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Higher plasma MMP-9 level in breast cancer patients with MMP-9 promoter T allele

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Matrix metalloproteinase-9 (MMP-9) in blood is a promising new tumor marker. Previously, a correlation between C/T–1562 MMP-9 polymorphism and tumor progression of breast cancer was reported. In the present study, we examined the association between the C/T polymorphism and plasma MMP-9 level in breast cancer patients. This study included 124 breast cancer patients, 52 of which with initial breast cancer and 72 with progressive breast cancer. Different allelic genotypes of MMP-9 promoter polymorphism were determined by PCR-RFLP and the plasma MMP-9 concentrations were measured using ELISA. Plasma MMP-9 levels were significantly increased in breast cancer patients with the MMP-9 promoter T allele compared with patients with the MMP-9 promoter C allele ($p < 0.001$). The plasma MMP-9 levels were correlated with progression of breast cancer and lymph node involvement ($p = 0.002$). According to our findings, individuals with MMP-9 promoter T allele are at a risk of higher plasma MMP-9 and they are more susceptible to breast cancer.

Key words: polymorphism, breast cancer, matrix metalloproteinase, plasma, metastasis.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of proteinase enzymes that breakdown a broad range of extracellular matrix (ECM) and basement membrane components. Invasion and metastasis of tumor cells require adherence to ECM and passage through its components. To invade, epithelial cancer cells need to penetrate through the basement membrane components (BM) and remove ECM tissue boundaries. In this context, MMPs play a key role, because they can either degrade or digest the ECM components and thereby support cancer cell metastasis to the other tissues (Nagase *et al*., 1999).

MMP-9 (gelatinase B) is the only member of the metalloproteinase family that plays a role in degrading components of the BM, the first vital barrier breached by tumor cells when they start invasion to the other tissues. This enzyme plays several specific roles in progression and invasion of some cancers (Kähäri *et al*., 1999; Stamenkovic, 2000; Egeblad & Werb, 2002; Ala-aho & Kähäri, 2005).

The MMP-9 gene is located at chromosomal location 20q13.2 and mechanisms involved in its expression are not clearly known. There are several factors which affect gene expression at different steps. A single nucleotide polymorphism (SNP) at the promoter causes change in RNA transcription rate. This SNP is caused by a cytosine (C) to thymine (T) base change, 1562 bases upstream the MMP-9 transcription initiation site (MMP-9–1562 C/T). The presence of the C allele has been associated with low levels of MMP-9 promoter activity while the presence of the T allele has been associated with high activity (Zhang *et al*., 1999). There are several reports on the relation between the presence of T allele and occurrence of some cancers, including breast cancer (Roehe *et al*., 2007).

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Also, in recent studies, plasma and serum MMP-9 levels have been reported as new tumor markers. Elevated levels of plasma MMP-9 have been found in a variety of malignant tumors such as breast cancer, colon cancer, lung cancer, and hepatocellular carcinoma (Zucker *et al*., 1993; Hayasaka *et al*., 1996; Torii *et al*., 1997; Hoikkala *et al*., 2006). High plasma MMP-9 levels in patients with breast cancer are considered as a good diagnostic marker in correlation with the features of the patients such as tumor progression and node invasion (Mannelo & Tonti, 2007). In a previous research effort, we found that the presence of the T allele at this gene promoter is correlated with tumor progression and lymph involvement of breast cancer patients in Iranian population (Sadeghi *et al*., 2009). With respect to the correlation between the MMP-9 promoter T allele and several cancers as well as recent reports about the plasma level of the enzyme and progression of cancer, we hypothesized that the T allele at this gene promoter may be correlated with gene expression and thus plasma level of the MMP-9. Therefore, we conducted a study to investigate the association between the different MMP-9 promoter alleles and plasma MMP-9 levels in breast cancer patients.

MATERIALS AND METHODS

Samples

This study included blood samples from 124 breast cancer patients; 52 of them without metastatic activity (all at stages I and II of disease) and 72 metastasis patients with invasion to the other tissues like wide bones and lymph nodes (stages IIIB and IV of disease). All subjects were middle-aged women (median age 45 ± 10.5 years). A structured questionnaire was used through person-to-person interviews to elicit information on demographic features, dietary habits, prior disease history, physical activities, weight, and family history of cancer. The buffy coats (CinnaGen, Iran) were stored at –70°C for subsequent DNA isolation. Cancer diagnosis for all patients was confirmed by two senior study pathologists through a re-examination of tumor slides. Genomic DNA was isolated from the peripheral blood.

PCR optimization and genotype analysis

To analyze the C/T–1562 polymorphism, we amplified a region of the MMP-9 promoter with forward primer 5′-GCCTGGCACATAGTAGGCCC-3′ and reverse primer 5′-CTTCCTAGCCAGCCGGCATC-3′ (CinnaGen, Iran). PCR-RFLP assay (for all samples) and sequencing (for half of the samples) were used to determine different MMP-9 promoter 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 10× reaction buffer (100 mM Tris-HCl pH 8.3 at 25°C, 500 mM KCl, 15 mM $MgCl_2$), 4 μl of a 1.25 mM solution of the four dNTPs (CinnaGen, Iran), 1 μl of *Taq* DNA polymerase (CinnaGen, Iran), 1 μl of genomic DNA (80 ng μl^{-1}), and 14.5 μl dH_2O. Conditions were: 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. The PCR products were separated by electrophoresis in a 1.5% agarose gel and subsequently stained with ethidium bromide. For RFLP analysis, the PCR products were digested with *Sph*I (New England BioLabs) overnight at 37°C. *Sph*I does not digest the C allele (435 bp) but generates two fragments, 188 and 247 bp, for the T allele. Digests were separated by electrophoresis on 1.5% agarose gels for 60 min at 100 V. Heterozygote samples had a combination of both alleles (435, 188, and 247 bp bands). Samples were PCR amplified and then purified with a purification kit following the manufacturer's instructions (CinnaGen, Iran). One to 10 ng from 62 purified samples were sequenced in the

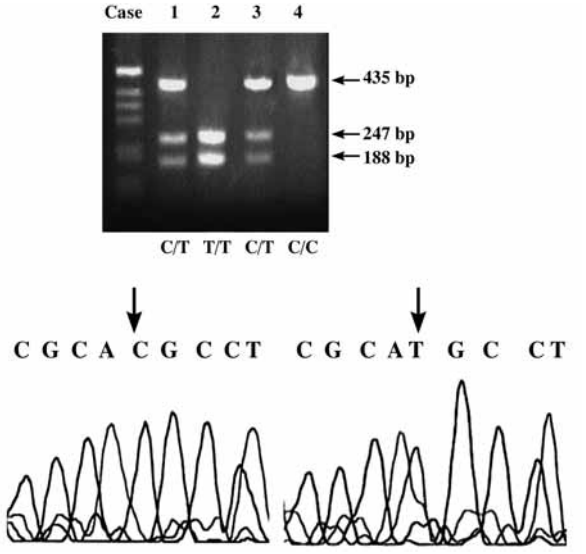

FIG. 1. PCR-RFLP analysis of the C/T–1562 polymorphism in breast cancer patients. The target region (435 bp) of the MMP-9 gene promoter was PCR-amplified and digested with *Sph*I. *Sph*I cleaved the T allele to generate two fragments (188 and 247 bp), while the C allele was not digested (435 bp). Numbers above the panel are case numbers. Genotypes are indicated below each case.

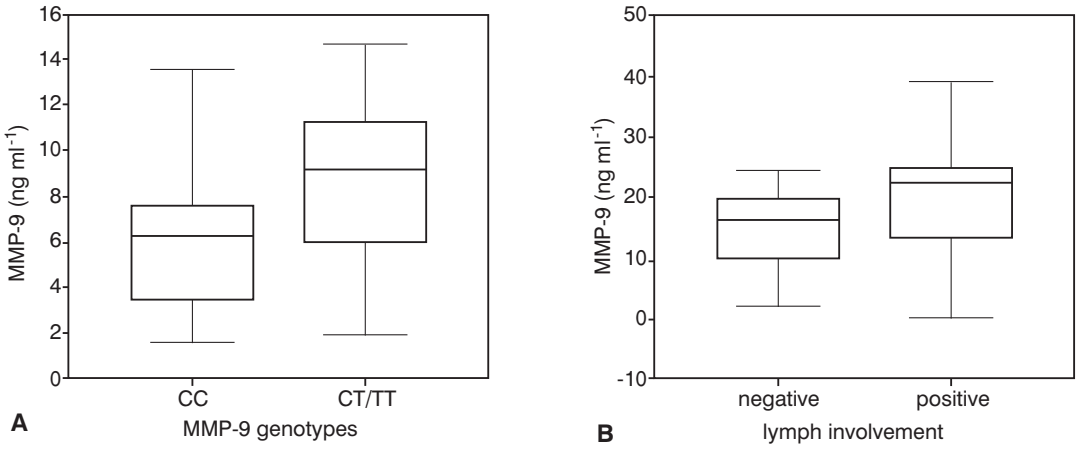

FIG. 2. Box plots of plasma MMP-9 level in breast cancer patients with different allelic genotypes and different stages of the disease. A: levels of MMP-9 between the two groups of patients with different genotypes is significantly different ($p < 0.001$); B: MMP-9 plasma level is significantly increased in breast cancer patients with lymph node metastasis ($p = 0.032$).

DNA Sequencing Core Facility at CinnaGen Institute Iran (Fig. 1).

Measurement of plasma MMP-9 concentration

Blood samples were collected in EDTA-coated plasma collection tubes (CinnaGen, Iran). The plasma of each person was obtained by centrifugation at 1300 g for 12 min and stored at –80°C until used. Plasma samples were allowed to thaw at room temperature just before being used. Plasma MMP-9 level was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine human total MMP-9 ELISA kit, Biorad). The detection limit of the assay was 0.156 ng ml^{-1}.

Statistical analysis

Total level of plasma MMP-9 was determined and correlated with different allelic genotypes. Measurements were done in duplicate and results were expressed as median (lower quartile Q25/upper quartile Q75). The assumption of normality was verified using the normal probability plot; the Shapiro-Wilk test was used for homogeneity of different genotypes. Differences in genotype distribution and consistency with Hardy-Weinberg equilibrium were tested by chi-square test. Differences in allele frequencies of the SNP were tested by a two-tailed Fisher exact test. Values of $p < 0.05$ were considered to be statistically significant. Statistical analyses were performed using Statistica for Windows (StatSoft Inc., USA).

RESULTS

MMP-9 plasma levels and different disease phenotypes

The plasma MMP-9 level was higher in patients with progressive breast cancer (n = 72) than in patients with initial breast cancer (n = 52, $p = 0.017$). Moreover, plasma MMP-9 was significantly higher in patients with MMP-9 promoter polymorphism T allele (CT/TT, n = 45, 8.9 ± 3.43 ng ml^{-1}) compared with C homozygotes (n = 79, 5.3 ± 3.03 ng ml^{-1}) ($p < 0.001$). Plasma MMP-9 was also higher in patients with lymph node metastasis (n = 52) than in noninvasive ones (n = 72, $p = 0.032$) (Fig. 2).

MMP-9 promoter (C/T–1562) allelic genotype analysis

A significant correlation was found between the presence of the MMP-9 promoter polymorphism T allele and progression of breast cancer. The allelic distribution of MMP-9 promoter polymorphism in patients was CC/CT/TT: 64/29.5/6.5%. The overall frequency of allele T was 0.215. The frequency of MMP-9 allele T was higher (0.276) in patients with progressive breast cancer than in patients with early breast cancer ($p = 0.043$). Plasma MMP-9 was significantly higher in breast cancer patients with the T allele than in patients with the CC genotype ($p = 0.001$) (Table 1).

DISCUSSION

Cancer is a multistage process disease and there are several genes and environmental factors affecting its development. The identification of patients at risk

TABLE 1. Correlation between the different genotypes of MMP-9 promoter polymorphism and MMP-9 plasma levels in breast cancer patients

	CC (%)	CT (%)	TT (%)	OR[a] (95% CI)	*p*
Patients	79 (64)	37 (29.5)	8 (6.5)		
Lymph involvement					
Positive	31 (59.6)	15 (28.8)	6 (11.5)	4.72 (1.970-11.383)	0.001
Negative	63 (87.5)	9 (12.5)	0		
Disease stages					
Advanced	52 (53)	42 (42.8)	4 (4.08)	2.94 (1.116-7.743)	0.043
Primary	19 (73)	6 (23)	1(3.8)		
MMP-9 plasma level	5.3 (ng ml^{-1})	10.1 (ng ml^{-1})	7.7 (ng ml^{-1})		< 0.001
Positive lymph involvement	5.7	6.03	4.5		0.032
Negative lymph involvement	4.9	4.07	3.2		

[a] Odds ratios are for C/T+T/T genotypes relative to C/C genotype with confidence interval CI = 0.95

and patients with breast cancer in the early stages is very important for treatment planning and better inhibition of cancer cell growth. MMP-9 is an enzyme with proteolytic activity. Over-expression of the MMP-9 has been reported in several cancers and other diseases. By its proteolytic activity, MMP-9 is also known as a potential diagnostic marker in several cancers. There are reports on the role of the T allele of MMP-9 promoter C/T polymorphism in the occurrence and progression of some cancers. In addition, recent studies have reported plasma and serum MMP-9 levels as potential diagnostic markers for breast cancer. We come to the conclusion that a supplementary study is needed to find the correlation between the presence of different MMP-9 promoter alleles and the concentration of this enzyme in breast cancer patients' plasma. In the present study, the concentration of MMP-9 was measured in plasma of 124 breast cancer patients and also the correlation between the plasma MMP-9 level and presence of MMP-9 promoter T allele was investigated. The obtained results showed that patients with the MMP-9 promoter T allele displayed higher MMP-9 concentration compared with that of patients with the MMP-9 promoter C allele. This observation could be a confirmation for the previous findings about the function of MMP-9 promoter polymorphism or the MMP-9 plasma levels in breast cancer cases. Also, we have found a correlation between the increased plasma MMP-9 and lymph node metastasis in breast cancer patients. Elevated levels of plasma MMP-9 have been found in a variety of malignant tumors, such as breast cancer, lung cancer, hepatocellular carcinoma, and gastric cancer (Zucker *et al*., 1993; Hayasaka *et al*., 1996; Torii *et al*., 1997; Hoikkala *et al*., 2006). Also, there are several reports on the relation between the presence of the T allele and occurrence of different cancers including colorectal cancer, renal cell cancer, melanoma, lung cancer, and breast cancer (Awakura *et al*., 2006; Cotignola *et al*., 2007; Enping *et al*., 2007; Roehe *et al*., 2007).

As circulating MMP-9 has different forms (active and proenzyme), it is crucial to characterize the activity of this gelatinase with a sensitive method. ELISA level of MMP-9 is significantly correlated with both the plasma and serum levels of this enzyme. ELISA level of MMP-9 is also significantly correlated with both active and pro-MMP-9 forms (Kubben *et al*., 2006). In the present study, we used ELISA and PCR-RFLP to investigate the association between the presence of T allele and plasma MMP-9 level in breast cancer patients. For the first time, it is observed that the circulating levels of MMP-9 were higher in the plasma of breast cancer patients with MMP-9 promoter T allele. This finding can be a confirmation of previous studies on the function of MMP-9 promoter T allele and occurrence of the cancer. Also, we have observed that plasma MMP-9 levels were higher in patients with progressive breast cancer than in those with early stages of the disease.

It has been reported that the presence of T allele is correlated with the progression of breast cancer. Our results are in line with the observations made by Roehe *et al.* (2007) who observed that the MMP-9 promoter T allele is correlated with progressive breast cancer in patients.

Several other reports suggest that plasma MMP-9 may play a significant role in the development of breast cancer (Liu *et al*., 2006; Somiari *et al*., 2006). Liu *et al.* (2006) reported that the MMP-9 protein levels were higher in breast tumor tissues when compared with normal tissues ($p < 0.01$) and MMP-9 was significantly increased in larger tumors. Somiari *et al.* (2006) reported that in breast cancer patients, plasma activity of MMP-9 is associated with progression of this disease. These parallel results indicate that the level of active MMP-9 and the presence of the T allele at this gene promoter are related to breast cancer progression and should be considered in breast cancer patients for better early diagnosis of the disease.

In our study, lymph node metastasis of breast cancer was associated with high plasma MMP-9 level and this elevated level was in correlation with the presence of T allele. The present study is the first to report an association of plasma MMP-9 with the MMP-9 promoter T allele. Our most intriguing finding is the association of higher plasma MMP-9 levels with presence of the T allele. These results support the expressional role of the T allele at the MMP-9 promoter and may even indicate that MMP-9 exhibits greater transcription in the presence of the T allele. 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 transcription repressor protein binding at this region (Galateau-Salle *et al*., 2000).

Establishment of metastasis requires the serial processes of 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 C/T–1562 polymorphism may have a significant role on the gene expression.

In conclusion, this study demonstrates that breast cancer patients with MMP-9 promoter T allele display significantly different concentrations of MMP-9 in plasma compared to breast cancer patients with the MMP-9 promoter C allele. Also, the plasma MMP-9 level is increased in breast cancer patients with lymph node metastasis. According to our findings MMP-9 over-expression in clearly associated with the presence of the T allele at the gene promoter and this polymorphism can serve as a diagnostic marker in breast cancer. Nonetheless, further studies are required to validate our results and determine if there is an association with the age of the patients or their hormonal status.

REFERENCES

Ala-aho R, Kähäri VM, 2005. Collagenases in cancer. *Biochimie*, 87: 273-286.

Awakura Y, Ito N, Nakamura E, Takahashi T, Kotani H, Mikami Y, Manabe T, Kamoto T, Habuchi T, Ogawa O, 2006. Matrix metalloproteinase-9 polymorphisms and renal cell carcinoma in a Japanese population. *Cancer letters*, 241: 59-63.

Chakrabarti S, Zee JM, Patel KD, 2006. Regulation of matrix metalloproteinase-9 (MMP-9) in TNF-stimulated neutrophils: novel pathways for tertiary granule release. *Journal of leukocyte biology*,79: 214-222.

Cotignola J, Reva B, Mitra N, Ishill N, Chuai S, Patel A, Shah S, Vanderbeek G, Coit D, Busam K, Halpern A, Houghton A, Sander C, Berwick M, Orlow I, 2007. Matrix metalloproteinase-9 (MMP-9) polymorphisms in patients with cutaneous malignant melanoma. *BMC medical genetics*, 8: 10.

Egeblad M, Werb Z, 2002. New functions for the matrix metalloproteinases in cancer progression. *Nature reviews cancer*, 2: 161-174.

Enping X, Xiaoping X, Bingjian L, Xiaoming X , Qiong H, Yu M, Wei W, Maode L, 2007. Association of matrix metalloproteinase-2 and -9 promoter polymorphisms with colorectal cancer in Chinese. *Molecular carcinogen*, 46: 924-929.

Galateau-Salle FB, Luna RE, Horiba K, Sheppard MN, Hayashi T, Fleming MV, Colby TV, Bennett W, Harris CC, Stetler-Stevenson WG, Liotta L, Ferrans VJ, Travis WD, 2000. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in bronchial squamous preinvasive lesions. *Human pathology*, 31*:* 296-305.

Hayasaka A, Suzuki N, Fujimoto N, Iwama S, Fukuyama E, Kanda Y, Saisho H, 1996. Elevated plasma levels of matrix metalloproteinase-9 (92-kd type IV collagenase/gelatinase B) in hepatocellular carcinoma. *Hepatology*, 24: 1058-1062.

Hoikkala S, Pääkkö P, Soini Y, Mäkitaro R, Kinnula V, Turpeenniemi-Hujanen T, 2006. Tissue MMP-2/TIMP-2-complex are better prognostic factors than serum MMP-2, MMP-9 or TIMP-1 in stage I-III lung carcinoma. *Cancer letters*, 236: 125-132.

Kähäri VM, Saarialho-Kere U, 1999. Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Annals of internal medicine*, 31: 34-45.

Kubben FJGM, Sier CFM, van Duijn W, Griffioen G, Hanemaaijer R, van de Velde CJH, van Krieken JHJM, Lamers CBHW, Verspaget HW, 2006. Matrix metalloproteinase-2 is a consistent prognostic factor in gas-

tric cancer. *British journal of cancer*, 94: 1035-1040.

Liu S-C, Yang S-F, Yeh K-T, Yeh C-M, Chiou H-L, Lee C-Y, Chou M-C, Hsieh Y-S, 2006. Relationships between the level of matrix metalloproteinase-2 and tumor size of breast cancer. *Clinical chimica acta*, 371: 92-96.

Mannelo F, Tonti GA, 2007. Gelatinase concentrations and zymographic profiles in human breast cancer: Matrix metalloproteinases circulating in plasma are better markers for the subclassification and early prediction of cancer: The coagulation/fibrinolysis pathways alter the release, activation and recovery of different gelatinases in serum. *International journal of cancer*, 121: 216-218.

Nagase H, Woessner JF, Jr, 1999. Matrix metalloproteinases. *Journal of biological chemistry*, 274: 21491-21494.

Roehe AV, Frazzon APG, Agnes G, Damin AP, Hartman AA, Graudenz MS, 2007. Detection of polymorphisms in the promoters of matrix metalloproteinases 2 and 9 genes in breast cancer in South Brazil: preliminary results. *Breast cancer research and treatment*, 102: 123-124.

Sadeghi M, Motovali Bashi M, Hojati Z, 2009. MMP-9 promoter polymorphism associated with tumor progression of breast cancer in Iranian population. *International journal of integrative biology*, 6: 33-37.

Somiari SB, Shriver CD, Heckman C, Olsen C, Hu H, Jordan R, Arciero C, Russell S, Garguilo G, Hooke J, Somiari RI, 2006. Plasma concentration and activity of matrix metalloproteinase 2 and 9 in patients with breast disease, breast cancer and at risk of developing breast cancer. *Cancer letters*, 233: 98-107.

Stamenkovic I, 2000. Matrix metalloproteinases in tumor invasion and metastasis. *Seminars in cancer biology*, 10: 415-433.

Torii A, Kodera Y, Uesaka K, Hirai T, Yasui K, Morimoto T, Yamamura Y, Kato T, Hayakawa T, Fujimoto N, Kito T, 1997. Plasma concentration of matrix metalloproteinase 9 in gastric cancer. *British journal of surgery*, 84: 133-136.

Zhang B, Ye S, Herrmann SM, Eriksson p, Maat MD, Evans A, Arveiler D, Luc G, Cambien F, Hamsten A, Watkins H, Henney AM, 1999. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. *Clinical investigation and reports*, 99: 1788-1794.

Zucker S, Lysik RM, Zarrabi MH, Moll U, 1993. M_r 92,000 type IV collagenase is increased in plasma of patients with colon cancer and breast cancer. *Cancer research*, 53: 140-146.