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TIMP1 and TIMP3 circulating levels and promoter polymorphisms in breast cancer

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Breast cancer is one of the most prevalent cancers and the second cause of cancer-related deaths among women. Both the cancer-related deaths and cancer incidence rates are rising globally, and a major aetiology is genetics and gene polymorphisms [1,2]. Matrix metalloproteinases (MMPs) degrade the extracellular matrix, and their relationship with cancer invasion and metastasis has been demonstrated in numerous studies. MMPs can be activated by proteinase cleavage, and their activities are regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). The balance between MMPs/TIMPs is important in the regulation of the basement membrane and extracellular matrix turnover. Losing this balance may lead to diseases as a result of unrestrained extracellular matrix function, cell migration, growth, and inflammation. Changes in the gene polymorphism and the expression of MMPs or TIMPs may be involved in different kinds of cancers including breast cancer [3–5].

There are four human paralogous genes encoding TIMPs (TIMPs-1 to 4) [5]. *TIMP1* and *TIMP3* are two important members of this family and may be related to the development of different cancers [6]. One of the most important variants of *TIMP1* is 372T/C polymorphism, which seems to be related to the incidence and development of breast cancer [7]. *TIMP3*, another member of the *TIMPs* family, is located on chromosome 22q12.1, and a polymorphic variation in the *TIMP3* promoter region is reported to be related to the survival of human cancers, including breast cancer [8]. Therefore, TIMPs have been shown to be important in cell migration, metastasis, and pathogenesis of breast cancer. As the most important polymorphisms identified within the promoter of *TIMP1* and *TIMP3* are rs4898 and rs9619311, respectively, these may regulate the expression of their protein products. We hypothesized links between *TIMP1* (rs4898) and *TIMP3* (rs9619311) SNPs and their serum levels with breast cancer.

The analyses were performed on blood and serum samples from 100 breast cancer cases and 100 controls. Patients were women diagnosed with breast cancer, with a mean/SD age of 50 ± 11.7. Controls were matched for age (50.8 ± 12.3 years, $P = 0.65$). This study was approved by the University of Guilan Ethics Committee and has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All the participants consented to be involved in this study.

Genomic DNA was isolated by phenol/chloroform extraction from peripheral blood cells collected into EDTA. A ratio of A260/A280 absorbance was used to qualify the extracted DNA. The promoter SNPs in *TIMP1* and *TIMP3* were genotyped using PCR-RFLP assay. The PCR amplification reactions were carried out in 20 µl aliquots included 4 µl of distilled water, 10 µL of PCR Master Mix (Cinnagen, Iran), 2 µl for each primer and 4 µl of genomic DNA. The TIMP1 (rs4898) and TIMP3 (rs9619311) polymorphisms were detected using the following forward (F) and reverse (R) primers (Generay Biotech, Shanghai, China): TIMP1: F: 5′-GCACATCAC TACCTGCAGT-3′, R: 5′-GAAACAAGCCCACGATTTAG-3′. TIMP3: F: 5′-TGGCCACCAATCATCCCATC-3′, R: 5′-TCCTC GCTGAGAAGTGGACAA-3. The PCR cycling conditions for *TIMP1* SNP were as follows: an initial denaturation step of 95° for 5 min and followed by 35 repeated cycles at 95° for 30 s in denaturation step, 62° for 45 s in annealing step, 1 min of extension at 72° and a final extension at 72° for 10 min. The PCR conditions for *TIMP3 SNP (rs9619311)* were similar to *TIMP1* SNP (except annealing step at 60° for 45 s). The digestion of *TIMP1 (rs4898)* and *TIMP3 (rs9619311)* polymorphisms was performed with *BssSI* and *SacI* restriction enzymes (New England BioLabs, Hitchin, UK) for 3 hours in 37°, respectively. Finally, products were visualized under UV transilluminator on 2% agarose gels. Genotypes were determined as C/C (homozygous mutant genotype) (175 bp), T/T (wild-type genotype) (152, 23 bp) and CT (175, 152, 23 bp) for *TIMP1* variation and CC (wild-type genotype) (458 bp), TT (homozygous mutant genotype) (298, 160 bp) and CT (458,

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298, 160 bp) for *TIMP3* variation.

In order to compare the results of case–control samples and their allele-genotype frequencies, Hardy–Weinberg equilibrium (HWE) of the genotypes was analysed by the chi-square (χ^2) test. The Chi-square test was calculated by using MedCalc statistical software (version 14.8.1; Ostend, Belgium). A value of $P < 0.05$ was considered to be statistically significant. Analysis of variance (ANOVA) was used to test for differences among the groups. Serum TIMP1 and TIMP3 were measured by ELISA (ab187394 and ab119608, Abcam, Cambridge, UK).

The genotype and allele frequencies of *TIMP1* (rs4898) and *TIMP3* (rs9619311) are summarized in Table 1. There were no statistically significant differences in either of gene analyses. Serum TIMP1 and TIMP3 levels were lower in patients (96 ± 17 ng/ml and 44 ± 7 ng/mL) compared to controls (127 ± 20 ng/ml, $P = 0.008$ and 31 ± 6 ng/ml, respectively, $P = 0.004$). The levels of both TIMPs were strongly linked to their respective genotypes (Figure 1). For TIMP1, the serum levels of C/C, C/T and T/T genotypes were 118 ± 11 ng/ml, 99 ± 11 ng/ml and 78 ± 11 ng/ml, respectively, and for TIMP3, the serum levels for C/C, C/T and T/T genotypes were 34 ± 4 ng/ml, 44 ± 6 ng/ml and 56 ± 10 ng/ml, respectively. The results showed that patients with C/C genotype have increased levels of serum TIMP1 when compared to other genotypes, and for TIMP3, we observed higher serum level of TIMP1 in mutant homozygous cases (T/T) than other genotypes.

The results of this study showed no association between *TIMP1* and *TIMP3* SNPs and breast cancer. However, a significant increase in serum TIMP1 and TIMP3 was seen in both groups in certain genotypes. We suggest that the T allele for *TIMP3* may affect the amount of transcription and expression of TIMP3 circulating level. For *TIMP1*, patients with C/C genotype had increased levels of serum when compared to other genotypes, which means C allele may influence the expression level of serum TIMP1. In some studies, downregulation of TIMP3 has been reported in various

Table 1. TIMP1 and TIMP3 genotype and allele frequencies.

	Controls (n%)(n = 100)	Patients (n%)(n = 100)	P-value
TIMP1			
Genotypes			
C/C	26 (26%)	25 (25%)	0.56
C/T	39 (39%)	46 (46%)	
T/T	35 (35%)	29 (29%)	
Alleles			
C	91 (45.5%)	96 (48%)	0.61
T	109 (54.5%)	104 (52%)	
TIMP3			
Genotypes			
C/C	22 (22%)	18 (18%)	0.63
C/T	37 (37%)	43 (43%)	
T/T	41 (41%)	39 (39%)	
Alleles			
C	81 (40.5%)	79 (39.5%)	0.83
T	119 (59.5%)	121 (60.5%)	

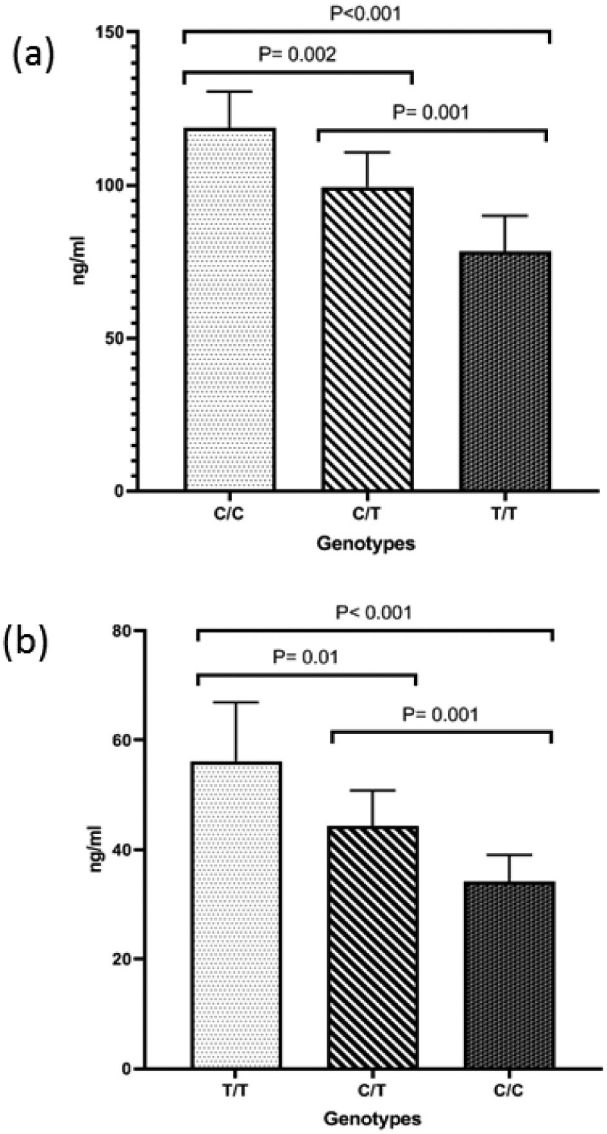

Figure 1. Association of *TIMP1* (a) and *TIMP3* (b) genotypes and serum concentration breast cancer patients. Data are shown as mean ±SD of three genotypes of each protein. The number of 100 serum samples from patients with breast cancer were used (n = 100).

cancer types such as thyroid cancer, which is a result of aberrant promoter hypermethylation. Silencing of *TIMP-3* is linked with 5′ CpG island hypermethylation, suggesting that upregulating TIMP3 expression directly may only have a restricted impact on its anti-tumor capability. Moreover, the effect of other TIMP proteins, including TIMP2 and TIMP4 on carcinogenesis, should be also considered [9,10].

The link between *TIMP* SNPs and their circulating levels in breast cancer has been investigated in numerous studies. A 2009 meta-analysis reported a significant relationship between *TIMP2* and *TIMP3* SNPs and cancer [11]. Research from Sweden showed that MMP9–1562 C/T and TIMP3–1296 T/C variants are significantly related to the breast cancer [4], whilst a study from Taiwan reported a relationship between the *TIMP1* (rs4898) SNP and breast cancer [12]. Moreover, changes in the serum MMP-9 and

TIMP-1 in breast cancer patients were seen as compared to controls [13]. Changes in TIMP1 serum level have been shown to be significantly related to breast cancer among the Taiwanese population [14], whilst a study from Poland reported a link between TIMP1 and MMP/TIMP expression ratio and breast cancer [15].

The results of this project should be interpreted considering some limitations. The sample size has been quite small; the result should be interpreted with caution. As only one SNP in *TIMP1* and *TIMP3* has been studied, we cannot exclude the possibility that other variants could have a role. Finally, several factors act individually and together to influence the risk of breast cancer.

We conclude that there is no significant association between *TIMP1* (rs4898) and *TIMP3* (rs9619311) SNPs with breast cancer in our population. Moreover, CC and TT genotypes are linked with increased serum TIMP1 and TIMP3 levels, respectively. We speculate that serum TIMP1 and TIMP3 concentrations are associated with breast cancer. Further investigations are necessary to better understand the regulatory role of *TIMP1* and *TIMP3* in the pathophysiology of this disease. This paper represents an advance in biomedical science because it suggests that TIMP1 and TIMP3 serum levels may play a role in the diagnosis and pathogenesis of breast cancer.

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Disclosure statement

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