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IMPACT OF UTP23 GENE POLYMORPHISM (rs13250873) ON EXT1 GENE EXPRESSION IN DEVELOPING AND RELAPSING OF BREAST CANCER

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ABSTRACT

Background:

Breast cancer (BC) is a multifactorial disease with various genetic, epigenetic, and environmental causes. This study investigated association of the *UTP23* gene polymorphism (rs13250873) with *EXT1* mRNA expression in developing and relapsing (recurrence) of BC disease.

Methods:

Breast tissues obtained from 70 women assigned to three groups of primary BC (n=55), recurrent BC (n= 15), and healthy age-matched controls referring for mammoplasty (n= 25), were studied. Tissue DNA content was extracted; the *UTP23* gene region of interest was

amplified by PCR assay, and analyzed for the G→A SNP (rs13250873) by automated sequencing. The mRNA expression of the *EXT1* gene was measured by real-time PCR assay.

Results:

Frequency of the allele A was insignificantly and distribution of the AA-homozygosity markedly higher among BC group compared to the control (0.471 vs. 0.340, P> 0.05; and 26% vs.4%, P= 0.020, respectively), with allelic odds ratio of 1.7. Also, frequency of the allele A and distribution of AA-homozygosity were insignificantly higher in the recurrent BC group compared to primary BC group (0.533 vs. 0.455 and 33.3% vs. 23.6%, respectively), with allelic odds ratio of 1.36. The *EXT1* expression was decreased in the recurrent BC compared to the primary BC and healthy groups (P= 0.018 and P= 0.002, respectively). The rs13250873 strongly associated with *EXT1*expression in the primary (r= 0.751; P< 0.001) and recurrent (r= 0.833; P< 0.001) BC groups.

Conclusions:

Simultaneous evaluating of rs13250873 and *EXT1* mRNA expression might be useful as a biomarker for identifying individuals who are at risk of BC development and relapse.

Key words: Breast neoplasm, Exostoses-1, SNP, Relapse, rs13250873

1- INTRUDUCTION

Breast cancer (BC) is a multifactorial disease with various genetic [1], epigenetic [2] and environmental [3] underlying causes. Since the clinical phenotype of the disease is quite homogenous, the intrinsically heterogonous nature of BC suggests genetic analysis, at either personal or population level, as a promising approach for prediction and early detection of BC as well as discrimination of different subtypes and stages of the disease [4-6].

Single nucleotide polymorphisms (SNPs) refer to any germline or somatic change in a single nucleotide at a specific position in an exon or intron part of a gene [7, 8]. While some SNPs exert no effect on the respective gene function in most cases [9], some may considerably influence the gene expression of same region or that of nearby genes at either transcriptional or translational level leading to a particular clinical outcome [10, 11]. Vast number of SNPs occurring in different genes has been detected to be implicated in occurrence and progression of BC [11-14].

The human exostoses-1 (*EXT1*) gene consists of 312457 bp located on 8q24.11 [15], and encodes exostosin glycosyltransferase 1 enzyme. It has been primarily known to be involved in the pathogenesis of hereditary multiple

exostoses, an autosomal dominant disorder of the bones [16].

Also, a tumor suppressive role has been described for this gene [17], an emerging notion supported by several studies [4, 18, 19]. The EXT1 is essentially involved in heparan sulfate biosynthesis [20] and the aberrant expression of the *EXT1* gene may disrupt heparan sulfate biosynthesis duringtumorogenesis process [21]. However, expression level of the *EXT1* gene and its underlying mechanisms remain unclear in many cancers.

A mutation of G→A (rs13250873) located within intronic region of the *UTP23* gene, encoding a predicted intracellular protein, has been previously reported to be associated with increased risk of breast cancer among Caucasian populations [22]. While it is uncertain whether this SNP affects the

UTP23 function, Sapkota et al. [22] proposed that rs13250873 might be involved in regulation of*UTP23* nearby genes such as *RAD21* and *MGMT*. We previously reported that the *EXT1* gene is down-regulated in relapse (recurrent) BC tissues compared to the normal healthy breast tissues [23]. Herein, we aimed to comparatively investigate the association between the rs13250873 of *UTP23* gene and *EXT1* mRNA expression in primary and recurrent BC patients compared to healthy individuals.

2-MATERIALS AND METHODS

Study population

The subjects consisted of 70 women with breast cancer who attended cancer the research center of ShahidBeheshti University of Medical Sciences, Tehran, Iran during 2012-2015 years; fifteen outof 70 the patients suffered disease relapse during five years after their curative surgery. Also, 25healthy age-matched women referring for mammoplasty surgery, with no history of cancer, infection,autoimmune diseases, and chemotherapy were recruited as control. Demographic and clinical features of the subjects are presented in Table 1.

In compliance with the ethical standards of Helsinki declaration, all participants signed a written informed consent. Also, an ethical committee based at the center approved the study procedure.

The UTP23 gene amplification and sequencing

First, 10-micron sections were prepared from normal and tumor paraffin-embedded tissues. Following deparaffinization by Xylene and tissue lysis by proteinase K digestion, their DNA content was extracted using DNA extraction kit (DNG-plus, Cinnagen, Tehran, Iran), according to manufacturer's instructions. The intronic

region of the *UTP23* gene was then amplified by polymerase chain reaction (PCR) assay using a pair of specific primers (Table 2). The PCR was performed using

Eppendorf thermocycler (Mastercycler gradient, Hamburg, Germany) in a final volume of 25 µlcontaining 2.5 µl of Buffer10x, 0.5 µl of DNTPs, 0.6 µl of Mgcl2, 0.4 µl of Taq polymerase, 1 µl (100pmol/ml) of forward primer, 1 µl (100 pmol/ml) of reverse primer, 1 µl (50 ng) of DNA, and 18 µl ofddH2O. Thermal cycling of the reaction included; primary denaturation at 93°C for 3 minutes,followed by 35 cycles of; denaturation at 93°C for 30 seconds, annealing at 55°C for 20 seconds andextension at 72°C for 30 seconds, and termination by final extension at 72°C for 30 minutes. The PCR product was detected and verified on 1.5% agarose gel electrophoresis. The amplified DNA was subjected to sequencing (Applied Biosystems 3730/3730xl DNA Analyzers Sequencing, Bioneer,South Korea) and analyzed by AutoAssembler software version 2.1 (Applied Biosystems, Foster City,

CA, USA).

Quantitative Real-time PCR of the EXT1 gene

Total RNA was extracted from deparaffinized and lyzed tissue using RNeasy FFPE kit(QIAGEN GmbH, Hilden, Germany), according to the manufacturer's guidelines. For cDNA synthesis, 1 µl of the extracted total RNA from each sample was separately subjected to reverse transcription PCR in a final reaction volume of 20 µl using RT2 PreAMP cDNA Synthesis Kit(QIAGEN, Germany), according to the manufacturer's protocol.

A SYBR green-based real-time PCR assay was carried out for measurement of mRNA copy numbers of the *EXT1* gene. The GAPDH gene was recruited as an internal control. The assay was performed using specific primers (Table 2) and 25 µl of Takara Bio SYBR Premix Ex Taq (Tli RNase

H Plus) master mix (Takara Bio Inc., Shiga, Japan), in a reaction volume of 25 µl, and using 7500

Fast Real-Time PCR System (Applied Biosystems®). Thermal cycling of the reaction included; aninitial denaturation at 95°C for 30 seconds, followed by 40 two-step cycles of; denaturation at 95°C for 5 seconds, annealing and extension at 60°C for 30 seconds, and termination in a dissociation stage by gradually increasing the temperature from 55 to 95 °C.

For each sample, the cycling threshold (Ct) value of the *GAPDH* gene was subtracted from that of the *EXT1* gene (Ct*EXT1*-Ct*GAPDH*) to calculate delta Ct (ΔCt). The mRNA expression of *EXT1* gene was then expressed as 2 to the power of –ΔCt [2(-ΔCt)] for each individual.

Data were analyzed using the GraphPad Prism software version 5.0 for Windows (San Diego,Ca, USA). The Mann–Whitney U test or Kruskal Wallis test followed by Dunn's pair-wise comparison test were performed for comparing the mean mRNA expression of *EXT1* between the groups of interest. The possibility of any association between the expression of *EXT1* and genotypedistribution of rs13250873 was examined by non-parametric Spearman correlation test. Thedifferences in the allele frequencies between the groups were examined by Chi-square test. Also,distribution of each genotype was compared between the groups by proportional test using STATAsoftware version 11 (StataCorp, College Station, TX). The differences were considered as statisticallysignificant wherever the *P* value was less than 0.05.

3-RESULTS

SNP identification

Sequencing of intronic region of the *UTP23* gene in 70 BC patients and 25 healthy women detected the G to A base change at the region of interest. The frequency of allele G among the study population was calculated as 56.32% and that of allele A as 43.68%; therefore, the former was considered as major allele and the latter as minor allele.

Comparison of G→A SNP between BC and control groups

Although there was no significant difference in the allele frequencies between the BC patients and healthy individuals (P=0.061), the frequency of the allele A was insignificantly higher among BC group compared to the control (0.471 vs. 0.340). In addition, as given in Table 3, the distribution ofAA-homozygosity among BC group was significantly higher compared to the control (26% vs. 4%;P= 0.020). As presented in Table 4, frequency of the allele A and also the distribution of AAhomozygositywere insignificantly higher in the recurrent BC group compared to the primary BCgroup (0.533 vs. 0.455 and 33.3% vs. 23.6%, respectively). Additionally, the distributions of AA homozygosity among primary and recurrent BC groups were significantly higher compared to thecontrol (P= 0.032 and P 0.012, respectively). Moreover and interestingly, the highest frequency of theallele A was observed among the recurrent BC patients and the lowest frequency among the healthy women

(0.533 vs. 0.340; P= 0.007). The allelic and genotypic odds ratios of G→A SNP for incidence of BC are shown in Table 5.

As seen, the risk of BC incidence in people carrying AA genotype seemingly was 9 and 7.5 folds higher compared to those with AG and GG genotypes, respectively. Also the risk of relapse seemed to be higher among population with higher frequency of the allele A (odds ratio= 1.36).

Comparison of the EXT1 mRNA expression between BC and healthy women

The mRNA expression of the *EXT1* among BC patients was insignificantly lower compared to the healthy group (P= 0.167; Fig1A). This decrease was also observed with respect to the primary and recurrent BC groups compared to the healthy control (P= 0.266 and P= 0.002, respectively; Fig1B).

Furthermore, the *EXT1* expression in the recurrent BC patients was significantly lower compared to the primary BC patients (P= 0.018).

Correlation between G→A SNP of the UTP23 gene and mRNA expression of the EXT1 gene

There was a strong correlation between G→A SNP genotype of *UTP23* gene and *EXT1* mRNA expression in the primary BC group (r= 0.751; P< 0.001) and also in the recurrent BC group (r=0.833; P< 0.001), but not in the control group (r= 0.251; P= 0.408); the AA, AG and GG genotypes were associated respectively with low, moderate and high expression of the *EXT1* gene (Fig. 2A).

Interestingly, frequency of AA genotype in both primary and recurrent BC groups was significantly higher, and in turn, these groups revealed lower *EXT1* expression compared to the healthy control

(Fig. 2B).

4-DISCUSSION

Although it was primarily described in pathology of hereditary multiple exostoses [16], the*EXT1* gene has been recently proposed as an oncogenic transformation-related gene [24]. The loss ofheterozygosity at the *EXT1* loci harboring either germline [25] or somatic [26] mutations, and also the presence of chromosomal abnormalities including this gene region [27] have been suggested to be involved in development of chondrosarcomas. Nevertheless, alterations in the *EXT1*expression/function under the influences of mutations in other genes regions located nearby the *EXT1*gene region and their relationship with tumorogenesis have not yet been studied. We had previously reported decreased expression of *EXT1* among Iranian BC patients withrecurrent compared to

primary disease [23]. Consistent with that, in the present study, we re-observedthat expression of the *EXT1* gene in the recurrent BC patients was significantly lower than that in theprimary BC patients. However, unlike our previous report [23], in the present study the *EXT1*expression in primary patients was insignificantly declined compared to the healthy individuals, aswell. To the best of our knowledge, we are first to demonstrate the reduced expression of *EXT1* genein human breast cancer tissues. Our results in line with our previous study corroborate reducedexpression of the *EXT1* gene as a predictive value for risk of BC incidence as well as of recurrence ofthe disease after curative surgery.

Our findings are in good agreement with previous studies suggesting a tumor suppressive role for the *EXT1* gene [17, 27]. Hameetman et al. [28], for instances, found that decreased expression of this gene leads to intracellular accumulation of heparansulphate proteoglycan implicated in the pathogenesis of chondrosarcomas. Additionally, epigenetic transcriptional inactivation of the *EXT1*gene, due to CpG island promoter hyper-methylation, has been suggested to abrogate heparin sulfate biosynthesis exerting a tumorogenesis effect [4]. However, the cell-surface or intracellular status of heparin sulfate was not evaluated in our study, and it remains yet unclear whether the diminished *EXT1* expression exerts its effect through alterations in heparin sulfate biosynthesis or there is/are other mechanism(s) of action for this gene during tumorogenesis process of BC.

Another important finding of the present study was that frequency of the allele A and also AA genotype of G→A SNP (at the *UTP23* gene region) was higher among the BC patients compared to healthy population. Previously, Sapkotaet al.[22], in a two-stage association study found that thisG→A SNP was associated with increased risk of breast cancer with allelic odds ratio of 1.17; however, they did not explain any relationship between this SNP and gene expression regulation or any other underlying events involved in the BC pathogenesis. Consistent with Sapkotaet al.[22], in the present study we observed that the allelic odds ratio of the G→A SNP was 1.7, which demonstrates that the risk of BC is perhaps increased in individuals carrying the Allele A. Moreoverand notably, we for the first time observed that in BC patients possessing the Allele A of the G→ASNP, the risk of recurrent BC was increased with odds ratio of 1.36.

Interestingly, in our study, the risk of BC in people having the AA genotype was 9 and 7.5 folds higher compared to those

with AG and GG genotypes, respectively. Although, there is no comparable evidence to support this finding at this time, it seems that the G→A SNP plays a pivotal role in determining susceptibility to BC development as well as to the disease recurrence. More interestingly, in the present study, the AA genotype strongly correlated with the expression of the *EXT1* gene in both groups of the BC patients manifesting primary and recurrent phenotypes of the disease. From another analytical view, the expression of the *EXT1* gene in people with the AA genotype was significantly lower than those with the AG genotype, and also in the latter was significantly lower than those with the GG genotype. This is the first report of an association between G→A SNP of the *UTP23* gene and expression of the *EXT1* gene in BC patients.

The present study provided novel data regarding molecular basis of breast cancer. In addition, one of the strength of our study was use of breast normal and cancerous tissues, instead of peripheral blood cells, for the measurements. Despite these advantages, the present study had some major limitations that should be considered in future investigations; the major weakness of this study was its short sample size. Also, we did not assess expression of heparansulfate to examine its possible relationship with alterations in expression of the *EXT1* gene.

5-CONCLUSION

The present study indicated the G→A SNP of the *UTP23* gene affects the mRNA expression of its nearby *EXT1* gene. This finding suggests that simultaneous screening for G→A SNP and measuring the mRNA expression of the *EXT1* gene might be useful for identifying not only individuals at risk of BC development, but also treated BC patients who are likely to redevelop the disease. However, this notion needs to be further corroborated through future investigations with larger sample sizes and among other ethnically different population.

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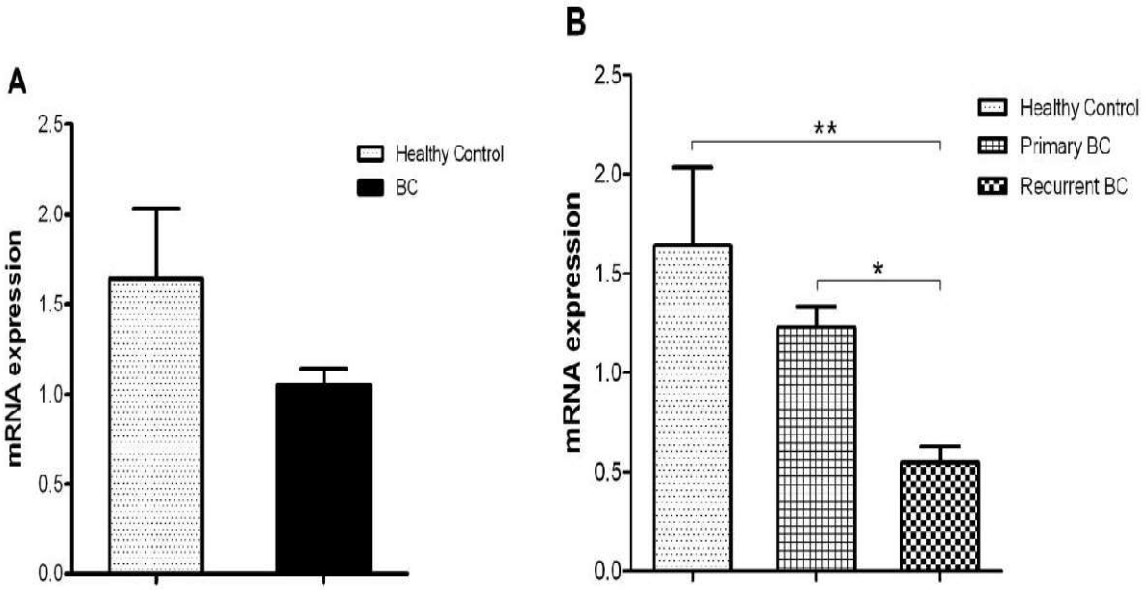

Figure 1 comparison of EXT1 expression between; A) healthy control and breast cancer (BC) groups, and B) healthy control, primary and recurrent BC groups; the signs * and ** represent *P* values < 0.05 and <0.01, respectively.

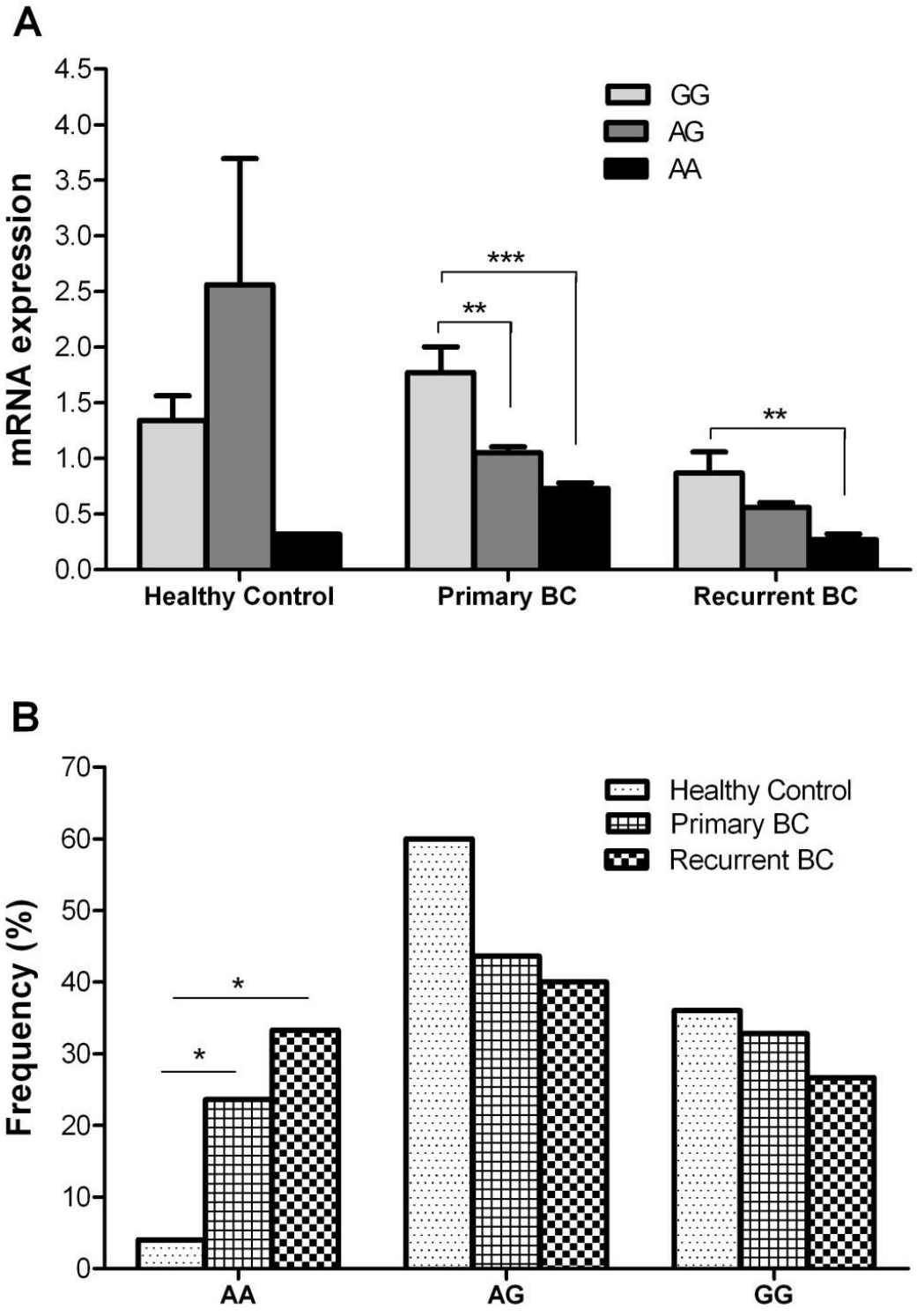

Figure 2 Correlation between genotypes of rs13250873 and *EXT1* expression. A) The AA genotype was associated with low *EXT1* expression in both primary and recurrent breast cancer (BC) patients.
B) Frequency of AA genotype was significantly higher in both primary and recurrent BC groupscompared to the healthy control group. The signs *, **, and * represent *P* values < 0.05, < 0.01, and<0.001, respectively.**

Table 1: Demographic and pathological characteristics of the control and breast cancer (BC) groups

Variable		Primary BC (n=55)	Recurrent BC (n=15)	Healthy Control (n=25)
Age (Year), Mean ± SD		55.0 ± 11.9	47.1 ± 15.0	41.9 ± 7.3
Gender		Women	Women	Women
Race		Caucasian	Caucasian	Caucasian
Pathological Type	IDC*	52 (94.6%)	9 (60.0%)	-
	ILC**	2 (3.6%)	4 (26.7%)	-
	Other	1 (1.8%)	2 (13.3%)	-
Stage	I	41 (74.5%)	7 (46.7%)	-
	II	14 (25.5%)	8 (53.3%)	-
Grade	G1	5 (9.1%)	1 (6.7%)	-
	G2	44 (80.0%)	13 (86.6%)	-
	G3	6 (10.9%)	1 (6.7%)	-
ER+		55 (100%)	15 (100%)	-
PR+		53 (96.4%)	14 (93.3%)	-
HER2+		19 (34.5%)	5 (33.3%)	-

*** IDC: Invasive ductal carcinoma**

****ILC: Infiltrating lobular carcinoma**

Table 2: Oligonucleotide sequences of the primers used in the current study.

Primer		Sequence (5'->3')	Product length	Assay
Chromosome 8, GRCh38.p7 Primary Assembly	Forward	5'-TAGTGCAGGGATCGTGTCTT-3'	236 bp	Conventional PCR
	reverse	5'-ACAACCATTCTCCCACCTTG-3'		
EXT1	Forward	5'-CTTCGTTCCTTGGGATCAAT-3'	95 bp	Real-time PCR
	Reverse	5'-TGCCTTTGTAGATGCTGGAG-3'		
GAPDH	Forward	5'-ATGGAGAAGGCTGGGGCT-3'	125 bp	Real-time PCR
	Reverse	5'-ATCTTGAGGCTGTTGTCATACTTCTC-3'		

Table 3: Genotypes distribution and allele frequency of G→A SNP (rs13250873) among healthy control and breast cancer groups, detected by automated sequencing

Variable	Healthy Control (n=25)	Breast cancer (n=70)	*P* value
Allele			
A	0.340	0.471	0.061 §
G	0.660	0.529	
Genotype			
AA	1 (4%)	18 (26%)	0.020 ┼
AG	15 (60%)	30 (43%)	0.141 ┼
GG	9 (36%)	22 (31%)	0.676 ┼

§ Chi-square test, and ┼ proportional test

Table 4: Genotypes distribution and allele frequency of G→A SNP (rs13250873) among primary and recurrent breast cancer (BC) groups, detected by automated sequencing

Variable	Primary BC (n=55)	Recurrent BC (n=15)	*P* value
Allele			
A	**0.455**	**0.533**	**0.322** §
G	**0.545**	**0.467**	
Genotype			
AA	**13 (23.6%)**	**5 (33.3%)**	**0.446** ┼
AG	**24 (43.6%)**	**6 (40.0%)**	**0.801** ┼
GG	**18 (32.8%)**	**4 (26.7%)**	**0.654** ┼

§ Chi-square test, and ┼ proportional test

Table 5: Allelic and genotypic odds ratio of G→A SNP (rs13250873) for incidence and recurrence of breast cancer (BC).

Odds Ratio		BC/Control	Primary BC/Recurrent BC
Allelic	**A vs. G**	**1.7**	**1.36**
Genotypic	**AA vs. AG**	**9.0**	**1.5**
	AA vs. GG	**7.5**	**1.7**
	AG vs. GG	**0.85**	**1.2**