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# Evaluation of UDP-glucuronosyltransferase 2B17 (UGT2B17) and dihydrofolate reductase (DHFR) genes deletion and the expression level of NGX6 mRNA in breast cancer

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**Abstract** The present study was aimed to investigate the possible association between 19-base pair (bp) deletion polymorphism of the DHFR gene (rs70991108), null genotype of UDP-glucuronosyltransferase 2B17 (UGT2B17) as well as the expression level of nasopharyngeal carcinoma-associated gene 6 (NGX6) with the risk of breast cancer. This case–control study was done on 236 patients with breast cancer and 203 cancer free women. Detection of 19-bp del of DHFR was done using bi-directional PCR allele-specific amplification and UGT2B17 genotyping was performed using multiplex PCR assay. NGX6 mRNA expression level was determined by quantitative reverse transcriptase PCR in 62 breast cancerous and 62 adjacent non-cancerous tissues. Our finding showed an association between null genotype of UGT2B17 and risk of breast cancer and the null genotype increased susceptibility to breast cancer (OR: 2.99; 95 % CI: 1.94–4.60; $p < 0.0001$). However, no statistically significant difference was found between breast cancer patients and cancer free normal women regarding 19-bp ins/del of DHFR ($\chi^2 = 0.91$, $p = 0.63$). Real-time PCR data showed that the relative expression level of NGX6 mRNA was significantly lower in cancerous than that in non-cancerous breast tissue specimens ($0.936 \pm 0.042$ and $1.042 \pm 0.039$, respectively). However, NGX6 mRNA expression was not correlated with tumors grade ($p > 0.05$). In conclusion, the null genotype of UGT2B17 revealed to be a risk factor for breast cancer in a sample of Iranian population. Furthermore, down-regulation of NGX6 mRNA expression in breast carcinoma confirms the growing proof regarding the tumor suppressor role of NGX6.

**Keywords** Breast cancer · DHFR · UGT2B17 · Gene deletion · NGX6 expression

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## Introduction

Breast cancer is the most common malignancy in women worldwide and after lung cancer is the second most frequent cancer (10.4 % of all cancers) and cause of death in industrialized countries [1–3]. The cause of breast cancer is unknown; however it develops when cells of the breast reproduce uncontrollably as a result of both genetic and environmental factors [3]. Enzymes involved in the detoxification of carcinogenic compounds as well as DNA synthesis may play a role in the predisposition to this and other forms of cancer [4, 5]. Thus, it would be worth elucidating the connection between interindividual variability

in genes that code for these enzymes and altered risk for cancer susceptibility.

UDP-Glucuronosyltransferases (UGTs) are the major phase II metabolism enzymes that detoxify several endogenous and exogenous compounds in humans and other mammalian species [6–8]. In phase II metabolic reaction pathways, UGTs transfer the glucuronosyl group from the UDP-glucuronic acid (UDPGA) to several lipophilic compounds including bile acids, bilirubin, steroid hormones, drugs and environmental carcinogens [9, 10]. An additional role of UGTs is to catalyze glucuronidation of sex hormones including endogenous and exogenous estrogens and androgens, and metabolize them to less active compounds [11]. The resulting glucuronide products are more polar, generally water soluble, less toxic and more easily eliminated from the body through bile and urine [12, 13].

Based on evolutionary divergence and substrate specificity, UGT proteins have been divided into two families; UGT1 and UGT2 [14]. Isoforms of the UGT2 family are further categorized into two subfamilies; UGT2A and UGT2B. UGT2A enzymes are present in the olfactive epithelium, whereas UGT2B are expressed in hepatic tissues as well as several non-hepatic tissues including kidney, skin, brain, breast, uterine, and prostate [15]. The human UGT2B genes are clustered on chromosome 4q13 [13, 15]. UGT2B17 has high homology with other UGT2B enzymes in primary structure, and is 90 % identical with UGT2B15 [16].

A 150-kb deletion polymorphism spanning the whole UGT2B17 gene has been identified, with the approximate frequency of 10 and 67 % in Caucasians and Asians, respectively [17, 18]. It has been proved that individuals with the null genotype for this gene have no protein expression and enzyme activity for UGT2B17 [10, 19, 20]. Several studies suggested the possible function of UGT2B17 deletion polymorphism in individual response to drugs used for treatment of multiple cancers including suberoylanilide hydroxamic acid (SAHA) and calcitriol [9, 21–23].

It is believed that variations in metabolic genes can alter the performance of phase I and phase II detoxification mechanisms and also other metabolic pathways of micronutrients, like folate [24]. Folate metabolism performs a critical role in the processes of DNA synthesis, repair and methylation [25]. Dihydrofolate reductase (DHFR) is responsible for the conversion of dihydrofolate (DHF) into tetrahydrofolate (THF) and can stabilize the fully oxidized folic acid, thus making the ingested folic acid consumable for the cells. Reduction of endogenous substrate, dihydrofolate (FH2) into tetrahydrofolate (FH4) by DHFR is a process necessary for the de novo biosynthesis of purines, thymidylate, and some amino acids [26]. Given the critical function of DHFR in purine synthesis, certain anticancer drugs like methotrexate (MTX) are designed to inhibit DHFR, thus emptying FH4 and decelerating the rate of de novo DNA synthesis and cell proliferation [26, 27].

Recently a 19-bp deletion polymorphism (D-allele) in intron 1, 60 bases from the splice donor site of the DHFR gene has been identified [28]. The deletion removes a putative transcription factor binding site, which may influence gene expression. It has been shown that the expression level of DHFR in individuals with the DD-genotype is much higher than subjects carrying the WW-genotype [29]. Several studies have correlated the deletion polymorphism of DHFR with greater risk of various diseases including adult acute lymphoblastic (ALL) [27, 29, 30] and spina bifida (SB) [28]. However, DHFR deletion polymorphism has not been well studied in relation to predisposition to breast cancer. To the best of our knowledge, only one study has investigated the relationship between DHFR genotype and breast cancer risk. In this study, Xu et al. [29] found a positive association between DHFR Del-allele and breast cancer risk only in a subgroup of breast cancer women who were multivitamin users.

Gain-of-function of oncogenes and/or loss of expression of tumor suppressor genes are also believed to play a vital role in all types of cancers [31]. Nasopharyngeal carcinoma (NPC) progression-associated gene 6 (NGX6) is a novel candidate suppressor gene of tumor metastasis, that was originally cloned from the NPC high frequency heterozygosity loss region at chromosome 9p21–22 [31–33]. NGX6 is a transmembrane protein that contains an epidermal growth factor (EGF)-like domain. The role of growth factors-driven signaling in the pathogenesis of human cancer has been long established. The EGF-like domain-containing proteins appear to act as a suppressive membrane receptor or a negative growth factor [34].

A number of independent studies have evaluated the expression levels of NGX6 gene in various malignancies. In NPC, hepatocellular carcinoma (HCC) and colorectal carcinoma (CRC), the NGX6 was found to be down-regulated, and loss of NGX6 mRNA in tumor tissues reported to be associated with lymph node metastasis or distant metastases in NPC and CRC [31–33, 35, 36]. In fact, the NGX6 is able to suppress both NPC and (CRC) tumorigenesis [37] possibly through altering the cell metabolism, inhibiting cell proliferation, inducing cell apoptosis and preventing tumor invasion [37]. To the best of our knowledge there is no report regarding expression profile of NGX6 in breast carcinoma. Therefore, this study was conducted to evaluate the expression levels of NGX6 gene in breast carcinoma. Furthermore, since it is hypothesized that the genetic variations in the genes encoding UGT2B17 and DHFR could account for decreased carcinogen detoxification and DNA repair, respectively, this study was also aimed to find out whether the UGT2B17 and DHFR gene deletions are associated with the risk of breast cancer.

## Materials and methods

### Patients

DNA was extracted from peripheral blood of 174 patients with breast cancer, and also from 62 unrelated paraffin-embedded tissue specimens from breast cancer patients whom their blood samples were not available. In most cases, the histologic type was infiltrative ductal carcinoma. DNA of blood of 221 ages matched females with no history of cancer of any type, were selected as a control group. Ethical approvals for recruitment were obtained from local Ethics Committee of Zahedan University of Medical Sciences, and informed consent was obtained from all patients and healthy individuals. Blood samples were collected in EDTA-containing tubes from patients and healthy controls and DNA were extracted using salting out method as described previously [38]. DNA from formalin-fixed paraffin embedded (FFPE) tissues was extracted using standard procedures. Briefly, 10 sections (10 μm) of FFPE samples were cut into eppendorf tubes. Then, 1 ml xylene was added to remove the paraffin from each FFPE sample. Next, pellet was washed with 1 ml of 100 % ethanol and after vortex and centrifugation at 12,000 rpm for 2 min, ethanol poured off and the pellet was incubated in 37 °C for 10 min. Afterwards, 10 μl of Proteinase K, 300 μl lysate buffer and 100 μl SDS 10 % was added and incubated at 55 °C for 2 h to complete protein digestion. Other next steps are the same as the protocol for DNA isolation from blood samples [38]. The quality of the isolated DNA was checked by electrophoresis on 1 % agarose gel, quantitated spectrophotometrically and stored at −20 °C till further use.

### RNA isolation, preparation and real-time PCR

Total RNA was isolated from formalin fixed paraffin-embedded tissue samples using a RNeasy® FFPE Kit (QIAGEN) according to manufacturer instructions. CDNA synthesis performed applying RevertAid™ First strand cDNA synthesis Kit (Fermentas) based on the manufacturer procedure. Quantitative reverse transcriptase PCR (qRT-PCR) for NGX6 was performed using the Light-Cycler ABI 7500 system and Maxima®SYBR Green/Rox (Fermentas). Specific primers were designed for mRNA amplification of NGX6 and GAPDH that are listed in Table 1. Reaction volumes of 20 μl consisted of 10 μM forward primer, 10 μM reverse primer, 12.5 μl Maxima®SYBR Green/Roxand and 3 μl of cDNA as PCR template. Gene expression was quantified by the comparative Ct method, normalizing Ct-values to the housekeeping gene *GAPDH* and calculating relative expression values. The following program conditions were applied for qRT-PCR running: 95 °C for 10 s followed by 40 cycles of 95 °C for 10 s, 60 °C for 1 min.
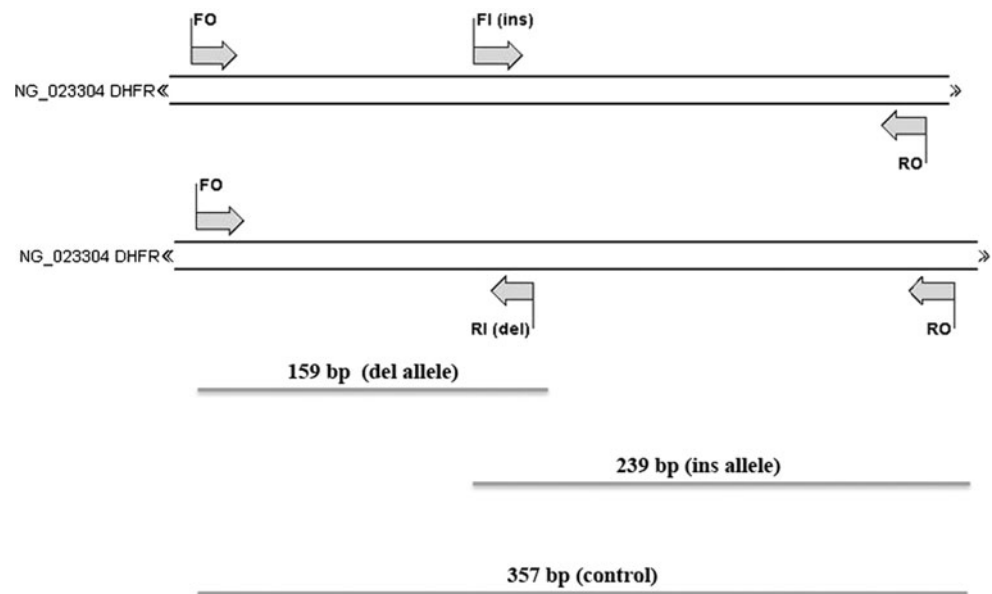
### DHFR genotyping

For detection of 19-bp deletion of DHFR, we designed bi-directional PCR allele-specific amplification (bi-PASA). The principle of the bi-PASA method was used is schematically demonstrated in Fig. 1 and a list representing the sequence of all primers is provided in Table 1. A one-tube PCR reaction enables the distinction between the different genotypes. PCR was performed by using commercially available PCR premix (AccuPower PCR PreMix; BIO-NEER, Daejeon, Korea) according to the manufacturer's

**Table 1** The list of primers used for genotyping of the UGT2B17 and DHFR genes and qRT-PCR analysis of NGX6 and GAPDH genes

| Primers | Sequence (5′–3′) | Amplicon size |
|---|---|---|
| DHFR-FO | CTGTCATGGTTGGTTCGCTAAAC | 357 bp |
| DHFR-RO | TGGGTAGGGGTGAGGTGTTTTC | |
| DHFR-FI (Del allele) | CCACGGTCGGGGTACCTGGG | 159 bp |
| DHFR-RI (Ins allele) | GAGTCGGCCACCCCGACCGT | 239 bp |
| DHFR-F1 | TCGGGGTACCTGGG | 113 and 92 bp |
| DHFR-F2 | ACGGTCGGGGTGGCCGACTC | |
| DHFR-R | AAAAGGGGAATCCAGTCG | |
| UGT2B17-FO | GGAGTTGTGGAAAGGTGCTG | 350 bp |
| UGT2B17-RO | CACAGAGCTTTATATTATAGTCAG | |
| TLR2-FO | GATGCATTTGTTTCTTACAGTGAGCG | 259 bp |
| TLR2-RO | TCTCATCAAAAAGACGGAAATGGG | |
| NGX6-FO | TGCGCCAGGAAAACGTGA | 163 bp |
| NGX6-RO | GTCTGCGGGAATGGGATT | |
| GAPDH-FO | TTGCCATCAATGACCCCTTCA | 173 bp |
| GAPDH-RO | CGCCCCACTTGATTTTGGA | |

**Fig. 1** Schematic illustration of designed bi-directional PCR allele-specific amplification (bi-PASA) assay for detection of 19-bp insertion/deletion of DHFR. Two outer primers and two specific primers [insertion allele (FI); deletion allele (RI)] are used to produce three potential products. Product sizes were; 159 bp for deletion allele, 239 bp for insertion allele and 359 bp for two outer primers (control band)



instructions. Briefly, 1 µl template DNA (~100 ng/ml), 1 µl of each primer (10 pmol/ml), and 15 µl DNase-free water were added to AccuPower PCR PreMix. Amplification was done with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 25 s with a final extension at 72 °C for 10 min. Each reaction was verified on a 2 % agarose gel. Product sizes were 159 bp for deletion allele and 239 bp for insertion allele; whereas the product size of the two outer primers (control band) was 357 bp (Fig. 2a). The bi-PASA method was effectively applied to determine 19-bp deletion of DHFR. To certify genotyping quality, all polymorphisms in random samples were regenotyped and found no genotyping mistake. We also genotyped 19-bp DHFR deletion by using two different allele-specific forward primers, F1 and F2, for detection of nondeleted and deleted polymorphic alleles respectively, and one common reverse primer (R) as described by Gemmati et al. [30] (the sequence for the corresponding primers are listed in the Table 1). The product sizes were 113 bp for wild and 92 bp for del allele (Fig. 2b). The results were 100 % concordant with the findings of bi-PASA.

## UGT2B17genotyping

The multiplex PCR method was used to detect the presence or absence of UGT2B17 with concomitant amplification of internal controls in the same tube as described by Park et al. [10] with some modifications. The presence of the UGT2B17del allele was established using primers listed in the Table 1 that spanned the known deleted region (UGT2B17-FO and UGT2B17-RO). Simultaneously, Toll-Like receptor 2
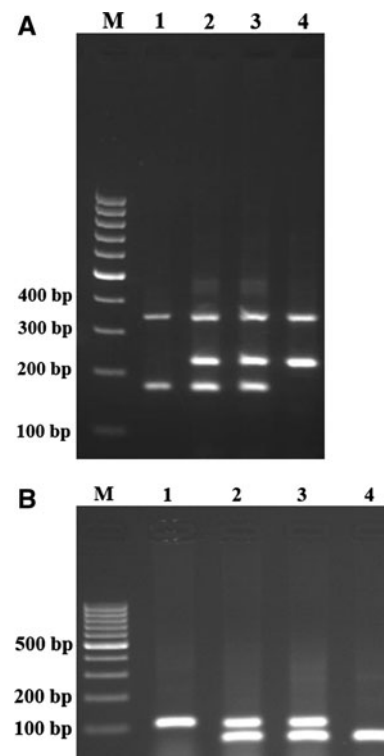


**Fig. 2** Photograph of the PCR products of the 19-bp ins/del of DHFR using bi-PASA (**a**). *M* DNA marker, *Lane 1* Del/Del, *Lanes 2, 3* Del/Ins, *Lane 4* Ins/Ins. The photograph of the PCR product of DHFR genotype by using two different allele-specific forward primers and one common reverse primer. The product sizes were 113 bp for insertion and 92 bp for deletion allele (**b**). *M* DNA marker, *Lane 1* Ins/Ins, *Lanes 2, 3* Ins/Del, *Lane 4* Del/Del

(TLR2) primers (TLR2-FO and TLR2-RO), were added to the same tube and used as the internal control (primers shown in the Table 1).
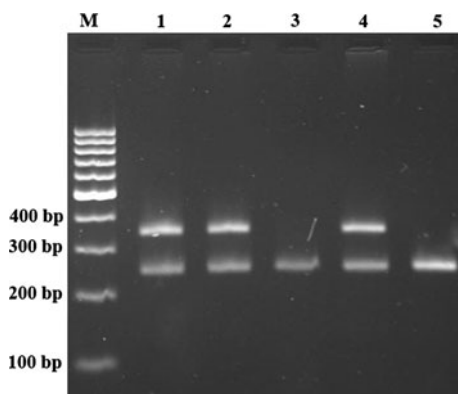
**Fig. 3** Representative multiplex PCR products resolved by agarose gel electrophoresis to identify the presence or absence of the UDP-glucuronosyltransferase 2B17 (UGT2B17) gene in the presence of an internal control. UGT2B17: 350 bp, Toll-like receptor 2: 259 bp (control). *M* DNA marker, *Lanes 1, 2, 4* wild, *lanes 3, 5* null

**Table 2** Distribution of UDP-glucuronyltransferase 2B17 (UGT2B17) and dihydrofolate reductase (DHFR) genotypes between breast cancer and normal cancer free women

| | Patients $n$ (%) | Control $n$ (%) | OR (95 %CI)[a] | $p$ value |
|---|---|---|---|---|
| UGT 2B17 | | | | |
| Wild | 136 (57.6) | 163 (80.3) | | |
| Null | 100 (42.4) | 40 (19.7) | 2.99 (1.94–4.60) | <0.0001 |
| DHFR | | | | |
| WW | 94 (39.8) | 90 (44.3) | Reference | – |
| WD | 123 (52.1) | 98 (48.3) | 1.20 (0.81–1.78) | 0.359 |
| DD | 19 (8.1) | 15 (7.4) | 1.21 (0.58–2.53) | 0.608 |
| Alleles | | | | |
| W | 161 (34.1) | 128 (31.5) | | |
| D | 311 (65.9) | 278 (68.5) | 0.88 (0.67–1.18) | 0.429 |

[a] Adjusted for age

Amplification consisted of an initial cycle with denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 25 s with a final step at 72 °C for 10 min.

The band sizes corresponding to 350 bp (UGT2B17) and 259 bp (internal control) were separated on 2 % agarose gel (Fig. 3). The samples in which only the TLR2 gene is amplified were considered a deletion for UGT2B17.

Statistical analysis

The risk for breast cancer in relation to UGT2B17 and DHFR genotypes were estimated with logistic regression. The expression analyses were performed by independent samples $T$ test, and Mann–Whitney test according to the data. All computational analyses were executed using statistical software package SPSS 18. $P < 0.05$ (two sided) was considered to be statistically significant.

Results

A total of 236 cases (age; 47.04 ± 12.30 years) and 203 controls (45.25 ± 12.78 years) were used to evaluate the genotype frequency of UGT2B17 and DHFR genes. There was no significant difference between group regarding age ($p = 0.136$). To determine whether these genetic variants in the UGT2B17 and DHFR were associated with increased risk for breast cancer, we compared genotypes in breast cancer cases and controls (Table 2). The frequencies of the UGT2B17 null genotype among the case and control subjects were 42.4 and 19.7 %, respectively. The results showed that the null genotype is a risk factor for susceptibility to breast cancer (OR = 2.99, 95 %CI = 1.94–4.60,

$p < 0.0001$). However, we found no statistically significant difference between two groups regarding DHFR genotypes ($\chi^2 = 0.91$, $p = 0.63$).

The NGX6 mRNA expression level was determined in 62 neoplastic and 62 non-neoplastic breast tissues by qRT-PCR. The breast non-cancerous tissues expressed higher levels of NGX6 mRNA than that of cancerous tissue samples ($p = 0.001$). The ratios of GAPDH/NGX6 mRNA were 0.936 ± 0.042 and 1.042 ± 0.039 in breast cancerous and adjacent non-cancerous tissues, respectively. However, NGX6 downregulation revealed not to be correlated to tumors grade ($p > 0.05$).

Discussion

In the present study, we examined the possible association between UGT2B17 or DHFR polymorphisms with breast cancer in a sample of Iranian population. We found no significant difference between breast cancer and normal individuals with regard to the 19-bp deletion of DHFR. While, null genotype of UGT2B17 was associated with breast cancer risk. Our finding is consistent with the fact that this enzyme plays a pivotal role in the detoxification of carcinogens, and that reduction in its function may possibly increase susceptibility to breast cancer.

Cancer risk caused by human exposure to exogenous chemicals, like environmental carcinogens and drug metabolites, may vary according to the capacity to eliminate the xenobiotics from the body. Polymorphisms in the genes that encode enzymes involved in the metabolism of xenobiotic (xenobiotic-metabolizing enzymes), such as

UGT2B17, bring about varying activity levels of this enzymes, which can then influence xenobiotic clearance [39].

Several studies have estimated the influence of UGT2B17 deletion polymorphism on the risk of various cancers. Gallagher et al. [15] reported a positive correlation between UGT2B17 deletion polymorphism and increased risk for lung cancer. Additionally, Park et al. [10] showed that deletion polymorphism of the UGT2B17 gene results in greater risk for prostate cancer in Caucasian patients. However, Seltur et al. [40] and Olsson et al. [41] found no association between UGT2B17 deletion polymorphism and susceptibility to prostate cancer. Several other studies have estimated the role of UGTs including UGT1A1, UGT2B15, and UGT2B7 in breast carcinogenesis [42–44], but to date less is known about importance of UGT2B17 deletion polymorphism in this malignancy.

Glucuronidation has been implicated as a major detoxification pathway for a variety of compounds including endogenous compounds like bilirubin and steroid hormones, as well as xenobiotics including drugs and environmental carcinogens. UGT2s catalyze the transfer of glucuronic acid from UDPGA to endogenous and exogenous molecules with oxygen, nitrogen, and sulfur functional groups to several lipophilic compounds such as bile acids, bilirubin, steroid hormones, environmental pollutants and phenolic drugs [12, 45].

Exogenous and endogenous estrogen may also be a factor in breast carcinogenesis. Estrogen exerts pleotropic effects on lymphocyte activation, proliferation, cell cycle progression and apoptosis in steroid target tissues [46]. In estrogen target tissues like the breast, $17\beta$-estradiol is extensively converted by UGT enzymes before being released into the circulation. Recently, Hu and Mackenzie [47] has demonstrated that removal of endogenous catechol estrogens, such as 4-hydroxyestrone and 4-hydroxyestradiol, in breast tumors proceeds in a UGT2B-dependant manner, and that the $17\beta$-estradiol-enhanced UGT2B15 and UGT2B17 activity could minimize the exposure of breast tissues to these metabolites.

Moreover, a critical route of removal and detoxification of tamoxifen (TAM) and its metabolites is via glucuronidation, and a number of pharmacogenetics studies have demonstrated the possible role of UGTs in individual response to TAM. Blevins-Primeau et al. [48] and Sun et al. [23] have shown that metabolites of TAM are cleared from the body through the bile mainly by conjugation with glucuronic acid by UGTs function, and also reported that UGTs including UGT1A10, UGTs2B7 and UGT1A8 displayed higher levels of *O*-glucuronidating activity than other UGTs against *trans*- and *cis*-4-OH-TAM. Sun et al. [49] have also suggested the probable function of UGTs in individual pharmacological response to TAM therapy.

They found a positive association between UGT1A4 codon 48 polymorphism and altered rates of glucuronidation against TAM and its active hydroxylated metabolites [49].

Overall, given the pivotal role of UGT2B17 in detoxification of breast cancer carcinogens, interindividual variability in the genotypes of UGT2B17 gene may probably account for predisposition to this malignancy.

On the other hand, we did not found any association between DHFR deletion allele and susceptibility to breast cancer in our population. A 19-bp deletion polymorphism in intron 1 of the DHFR gene removes a putative transcription factor binding site, which may affect gene regulation [50]. A dose dependent relation between DHFR expression and the deletion was observed. Subjects with the DD-genotype had higher DHFR mRNA levels than subjects carrying the homozygous counterpart genotype (WW-genotype) [29].

Our results are consistent with multiple studies on cancer. Eroglu et al. [51] reported no association between DHFR genotypes and venous thromboembolism (VTE) in cancer patients. Moreover, Xu et al. [29] have found no overall correlation between breast cancer susceptibility and DHFR genotypes, but they reported this relation to be significant in multivitamin users. However, some other studies reported a positive association between DHFR del-allele and predisposition to ALL and SB [27, 30]. The discrepancy in the result of these studies might be the result of differences in the studied populations and their exposure to diverse carcinogens.

Considering the NGX6 expression profile, it was revealed that NGX6 mRNA expression in breast tumor tissues was significantly lower in comparison with the normal tissues but its down-regulation was not correlated with tumors grade. In agreement to our findings, Wang et al. [31] reported that NGX6 is extensively down-regulated in NPC and is associated with tumor metastasis. Moreover, Gue et al. [32, 52] demonstrated that NGX6 inhibits cell invasion, adhesion and proliferation of colon cancer cells through attenuation of the WNT/ss-catenin signaling pathway. They showed that NGX6 was down-regulated, and the frequency of downregulation of NGX6 was significantly greater in CRC tissues with lymph node involvement or distant metastases than that in patients without metastases [53].

It is suggested that the NGX6 regulates cell proliferation by modulating the function of EGFR pathway and increasing the length of G1 phase of cell cycle through down-regulating the expression of cyclin D1, A, and E. [54]. The EGFR pathways are implicated in invasion and metastasis in several different types of rodent and human cancer cells, including human breast cancer cells [34]. NGX6 as an antagonist receptor is able to increase cell–matrix adhesion and inhibit cell migration and invasion

[35] through blocking distribution and aggregation of EGFR on the plasma membrane, or depleting EGFR ligand binding/attenuating the sequential EGFR/Ras/Mek/MAPK signaling transduction [54]. The blockade of EGFR is reported to suppress growth of several different cell lines derived from human carcinoma of various histological types [34].

Overall, given the function of NGX6 in modulation of cell migration and metastasis and considering the fact that NGX6 is downregulated in breast carcinomas, any malfunction of NGX6 probably could predispose individuals to breast cancer. This study, for the first time, provided evidence regarding downregulation of NGX6 gene in breast carcinoma, and highlighted its role in pathogenesis of breast cancer as a tumor suppressor agent.

Additionally, our findings showed that null genotype of UGT2B17 may contribute to susceptibility to breast cancer in a sample of Iranian population. To the best of our knowledge, this is also the first report regarding the impact of UGT2B17 null genotype and risk of breast cancer. Larger studies with different ethnicity are required to validate our findings.

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