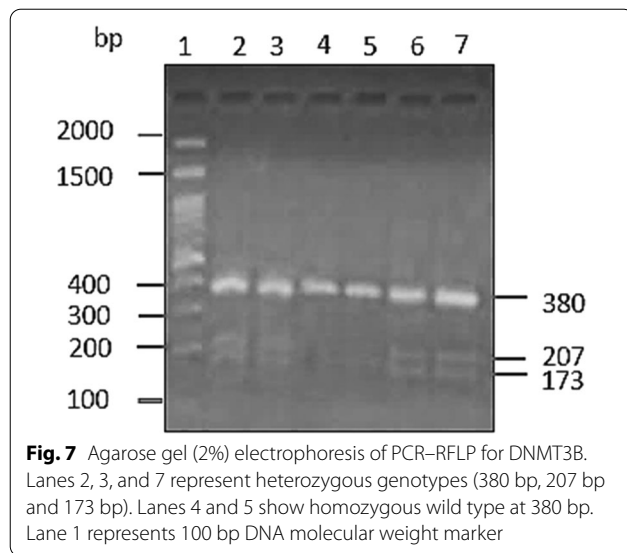
of the prepared RNA samples. Unique patterns of intact RNA were visualized as 28S and 18S rRNA bands on agarose gels (Fig. 11).

Total RNA was reverse-transcribed to cDNA using a GoScript™ Reverse Transcription System Kit (Promega, USA). The gene expression levels of *MTHFR*, DNMT3B *and TYMS* were determined using quantitative real-time PCR. The primer sequences and qPCR conditions are summarized in Table 2. The expression of targeted genes was normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression level within the same sample to determine ΔCT. Then, ΔCT for each sample was normalized to the expression of the targeted gene in the cells of the vehicle control to calculate ΔΔCT. The fold change in the expression of the target gene for each sample was calculated [27].

Results

The demographic data of the current study are shown in Table 3, where the age of the patients ranged from 24 to 74 years with a mean ± SD of 49.80 ± 11.22, while the age of the controls ranged from 21 to 74 years with a mean ± SD of 47.28 ± 10.58. Data revealed no significant differences between patients and controls regarding age ($P = 0.104$). The frequency of breast cancer family history,

Fig. 7 Agarose gel (2%) electrophoresis of PCR–RFLP for DNMT3B. Lanes 2, 3, and 7 represent heterozygous genotypes (380 bp, 207 bp and 173 bp). Lanes 4 and 5 show homozygous wild type at 380 bp. Lane 1 represents 100 bp DNA molecular weight marker

Fig. 8 Sequence analysis of the PCR product of 323 bp of a partial fragment of the MTHFR gene containing the C677T SNP. The chromatogram shows the homozygous genotype (CC) wild type (**a**), homozygous genotype (TT) (**b**) and heterozygous genotype (CT) (**c**). The arrow indicates the position of the SNP

smoking status and breast-feeding in the patient group was 82%, 26% and 72%, respectively.

Table 4 shows the histological grades and breast cancer types found among the studied patients, where 93% of patients had pathological grade II and 92% of them had invasive ductal carcinoma.
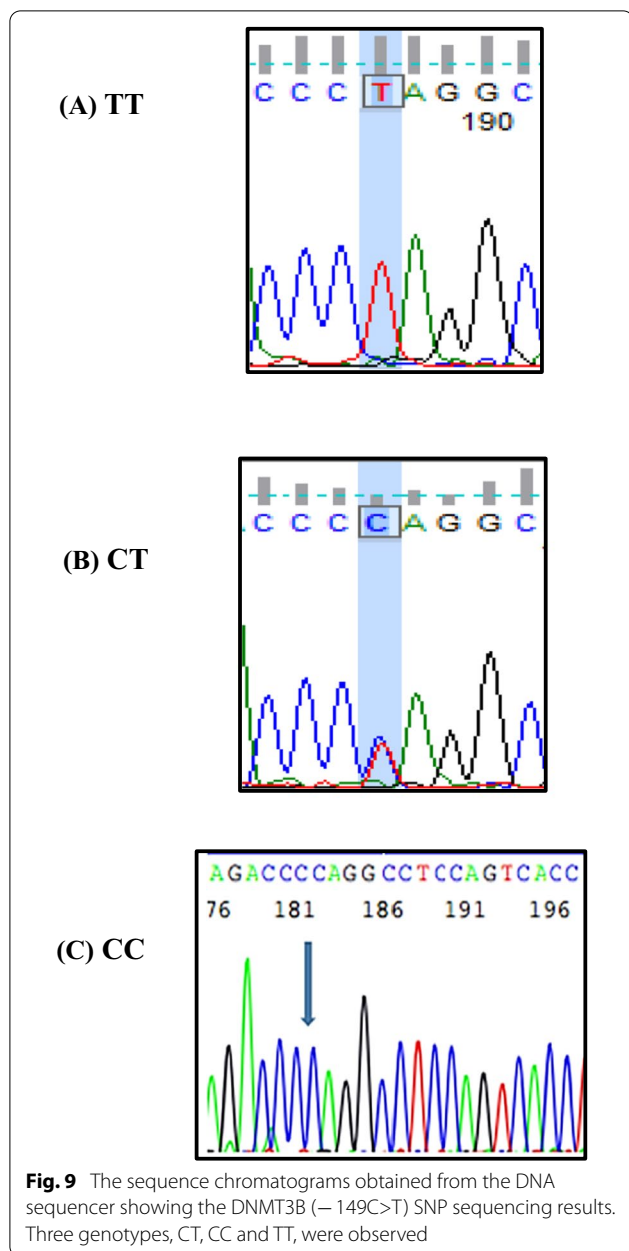
The frequencies of both studied genes (genotypes and alleles) in breast cancer patients and control subjects are shown in Table 5 and Fig. 12. The frequencies of the CC, CT and TT genotypes of MTHFR (C677T) were 68%, 29% and 3% in breast cancer patients, respectively. The CC, CT and TT genotypes of MTHFR (C677T) among the control subjects were 77%, 22% and 1%, respectively. There was no significant difference (OR 1.493; 95% CI 0.78–2.84; $P = 0.288$) in the frequency of the CT genotype between breast cancer patients and control subjects. The frequencies of the C and T alleles were 82.5% and 17.5%, respectively, in breast cancer patients. Conversely, the frequencies of C and T alleles in control subjects were 88% and 12%, respectively. There was no significant difference in the frequency of the mutant T allele between breast cancer patients and control subjects (OR 1.556; 95% CI 0.89–2.73; $P = 0.121$).

Regarding the TYMS genotypes and alleles for the tandem repeats (rs45445694), it was found that the frequencies of the 2R/2R, 2R/3R and 3R/3R genotypes were 23%, 77% and 0%, respectively, in breast cancer patients. However, the frequencies of the 2R/2R, 2R/3R and 3R/3R genotypes were 40%, 60% and 0%, respectively, in control subjects. The results indicated a significant difference (OR 2.232; CI 1.21–4.12; $P = 0.01$) in the frequency of the genotype 2R/3R among breast
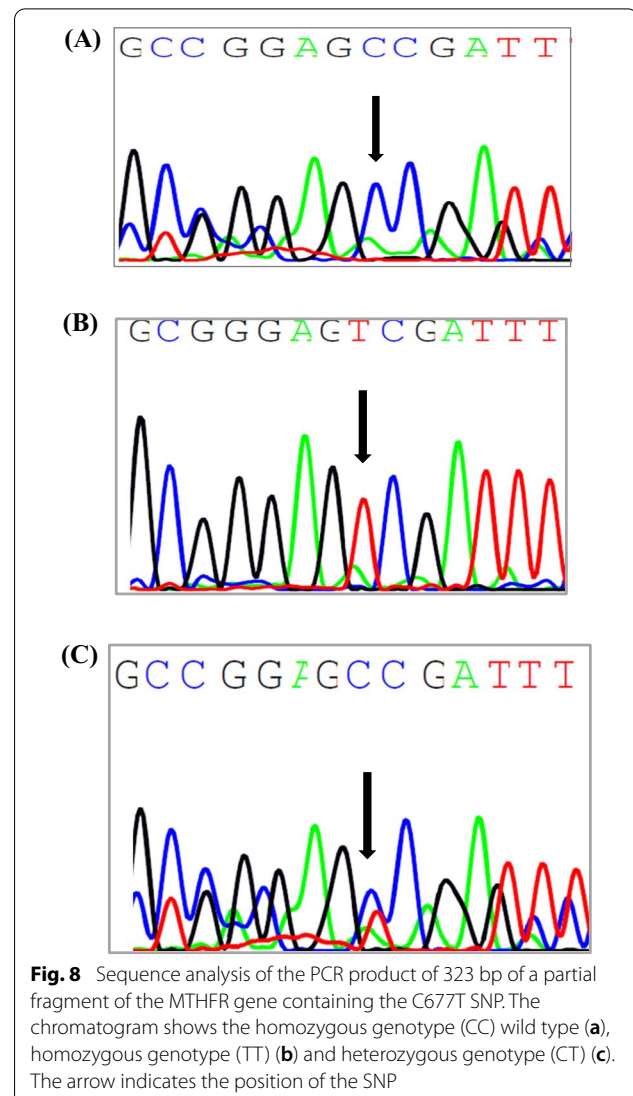
cancer patients and control subjects. On the other hand, the frequencies of 2R and 3R alleles were 61.5% and 38.5%, respectively, in breast cancer patients and 70% and 30%, respectively, in control subjects. The frequency of the 2R allele was not significantly different from that of the 3R allele (OR 1.461; 95% CI 0.96–2.21; $P = 0.073$).

Table 5 also shows the genotyping and allele frequency of DNMT3B $-149C>T$ in the studied groups. There was a significant difference in the distribution of C/C, C/T and T/T genotypes between the patients and control groups ($P \leq 0.001^*$). The genotype (C/T) had a significant association with increased breast cancer risk in the Alexandrian population (OR = 6.907, 95% CI = 3.58–13.32) compared to the C/C genotype. The mutant allele frequencies of DNMT3B 149 C>T in patients were 30% compared to 8.5% in the control subjects. The

Fig. 9 The sequence chromatograms obtained from the DNA sequencer showing the DNMT3B (− 149C>T) SNP sequencing results. Three genotypes, CT, CC and TT, were observed

corresponding *P* value, 95% CI and odds ratio (OR) were as follows for the mutant allele: *P* value ≤ 0.001, 95% CI = 2.58–8.25 and OR = 4.613.

The genotyping and allele frequency of TYMS 3′-UTR 1494del6 in both studied groups are also shown in Table 5. According to the results, using the (+6/+6) allele as a reference, there was a significant difference in the distribution of the (+6/+6), (+6/−6) and (−6/−6) genotypes between the patient and control groups (*P* ≤ 0.001*). This observation indicated that the genotype (−6/−6) had a significant association with an increased

risk of breast cancer in Alexandrian women (OR = 3.294, 95% CI = 1.57–6.91) compared to the (+6/+6) genotype. The mutant genotype (−6/−6) frequencies of TYMS 3′-UTR 1494del6 were 62% and 31% in patients and control subjects, respectively. The mutant allele frequencies of SNPs in patients were 72.5% compared with 51.5% in the controls. The corresponding *P* value, 95% CI and odds ratio (OR) were as follows for the mutant allele: *P* value ≤ 0.001, 95% CI = 1.64–3.76 and OR = 2.483.

Table 6 shows the gene expression analysis, where the expression of MTHFR was downregulated by 0.62-fold in all malignant tissues compared to normal adjacent tissues (mean ± SD) of 0.57 ± 0.20. Accordingly, the Mann–Whitney U test determined that there exists a significant difference (*P* < 0.001*) in the relative gene expression of MTHFR levels in breast cancer patients compared to normal adjacent tissue as a control (Fig. 13). On the other hand, statistical analysis using the Mann–Whitney test revealed that there was no significant difference (*P* = 0.143) in the expression level of TYMS between breast cancer patients and normal adjacent tissue as a control. Obviously, the level of gene expression of DNMT3B was upregulated by 1.65-fold in all breast cancerous tissue samples compared with the control normal adjacent tissue with mean ± SD of 1.81 ± 0.63. Statistical analysis using the Mann–Whitney test revealed a significant difference in gene expression (*P* < 0.001*).

Discussion

Methyl tetrahydrofolate reductase (*MTHFR*), thymidylate synthase (*TYMS) and* DNA methyl*transferase (DNMT)* enzymes play essential roles in folate metabolism. As folate plays a vital role in nucleic acid synthesis of DNA, polymorphisms in these coding genes are correlated with cancer risk [28, 29]. To our knowledge, the study of the expression of these genes in breast cancer and the combined effect of their allele frequency is still insufficient.

There was no significant difference (OR 1.493; 95% CI 0.78–2.84; *P* = 0.288) in the frequency of the CT genotype between breast cancer patients and control subjects. This inferred the conclusion stating that the CT genotype is not a risk factor for breast cancer in the tested sample size. Additionally, there was no significant difference in the frequency of the mutant T allele between breast cancer patients and control subjects (OR 1.556; 95% CI 0.89–2.73; *P* = 0.121).

There are several research studies that correlate the *MTHFR* (C677T) polymorphism to hyperhomocysteinemia, folate deficiency and cancer [30]. The results in the current study are in agreement with other studies that found no association between the *MTHFR* (C677T) SNP and breast cancer risk [31, 32]. Unlike
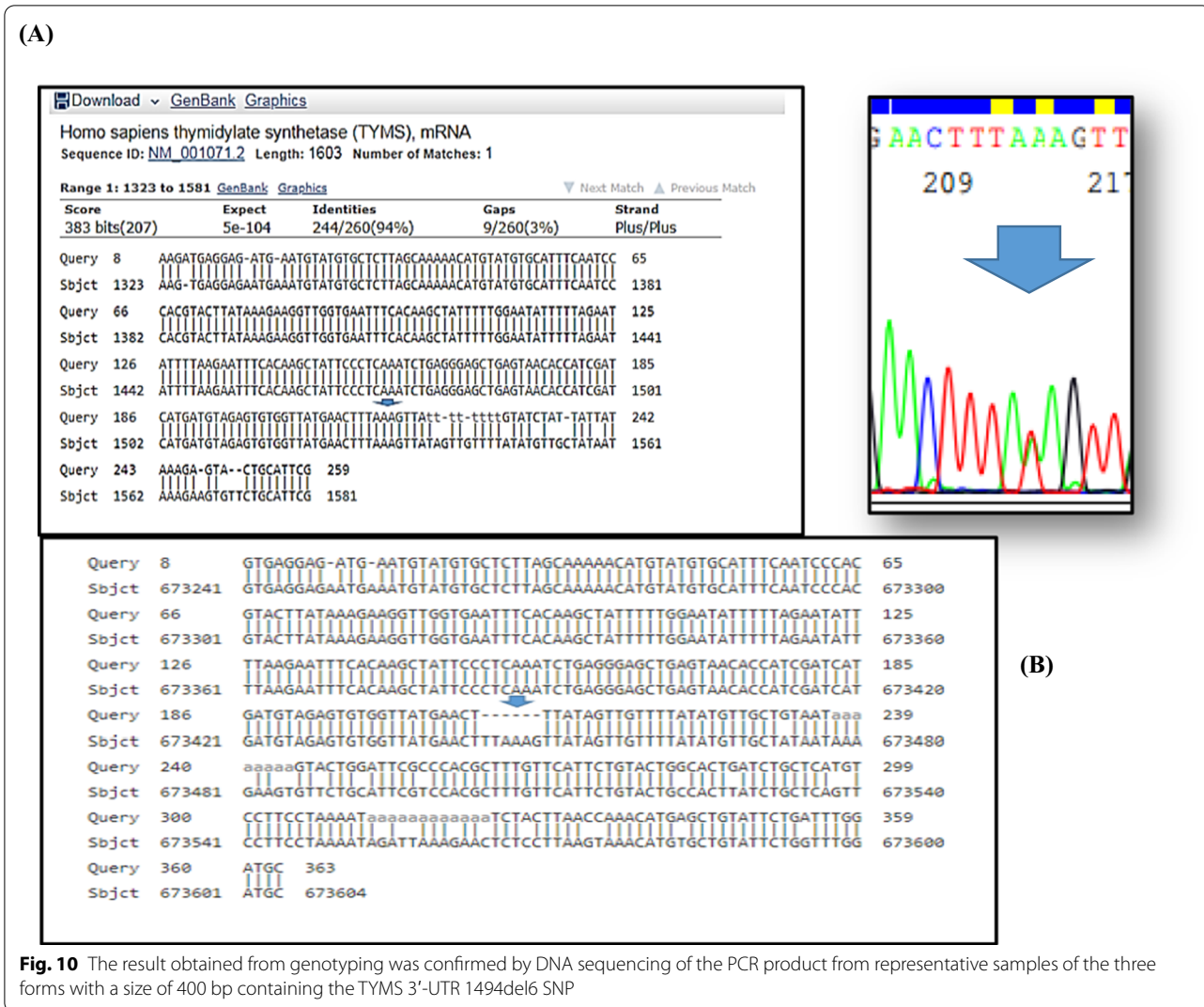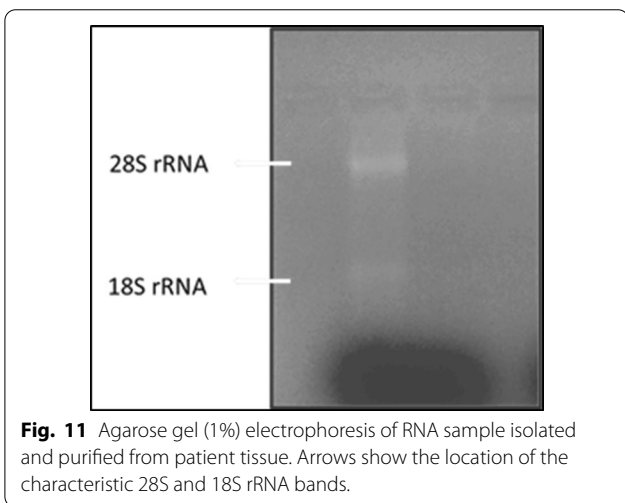
(A)

(B)

Fig. 10 The result obtained from genotyping was confirmed by DNA sequencing of the PCR product from representative samples of the three forms with a size of 400 bp containing the TYMS 3'-UTR 1494del6 SNP

Fig. 11 Agarose gel (1%) electrophoresis of RNA sample isolated and purified from patient tissue. Arrows show the location of the characteristic 28S and 18S rRNA bands.

the present finding, a previous study stated a significant correlation between the *MTHFR* (C677T) SNP and breast cancer occurrence [33–35]. The discrepancy in findings regarding the possible role of the (C677T) SNP in the occurrence of breast cancer among different populations with different ethnic groups is mainly confined to the significant difference in genetic makeup among these populations.

Regarding the frequencies of the *TYMS* genotypes and alleles in breast cancer patients and control subjects for the tandem repeats (rs45445694) (Table 5), the results indicated a significant difference (OR 2.232; CI 1.21–4.12; $P=0.01$) in the frequency of the genotype 2R/3R among breast cancer patients and control subjects. Subsequently, the genotype 2R/3R is considered a risk factor for breast cancer occurrence in the tested sample size of Egyptian women. On the other hand, the frequency of

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Table 2 Primers conditions for quantitative RT-PCR

Genes	*MTHFR*	*TYMS*	*DNMT3B*	*GAPDH*
Primers:				
Forward	5'-GAAGCAGGGAGCTTTGAGG -3'	5'-CGCTACAGCCTG AGAGATGA-3'	5'-GGT TTG GCG ATG GCA AGT TC-3'	5'-GACCTGCCGTCTAGAAAAAC-3'
Reverse	5'-ACGATGGGGCAAGTGATG-3'	5'-TCACTCCCTTGG AAGACAGC -3'	5'-TCC CAC CCT AGC TTT CTC CA-3'	5'-TTGAAGTCAGAGGAGACCAC-3'
Thermal profile of qPCR	Initial denaturation at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s denaturation, annealing at 60 ℃ for 10 s and elongation at 72 ℃ for 10 s			

Table 3 Demographic and clinical data

Age (years)	Patients (*n* = 100)	Control (*n* = 100)	*t*	*P*
Min.–Max	24.0–74.0	21.0–74.0	1.634	0.104
Mean ± SD	49.80 ± 11.22	47.28 ± 10.58		
Median	48.0	48.0		
Family history of breast cancer		Patients (*n* = 100) No		Percentage (%)
Absent		82		82.0
Present		18		18.0
Smoking status				
Smoker		26		26.0
Non-smoker		74		74.0
Breast-feeding status				
Yes		72		72.0
No		28		28.0

Table 4 Tumor histological grade and breast cancer types

Tumor histological grade	Percentage (%)
2 (II)	93.0
3 (III)	4.0
4 (IV)	3.0
Types of breast cancer	Percentage (%)
Invasive ductal carcinoma (IDC)	92
Invasive lobular carcinoma (ILC)	3
Mucoid carcinoma	3
PEGET disease + IDC	1
IDC + ILC	1

the 2R allele was not significantly different from that of the 3R allele (OR 1.461; 95% CI 0.96–2.21; $P = 0.073$).

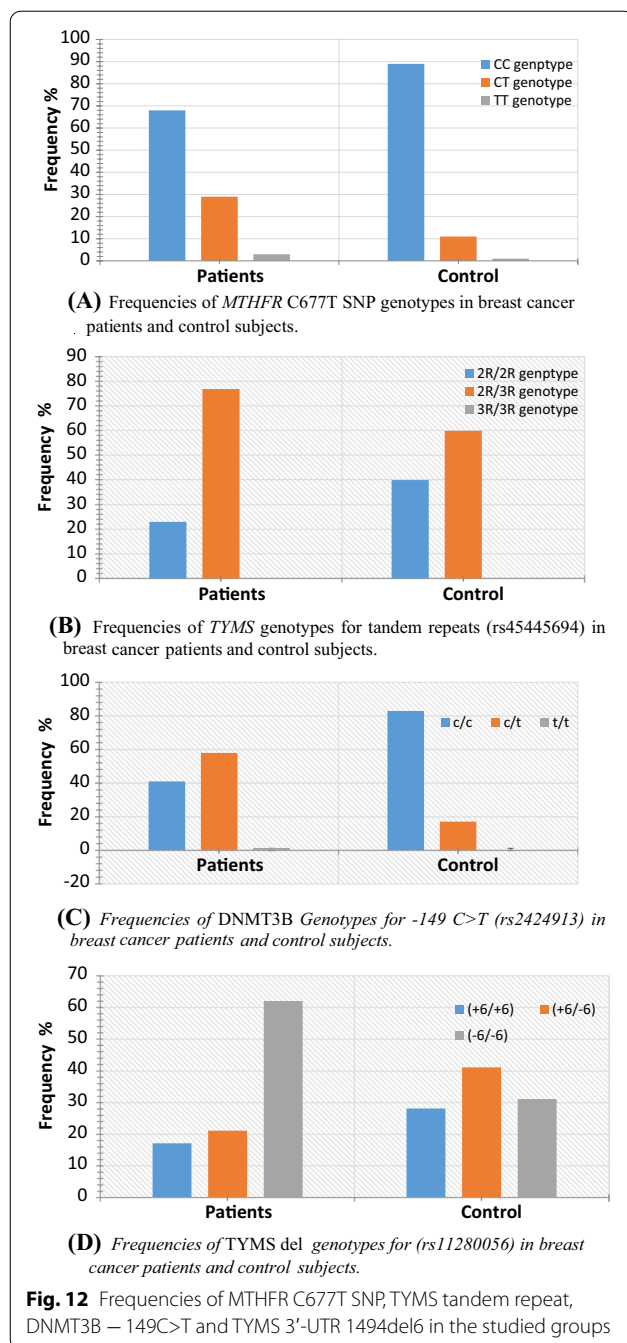Published data and research indicated that there were contradictory results regarding the possible role of the tandem repeats (rs45445694) of the *TYMS* gene in relation to breast cancer occurrence among different populations of different ethnicities. For instance, some studies did verify a positive correlation between the tandem repeats (rs45445694) of the *TYMS* gene and breast cancer occurrence [36, 37], while another study

Table 5 Associations between *MTHFR* C677T SNP, TYMS tandem repeat, DNMT3B − 149C>T and TYMS 3'-UTR 1494del6 with breast cancer risk in Egyptian women

	Patients		Control		χ^2	P	OR	95% CI
	No	%	No	%				
The MTHFR (C677T) genotypes and alleles frequency in the studied groups								
Genotype	(n = 100)		(n = 100)					
CC	68	68.0	77	77.0	2.446	MCp = 0.288	Ref	
CT	29	29.0	22	22.0			1.493	0.78–2.84
TT	3	3.0	1	1.0			3.397	0.35–33.43
CT+TT	32	32.0	23	23.0	2.03	0.154	1.575	0.84–2.95
Allele frequency	(n = 200)		(n = 200)					
C	165	82.5	176	88.0	2.406	0.121	Ref	
T	35	17.5	24	12.0			1.556	0.89–2.73

Genotype/allele	Patients		Control		χ^2	P	OR	95% CI
	No	%	No	%				
The TYMS tandem repeat genotypes and alleles frequency in the studied groups								
Genotype	(n = 100)		(n = 100)					
2R/2R	23	23.0	40	40.0	6.697*	0.010*	–	–
2R/3R	77	77.0	60	60.0			2.232	1.21–4.12
3R/3R	0	0.0	0	0.0			–	–
Allele frequency	(n = 200)		(n = 200)					
2R	123	61.5	140	70.0	3.208	0.073	–	–
3R	77	38.5	60	30.0			1.461	0.96–2.21

	Patients		Control		χ^2	P	OR	95% CI
	No	%	No	%				
The DNMT3B − 149C>T genotypes and alleles frequency in the studied groups								
Genotype	(n = 100)		(n = 100)					
CC®	41	41.0	83	83.0	38.671*	MCp <0.001*	Ref	
CT	58	58.0	17	17.0			6.907	3.58–13.32
TT	1	1.0	0	0.0			–	–
CT+TT	59	59.0	17	17.0	37.436	<0.001*	7.026	3.64–13.55
Allele frequency	(n = 200)		(n = 200)					
C	140	70.0	183	91.5	29.737	<0.001*	Ref	
T	60	30.0	17	8.5			4.613	2.58–8.25

	Patients		Control		χ^2	P	OR	95% CI
	No	%	No	%				
The TYMS 3'-UTR 1494del6 genotypes and alleles frequency in the studied groups								
Genotype	(n = 100)		(n = 100)					
(+6/+6)®	17	17.0	28	28.0	19.474*	<0.001*	Ref	
(+6/−6)	21	21.0	41	41.0			0.844	0.38–1.88
(−6/−6)	62	62.0	31	31.0			3.294	1.57–6.91
(−6/−6)+(+6/−6)	83	83.0	72	72.0	3.470	0.063	1.899	0.96–3.75
Allele frequency	(n = 200)		(n = 200)					
+6	55	27.5	97	48.5	18.718	<0.001*	Ref	
−6	145	72.5	103	51.5			2.483	1.64–3.76

χ^2, Chi-square test; *P*, *P* value for comparing between the two groups; OR, odds ratio; CI, confidence interval; (%), percentage of studied cases

*Statistically significant at $P \leq 0.05$

(A) Frequencies of *MTHFR* C677T SNP genotypes in breast cancer patients and control subjects.

(B) Frequencies of *TYMS* genotypes for tandem repeats (rs45445694) in breast cancer patients and control subjects.

(C) *Frequencies of DNMT3B Genotypes for -149 C>T (rs2424913) in breast cancer patients and control subjects.*

(D) *Frequencies of TYMS del genotypes for (rs11280056) in breast cancer patients and control subjects.*

Fig. 12 Frequencies of MTHFR C677T SNP, TYMS tandem repeat, DNMT3B − 149C>T and TYMS 3′-UTR 1494del6 in the studied groups

did not confirm this correlation with the variant alleles [38].

The genotyping and allele frequency of *DNMT3B* −149C>T in the studied groups showed that there were significant differences in the distribution of the C/C, C/T and T/T genotypes between the patient and control groups ($P \leq 0.001^*$). The genotype (C/T) had a significant association with increased breast cancer risk in the Alexandrian population (OR = 6.907, 95% CI = 3.58–13.32)

compared to the C/C genotype, and the mutant allele frequencies of *DNMT3B* 149 C>T in patients were 30% compared to 8.5% in the control subjects. The corresponding *P* value, 95% CI and odds ratio (OR) were as follows for the mutant allele: *P* value ≤ 0.001, 95% CI = 2.58–8.25 and OR = 4.613. (Table 5).

The present results are consistent with a previous study which stated that the *DNMT3B* -149C/T polymorphism was significantly associated with positive cancer risk in the Caucasian population but not in the African population [39].

In contrast, another study stated that the *DNMT3B* -149C/T polymorphism was relatively significantly increased head and neck cancer risk but showed no significantly elevated risk of colorectal cancer, breast cancer, hepatocellular cancer, gastric cancer or other cancers, so it was suggested that this SNP was not related to overall cancer risk [40]. This result was consistent with a study suggesting that for the −149C>T polymorphism, there was no evidence indicating that this SNP may increase the risk of cancer [41]. In addition, a study in southern China demonstrated that *DNMT3B* − 149C>T polymorphisms showed no significant associations with BC risk in the Chinese population [42]. A similar result was found in the Iranian population [43].

When using the (+6/+6) allele as a reference, there was a significant difference in the distribution of the (+6/+6), (+6/−6) and (−6/−6) genotypes of *TYMS* 3′-UTR 1494*del*6 between the patients and control groups ($P \leq 0.001^*$). This observation indicated that the genotype (−6/−6) had a significant association with an increased risk of breast cancer in Alexandrian women (OR = 3.294, 95% CI = 1.57–6.91) compared to the (+6/+6) genotype. The mutant genotype (−6/−6) frequencies of *TYMS* 3′-UTR 1494del6 were 62% and 31% in patients and control subjects, respectively. The mutant allele frequencies of SNPs in patients were 72.5% compared with 51.5% in the controls. The corresponding *P* value, 95% CI and odds ratio (OR) were as follows for the mutant allele: *P* value ≤ 0.001, 95% CI = 1.64–3.76 and OR = 2.483.

The current results seemed to be in agreement with a previous study that concluded that the *TYMS* 3′-UTR polymorphism may be a genetic determinant for developing BC in the Asian population [37]. The same result was found in subjects from northeastern Mexico [44]. In another study, they found that BC risk was associated with the *TYMS* 3′-UTR 1494*del*6 polymorphism in non-smoking and never drinking non-Hispanic white women regardless of tumor stage [45]. Moreover, this SNP has been correlated with BC risk among Asian populations [46]. Furthermore, it has been believed that the *TYMS* 3′-UTR polymorphism increases BC risk in Turkish

Table 6 Expression level of TYMS, MTHFR and DNMT3B genes in breast cancer tissues compared to normal adjacent tissue

Expression fold	Tumor (*n* = 10)	Normal adjacent tissue (*n* = 10)	U	P
Expression level of TYMS gene				
Min–Max	0.57–1.62	1.0–1.0	30.0	0.143
Mean ± SD	0.93 ± 0.39	1.0 ± 0.0		
Median	0.73	1.0		
Expression fold	**Breast cancer patients** (*n* = 10)	**Control**** (*n* = 10)	**U**	**P**
Expression level of MTHFR gene				
Min–Max	0.27–0.93	1.0–1.0	0.0*	< 0.001*
Mean ± SD	0.57 ± 0.20	1.0 ± 0.0		
Median	0.62	1.0		
Expression fold	**Tumor tissue** (*n* = 10)	**Normal adjacent tissue** (*n* = 10)	U	P
Expression level of DNMT3B gene				
Min–Max	1.02–2.95	1.0–1.0	0.0*	< 0.001*
Mean ± SD	1.81 ± 0.63	1.0 ± 0.0		
Median	1.65	1.0		

U: Mann–Whitney test

P: *P* value for comparing between the studied groups

*Statistically significant at $P \leq 0.05$

**Control: normal adjacent tissues from patients

women, particularly in the presence of high body mass index (BMI), age 55 years or older, early menarche, late age at first pregnancy and negative smoking history [47].
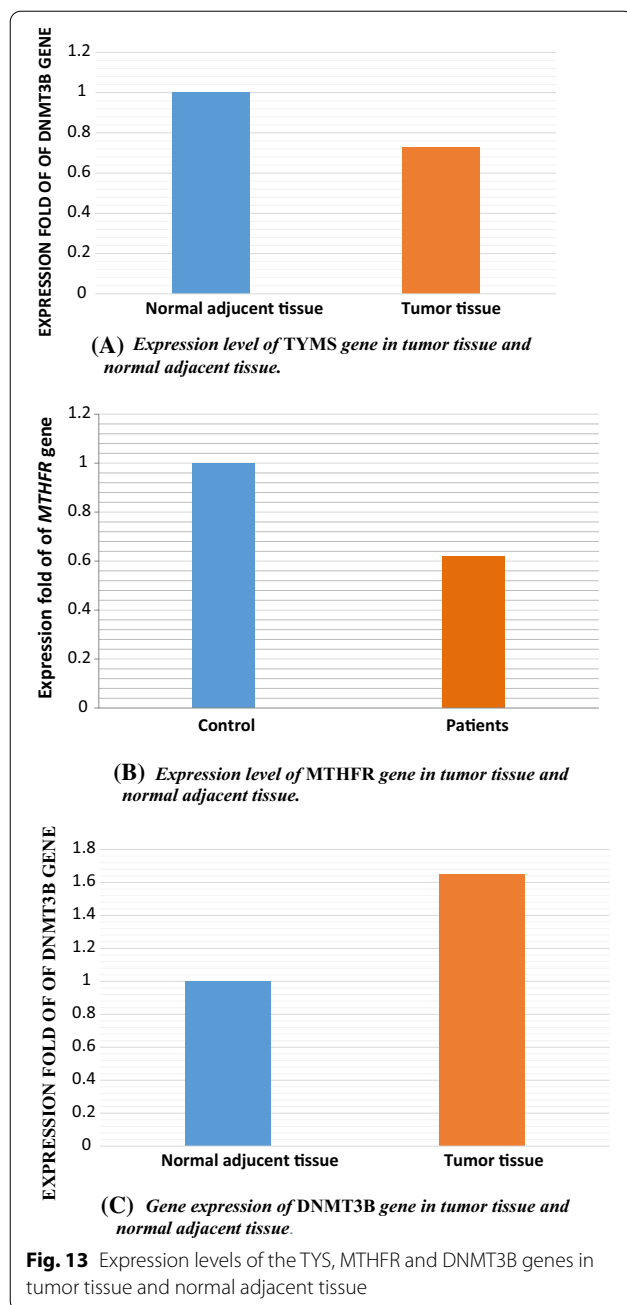
On the other hand, it has been found that 3′UTR 6-bp ins/del polymorphisms may not be associated with cancer risk according to a meta-analysis of 37 case–control studies [48].

Studied gene expression

The expression of *MTHFR* was downregulated by 0.62-fold in all malignant tissues compared to normal adjacent tissues (mean ± SD) of 0.57 ± 0.20 with a significant difference (*P* < 0.001*) in breast cancer tissue compared to the control. The data obtained were in agreement with a previous study [49] where a significant association between the rs1801133 (A222 V) genotypes and an increased risk of BC development was found. Regarding relative gene expression, we found significant mRNA sub-expression between the combined genotypes C/T + T/T (rs1801133) and triple-negative breast cancer (TNBC) (*P* = 0.034). In brief, the *MTHFR* gene and its protein could act as potential predictive biomarkers of BC [49].

The present study showed no significant difference (*P* = 0.143) in the expression level of *TYMS* between breast cancer patients and normal adjacent tissue. Many research groups have assessed the relationship between *TYMS* polymorphisms and *TYMS* gene expression;

however, the results have been inconclusive with scarce or no data on its expression in BC tissue. Many studies have found a positive correlation between the TSER 3R allele and TYMS protein levels. Horie and his coworkers in 1995 found that the expression activity of a reporter gene linked to the promoter region of the human *TYMS* genes with the two types of repeated sequences was examined, and the result showed that the expression activity of the gene with the double repeat was lower than that of the gene with the triple repeat in the transient expression assay. Thus, it appears that the unique repeated sequences in the 5′-terminal region of the human *TYMS* gene are polymorphic and contribute to the efficiency of gene expression [50]. Mauritz and his coworkers did not detect an association between TSER genotype and *TYMS* activity or mRNA levels in colorectal cancer (CRC) tissue; however, they detected correlations between genotype and *TYSM* activity/gene expression in normal tissues from the same patients. Nevertheless, this variation in the relationship between TSER genotype and *TYSM* expression in cancer versus normal tissues may be explained by the complications of gene expression in cancer tissue [51]. Because proliferation, tumor suppression and apoptosis-related protein levels are disturbed in colorectal carcinoma CRC tissue, it makes sense that *TYMS* genotype and gene expression are associated in normal tissues but not tumors [52].

(A) *Expression level of* **TYMS** *gene in tumor tissue and normal adjacent tissue.*

(B) *Expression level of* **MTHFR** *gene in tumor tissue and normal adjacent tissue.*

(C) *Gene expression of* **DNMT3B** *gene in tumor tissue and normal adjacent tissue.*

Fig. 13 Expression levels of the TYS, MTHFR and DNMT3B genes in tumor tissue and normal adjacent tissue

The level of DNMT3B gene expression showed a significant difference in BC tissue compared with control tissue ($P < 0.001*$) with upregulation by 165-fold in cancerous tissue. This result is in agreement with many previous studies that found that *DNMT3B* expression increases in different types of BC and is related to high histological grade [53–55]. *DNMT3B* overexpression is increased not only in BC but also in several types of primary tumors and cell lines of different cancers [56, 57], endometrioid cancers [58] and uterine cervical carcinoma [59].

Overexpression of *DNMT3B* is correlated with poor disease-free survival (DFS), overall survival (OS) and resistance to treatment [53, 60]. In contrast, *DNMT3B* expression is downregulated in serous endometrial cancer [58].

Although the level of *TYMS* gene expression was upregulated in BC tissue by 0.73-fold, there were no significant differences between BC tissue and normal control tissue. It was concluded previously that there was molecular diversity affecting the *TYMS* gene at the expression level [61]. As there are several functional polymorphisms that affect *TYMS* expression, the 3′-UTR 1494*del*6 polymorphism is one of them. Earlier, it had been suggested that the $+6$ bp/$+6$ bp genotype had significantly higher *TYMS* protein expression in breast cancer tissue than did those with other genotypes in breast cancer [62, 63]. An identical correlation between *TYMS* gene expression and the 3′-UTR 1494*del*6 polymorphism has been established, and all of them hypothesized that the -6 bp allele decreases the stability of TYMS mRNA, which influences protein expression [64, 65]. This hypothesis has been tested in a healthy population in Venezuela [66]. A previous study stated that the 3′-UTR 1494*del*6 polymorphism did not impose any statistically significant influence on mRNA expression levels [67].

On the other hand, not only do *TYMS* polymorphisms have an impact on the biosynthesis of *TYMS,* but polymorphisms of other enzymes, such as dihydropyrimidine dehydrogenase (*DPYD*) and methylenetetrahydrofolate reductase (*MTHFR*), also have an effect [61]. Moreover, it has been found that the enolase superfamily member 1 (ENOSF1) gene encodes a protein and RNAs antisense to *TYMS,* which suggests that *ENOSF1* regulates *TYMS* mRNA and/or protein expression [68]. *TYMS* expression is influenced by multiple factors and the diversity of gene polymorphisms.

In conclusion, the results obtained from the present study clearly indicated that there was no association between the *MTHFR* C677T genotype and the risk of breast cancer in Egyptian women. However, the 2R/3R *TYMS* allele and *DNMT3B* (-149C>T) SNP are associated with a high risk of breast cancer, and there is a correlation between the 3′-UTR 1494*del*6 polymorphism (genotype $-6/-6$) and breast cancer risk. At the level of gene expression, there was a significant reduction in the *MTHFR* gene expression level in breast cancer patients compared with control normal adjacent tissues, and the *DNMT3B* (-149C>T) SNP did affect the *DNMT3B* expression level. Finally, there were no significant differences in the *TYMS* expression levels in breast cancer patients and control normal adjacent tissues, and the correlation between the 3′-UTR 1494*del*6 polymorphism

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and gene expression of *TYMS* is uncertain and not yet fully understood. Further studies are needed on these associations with special stress on future personalized gene therapy trials.

Abbreviations
BC: Breast cancer; *BRCA1: Breast cancer gene 1; BRCA2: Breast cancer gene 2*; GWAS: Genome-wide association studies; SNPs: Single-nucleotide polymorphisms; MTHFR: Methyl tetrahydrofolate reductase; SAM: S-adenosylmethionine; TYMS: Thymidylate synthase; DNMTs: DNA methyltransferases; TS: Thymidylate synthase; TSER: Thymidylate synthase enhancer region; G/C SNP: G to C single-nucleotide polymorphism; UTR: 5′ untranslated region; MT: Malignant breast tissue; NT: Normal breast tissue; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; OR: Odds ratio; BMI: Body mass index; TNBC: Triple-negative breast cancer; CRC: Colorectal carcinoma; DPTD: Dihydropyrimidine dehydrogenase; ENOSF1: Enolase superfamily member 1 gene.

Acknowledgements
Not applicable.

Author contributions
All authors contributed in the research in various ways from collecting samples, tests performance, results analysis, statistical interpretation of results, paper writing and revision. MS and EE collected the patients' samples, performed analysis of samples and wrote the research article. HS wrote the paper and performed results interpretation and data analysis. AE performed isolation and purification of nucleic acids under investigation and, in addition, interpretation of the results. RR performed fresh breast tissue samples collection and verified the diagnosis of the case through histopathological examination of the tissue specimens. AF performed selection of patients according to the well-defined criteria and surgical operation with obtaining the suitable breast cancer and normal adjunct tissues. ME performed appropriate samples selection according to clinical and laboratory data and interpretation of the results according to clinical, medical impacts and guidelines. In addition, ME participated in writing the research article. All authors read and approved the final manuscript.

Funding
All authors declare they did not receive funding from any agency.

Availability of data and materials
All data are available and ready to share.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethical Committee of Medical Research Institute and Ethical Committee of Faculty of Medicine (Serial number0105660), Alexandria University. Verbal consent was taken from all participants in the current study as most participants were illiterate, and the purpose of the study was clarified to each participant.

Consent for publication
Not applicable.

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

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