MMP-9 promoter polymorphism associated with tumor progression of breast cancer in Iranian population

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Abstract
Matrix metalloproteinase-9 (MMP-9) gene plays an important role in several cancers development and progression including breast cancer. A single nucleotide substitution (C→T) in the MMP-9 promoter results in high expression of this gene and so affects susceptibility of tumor invasion and metastasis in some cancers. The aim of this study was to investigate the influence of C/T polymorphism on the occurrence and progression of breast cancer. This case-control study included 180 breast cancer patients and 100 healthy age-matched controls. Polymorphism in the promoter region (C→1562T) was genotyped by polymerase chain reaction-restriction fragment length polymorphism assay and sequencing. After data analyzing, a correlation was observed between the T allele (OR=3.27; P=0.004) and breast cancer occurrence. And also a high significant association was found between the occurrence of the T allele and progression and invasion of breast cancer (OR=5.85; P=0.000). The results suggest that the T allele of the C/T MMP-9 promoter polymorphism can be associated with the tumor development, progression and invasive phenotype of breast cancer, therefore it could be considered as a progression marker in this disease.

Keywords: Breast cancer, extracellular matrix (ECM), matrix metalloproteinase-9, metastasis.

INTRODUCTION
Breast cancer is the second leading cause of female death after heart diseases in Iran (Globacan et al., 2000). Treatment of breast cancer is usually better and easier if it is diagnosed in early stages. Increased awareness about this disease in recent years has led to the development of aggressive screening programs in most developed countries. However 10–15% of breast cancer patients are still not detected by standard procedures such as mammography. It is indicating more sensitive strategies for detection and diagnosis of breast cancers in early stages are very useful and needed (Gail et al., 1989). Development and progression of cancer is a complex, multistage process in which it needs to colonizing of transformed cells to distant sites. In this condition degradation of extracellular matrix and basement membrane barriers is a key step for invasive cells (Kohn and Liotta, 1995). The matrix metalloproteinases (MMPs) constitute a family of proteolytic enzymes that are capable of selectively degrading a wide range of both extracellular matrix and nonmatrix proteins (Werb, 1997). The major function of MMPs is digestion or modification of the extracellular matrix, and through this way normal and malignant cells get the opportunity to migrate from one tissue to another. It is generally believed that the MMPs play a specific role in tumor invasion and metastatic activity of many cancers and hence they are interested to study for their function in carcinogenesis (Stamenkovic, 2000; McCawley, 2000). However, recent works have also suggested that, in addition to the historically considered features of promoting invasion and metastasis, MMPs also play an important role in several steps of cancer development (Zhu et al., 2001; Galateau-Salle et al., 2000; Egeblad and Werb, 2002).

MMP-9 is one of the most important member of this family (known as Gelatinase-B or type IV collagenases), it degrades gelatin (denatured collagen) and type IV collagen, that are the most important components of basement membrane (Liotta et al., 1980). MMP-9 is stimulated by a variety of inducers such as tumor promoters, growth factors and cytokines (Van et al., 2002). Normal cells must pass through basement membrane to enter metastatic phase. Therefore, cells utilize the MMP-9 to penetrate into and exit out of tissues (Duffy et al., 2000). There is a

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functional polymorphism at site –1562 in the **MMP-9** promoter causes low-activity (C/C) and high-activity (C/T, T/T) promoter genotypes, and therefore influences expression of this gene (Park et al., 2000). With respect to the role of the C/T polymorphism in transcriptional activity of **MMP-9** we hypothesized that this polymorphism might also act as a genetic modifier in the development and progression of breast cancer. Therefore, we conducted a case-control study to investigate the association between the different **MMP-9** promoter alleles and the stages of breast cancer in Iranian population.

![Figure 1: PCR-RFLP analysis of the -1562 C/T polymorphism in five patients with breast cancer. The ethidium bromide stained 2.5% NuSieve 3:1 agarose gel used for genotyping is shown. Numbers above the panel are case numbers. Genotypes are indicated below each case.](image)

**Figure 1**: PCR-RFLP analysis of the -1562 C/T polymorphism in five patients with breast cancer. The ethidium bromide-stained 2.5% NuSieve 3:1 agarose gel used for genotyping is shown. Numbers above the panel are case numbers. Genotypes are indicated below each case.

**MATERIALS AND METHODS**

**Samples**

This case-control study include 280 blood samples from 100 healthy control subjects and 180 breast cancer patients involve 90 patients without metastatic activity (all at stages I and II of disease) and 90 metastasis patients with invasion to the other tissues like wide bones and lymph nodes (stages IIIB and IV of disease). Control subjects were randomly selected among individuals visiting hospitals for regular health checks. Breast cancer patients were provided from the Isfahan Omid Hospital cancer section. All these subjects were women with median age 47±10.5 yrs. A structured questionnaire was used during an in-person interview to elicit information on demographic features, dietary habits, prior disease history, physical activities, weight, and family history of cancer. In-person interviews were completed for 167 (92.7%) of the 180 eligible breast cancer cases and 98 (98%) of the 100 eligible controls. All of persons from the two groups donated blood samples. The Buffy coats were stored at −70°C for subsequent DNA isolation. Cancer diagnoses for all patients were confirmed by two senior study pathologists through a review of tumor slides. Genomic DNA was isolated from the peripheral blood.

**Polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) analysis and sequencing**

To analyze the -1562 C/T polymorphism, we amplified a region of the **MMP-9** promoter with forward primer 5’-GGC TGC ATA TGT GGC CC-3’ and reverse primer 5’-CTT CCT AGC CAG CCG GCA TC-3’. PCR-RFLP assay and sequencing were used to determine **MMP-9** genotypes. Each PCR reaction was carried out in a total volume of 25 µl consisting of 0.5 µl of a 10 µM solution of each primer, 2.5 µl of 10x reaction buffer (100 mM Tris–HCl pH 8.3 at 25°C, 500 mM KCl, 15 mM MgCl₂), 4 µl of a 1.25 mM solution of 4 dNTPs, 1 µl of MgCl₂, 0.3 µl of Taq DNA polymerase (Cinnagen, Iran), 1 µl of genomic DNA (80 ng/µl) and 15 µl H₂O under the conditions of an initial denaturation for 1 min at 95°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 58°C and 30 sec at 72°C followed 10 min at 72°C. The PCR products were separated by electrophoresis in a 1/5% agarose gel, and subsequently stained with ethidium bromide. For RFLP analysis, the PCR product fragments were digested with *SphI* restriction enzyme (New England BioLabs) overnight at 37°C. *SphI* does not digest the C allele.

![Figure 2: Confirmation of MMP-9 genotypes for the –1562 C/T polymorphism by sequencing. Genotype of the SNP was C/C in case 1 and T/T in case 2.](image)

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Statistical analysis

The \( \chi^2 \) test was used to evaluate case-control differences in the distribution of allele types and genotypes. Odds ratios (OR) and their 95% confidence intervals were used to measure the strength of the association between MMP-9 gene polymorphism and breast cancer development and progression (Breslow et al., 1980).

RESULTS

In this case-control study, we examined the -1562 C/T polymorphism distribution in the MMP-9 gene promoter by PCR-RFLP and DNA sequencing in breast cancer patients and control subjects. (Fig. 1) shows the pattern of genotype detection by PCR-RFLP. Patients 1, 4, and 6 had a single 460 bp band and so they are C/C genotype. Patients 2 and 3 had all three bands, indicating the heterozygous genotype (C/T) and patient 5 had 202 bp and 258 bp bands and so its genotype is T/T. Subsequent DNA sequencing of representative cases confirmed the genotypes of patients 1 and 5 (Fig. 2). Distribution of the -1562 C/T polymorphism genotypes in breast cancer patients with metastatic activity (acute) and control subjects is shown in Table 1. Distribution of genotypes in controls and non-metastatic activity breast cancer patients (benign) is shown in Table 2. Distribution of genotype in all 3 groups was in agreement with Hardy-Weinberg equilibrium.

Genotype frequencies of CC, CT and TT were observed in 91, 9, and 0% of the control group, and in 63.33, 31.11, and 5.56% of the metastasis subjects and in 75.5, 22.2, 2.3% of benign subjects, respectively. A significantly positive association was found between the CT genotype and the risk of developing the breast carcinoma (OR = 3.27; \( P = 0.004 \); Table 2). Moreover, lymphatic invasion was significantly enhanced in breast cancer patients carrying the T allele (OR = 5.85; Table 1).

DISCUSSION

Delay at detection of breast cancer is associated with lower survival so the identification of patients at risk and patients with breast cancer in early stages is important for better treatment and patient management (Ramirez et al., 1999; Richards et al., 1999).

### Table 1: Genotype distribution of breast cancer patients with metastasis and control subjects.

<table>
<thead>
<tr>
<th>Genotype/Allele</th>
<th>Metastasis (%)</th>
<th>Controls (%)</th>
<th>OR* (95% CI)</th>
<th>( P^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>( n = 90 )</td>
<td>( n = 100 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>57 (63.33)</td>
<td>91 (91)</td>
<td>0.171 (0.077-0.379)</td>
<td>0.000</td>
</tr>
<tr>
<td>CT</td>
<td>28 (31.11)</td>
<td>9 (9)</td>
<td>4.96 (2.215-11.107)</td>
<td>0.000</td>
</tr>
<tr>
<td>TT</td>
<td>5 (5.56)</td>
<td>0</td>
<td>-</td>
<td>0.006</td>
</tr>
<tr>
<td>CC+TT</td>
<td>33 (36.6)</td>
<td>9 (9)</td>
<td>5.85 (2.642-12.931)</td>
<td>0.000</td>
</tr>
<tr>
<td>C</td>
<td>142 (0.79)</td>
<td>191 (0.955)</td>
<td>0.170 (0.081-0.358)</td>
<td>0.000</td>
</tr>
<tr>
<td>T</td>
<td>38 (0.21)</td>
<td>9 (0.045)</td>
<td>5.68 (2.69-11.94)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*Odds ratios are for C/T+T/T genotypes relative to C/C genotype with confidence interval CI = 0.95.

**Association was analyzed by Fisher exact test. \( P \) values are for C/T+T/T genotype relative to C/C genotype.

### Table 2: Genotype distribution of breast cancer patients without metastasis and control subjects.

<table>
<thead>
<tr>
<th>Genotype/Allele</th>
<th>Metastasis (%)</th>
<th>Controls (%)</th>
<th>OR* (95% CI)</th>
<th>( P^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>( n = 90 )</td>
<td>( n = 100 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>57 (63.33)</td>
<td>91 (91)</td>
<td>0.306 (0.135-0.696)</td>
<td>0.004</td>
</tr>
<tr>
<td>CT</td>
<td>28 (31.11)</td>
<td>9 (9)</td>
<td>2.97 (1.294-6.815)</td>
<td>0.009</td>
</tr>
<tr>
<td>TT</td>
<td>5 (5.56)</td>
<td>0</td>
<td>-</td>
<td>0.19</td>
</tr>
<tr>
<td>CC+TT</td>
<td>33 (36.6)</td>
<td>9 (9)</td>
<td>3.27 (1.438-7.423)</td>
<td>0.004</td>
</tr>
<tr>
<td>C</td>
<td>142 (0.79)</td>
<td>191 (0.955)</td>
<td>0.306 (0.141-0.667)</td>
<td>0.002</td>
</tr>
<tr>
<td>T</td>
<td>38 (0.21)</td>
<td>9 (0.045)</td>
<td>3.265 (1.499-7.104)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*Odds ratios are for C/T+T/T genotypes relative to C/C genotype with confidence interval CI = 0.95.

**Association was analyzed by Fisher exact test. \( P \) values are for C/T+T/T genotype relative to C/C genotype.
The MMP-9 function is digestion of both extra and intra cellular components of basement membrane so the cellular level of this enzyme could influence cells malignancy. MMP-9 promoter with the T allele shows significantly higher transcriptional activity than the C allele, and so this polymorphism influences expression of the gene (Zhang et al., 1999). Thus, in the present study, we investigated the correlation between the different genotypes of C/T polymorphism in the MMP-9 promoter and the stages of breast cancer in 90 breast cancer patients without metastatic activity (benign group) and 90 breast cancer patients with metastasis activity (acute group). It was also investigated whether this polymorphism could serve as a diagnosis marker for detection of breast cancer in early or progression stages.

We found a significant association between this polymorphism and clinicopathological features, especially tumor invasion, and stage of the disease development. The T allele was detected more frequently in patients with benign breast cancer (OR=3.27). Moreover, lymphatic invasion was significantly enhanced in breast cancer patients carrying the T allele (OR = 5.85). We suggest that the MMP-9 polymorphisms might alter the expression and activity of the enzyme, increasing ECM degradation and invasion, leading to breast cancer progression.

Overexpression of MMP-9 has been observed in a variety of cancers including gastric cancer, colorectal cancer, renal cell cancer, melanoma, and lung cancer (Wu et al., 2007; Xu et al., 2007; Awakura et al., 2006; Cotignola et al., 2007; Rollin et al., 2007). Whereas gene transcription is the primary point of MPPs regulation, sequences change at the promoter of MMP-9 may have important role in the gene expression.

It has been reported that presence of T allele in this polymorphism is in correlation with good prognosis of breast cancer (Roehle et al., 2007). Liu et al reported that the MMP-9 level was higher in breast tumor tissues when compared to the corresponding normal tissues (p < 0.01) and MMP-9 was significantly increased in larger tumors, and in metastatic lesions (Liu et al., 2006). Also Somiari at another study reported that in breast cancer patients, plasma activity of MMP-9 is associated with progression of this disease (Somiari et al., 2006). These parallel results indicate the level of active MMP-9 and so presence of the T allele at the promoter are effective factors in breast cancer progression and these factors should be considered in breast cancer patient for better treatments. The MMP-9 promoter includes several transcription regulating binding sites for AP-1, NF-kB, Sp-1, and Ets transcription factors (Chakrabarti et al., 2006). The C/T polymorphism is exactly located within a transcription factor binding site, and the T allele may prevent from transcription repressor protein binding in this region and therefore, at person carrying the T allele; expression of MMP-9 could be increased (Galateau-Salle et al., 2000).

Establishment of metastasis requires the serial processes such as invasion, migration, implantation, and regrowth of cancer cells at the metastatic site. The MMP-9 polymorphism may affect the initial invasion step of lymph node metastasis. The present findings support our hypothesis that the –1562 C/T polymorphism may have a profound impact on progression and invasion of breast cancer.

CONCLUSION

In conclusion, our data demonstrated that the different allelic genotypes in promoter polymorphism of the MMP-9 gene are correlated with the susceptibility for invasion and tumor progression of breast cancer patients. With regard to our knowledge, this is the first study to investigate the association of the -1562 C/T polymorphism of MMP-9 with development and malignant phenotypes of breast cancer in Iran. We defined CT and TT genotypes of the MMP-9 gene as high-risk genotypes for solid tumor invasion and metastasis progression so this polymorphism could be considered as a progression marker in the breast cancer. However the increased risk of malignancy related to the occurrence of T allele suggests that further research in this field should be carried.

Acknowledgement

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References


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