Short communication

The role of matrix metalloproteinase-3 functional 5A/6A promoter polymorphism in tumor cell progression and metastasis of breast cancer

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Abstract

In the human genome, chromosome 11 contains a cluster of matrix metalloproteinase (*MMP*) genes. Single nucleotide polymorphisms in the promoter region of *MMP* genes are important for *MMP* expression. A common adenine deletion polymorphism (5A) at position -1171 of the *MMP*-3 gene promoter (5′-AAAAAACCAT-3′ change to 5′-AAAAACCAT-3′) facilitates transcriptional factor binding and *MMP*-3 promoter activity. A case-control study was performed including 120 breast cancer patients (60 patients with metastatic activity and 60 patients without metastatic activity); and 60 healthy controls. Whole blood samples were obtained from patients and healthy controls. Genomic DNA was extracted from samples and the *MMP*-3 5A/6A genotypes were determined using PCR-RFLP. *MMP*-3 genotype distributions between patients and controls were similar (OR= 0.89, 95%CI, 0.43-1.84, P= 0.047). It was observed that the 5A allele was more frequent among patients with metastatic activity than controls (OR= 2.9, 95%CI, 0.94-8.9, P= 0.074). Therefore, the 5A polymorphism in the *MMP*-3 promoter showed correlation with the metastasis group than patients without metastasis; both at the time of diagnosis. However our results do not show evidence for correlation between 5A/6A polymorphism and breast cancer susceptibility.

Keywords: Matrix metalloproteinase-3; Single nucleotide polymorphism; Breast cancer; Metastasis.

Matrix metalloproteinases (MMPs) are involved in all steps of tumor genesis, cancer invasion and metastasis (Sternlicht and Werb, 2001; Sternlicht *et al*., 2000; Declerck and Imren, 1994). Tumor invasion and metastasis formation always begin with blood lymphatic vessel inflammation. As these processes involve proteolytic digestion of the extracellular matrix, it is therefore suggested that MMPs play an important role in tumor progression (Somerville *et al*., 2003 and Shapiro, 1998). The basal expression of several MMPs such as *MMP*-1, *MMP*-3 and *MMP*-9 in cell culture have been detected at very low levels. However, their transcriptions are induced by a variety of extracellular stimuli including growth factors, cytokines, and chemical agents (Gotoh *et al*., 2004; Spurr *et al*., 1988). In the genome, chromosome 11 contains a cluster of *MMP* genes (Gatti *et al*., 1989; Pendas *et al*., 1996). The flanking regulatory regions of most inducible *MMP* genes exhibit common features, including the TATA box, activator protein-1 (AP-1) and regulatory elements in the proximal promoter region. In addition, *MMP* genes contain several potential AP-1 binding sites, and a transcriptionally important tumor suppressor P53 binding site (Posthumus *et al*., 2003), and also enhancing twenty six (ETS) family of oncoprotein binding polyoma enhancing activator-3 (PEA-3) site and nuclear factor of KB (Fang *et al*., 2005). Figure 1 shows these conserved elements upstream of the *MMP*-3 gene.

Besides the signal transduction pathway, single nucleotide polymorphisms (SNPs) in the promoter regions of *MMP* genes affect level of transcription by

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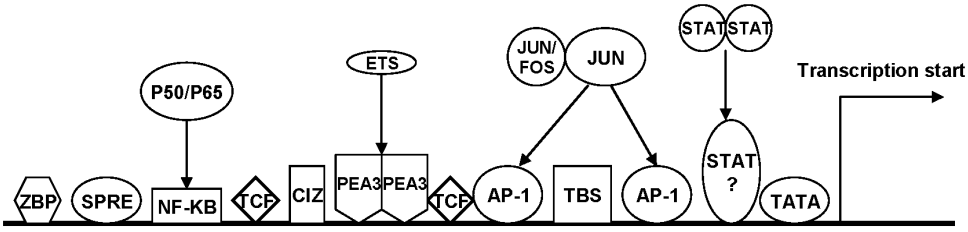

Figure 1. *MMP*-3 promoter region including transcription factor binding sites: ZBP-89: 89 kDa zinc binding protein. SPRE: Stromelysin 1 PDGF responsive element. NF-κB: Nuclear factor κB. TCF: T-cell factor. CIZ: Cos-interacting zing-finger protein. PEA-3: Polyomavirus enhancer A binding protein 3. AP-1: Activator protein-1. TBS: TEL (translocation-ETS-leukemia) binding site. STAT?: Possible binding site of signal transducers and activator of transcription. TATA: TATA box (Re-written and re-drawn from, Chamber *et al*., 2002).

creating a regulatory box (Kang *et al*., 2005; Ke *et al*., 2004; Posthumus *et al*., 2003). Approximately 90% of DNA polymorphisms are SNPs due to single base substitutions or base insertion/deletions (Posthumus *et al*., 2003). Promoter polymorphisms have been identified in a number of *MMP* genes such as *MMP*-1 and *MMP*-3. Some of them have been shown to influence *MMP* expression and are therefore associated with susceptibility and/or severity of disease such as cancer (Shan *et al*., 2005; Blons *et al*., 2004; Zhang *et al*., 2004; Lei *et al*., 2003; Ghilardi *et al*., 2002). A single adenine insertion polymorphism at position-1171 of the *MMP-3* promoter region modulates transcription and local expression of *MMP*-3. It has been demonstrated that the 6A allele expresses a roughly 2-fold lower amount of gene product as compared with the 5A allele (Kripple *et al*., 2004; Rhys, 2000). Additional studies have revealed binding of the putative transcriptional factors to this region. The difference in promoter activity is probably due to preferential binding of the transcriptional repressor to the 6A allele (Posthumus *et al*., 2003; Mercapide *et al*., 2003). Influence of this polymorphism has been assessed in breast cancer, head and neck cancer and atherosclerosis (Radisky *et al*., 2005; Blons *et al*., 2004; McGlinchey *et al*., 2004; Pollanen *et al*., 2002; Gnasso *et al*., 2000; Matt *et al*., 1999).

The presence of the 5A allele may be associated with breast cancer susceptibility and severity. Therefore, this study aimed to identify a subgroup of breast cancer patients who hadmore aggressive tumors or were prone to metastasis.

This case-control study included 120 female patients from Isfahan with confirmed diagnosis of breast cancer at different stages. Healthy women were included as controls and studied under the same conditions as the case studies. The median age of cases was 47 years old (age range, 27-68 years). The participants were also asked about any family history of cancer. Blood samples were collected from Omid hospital during 24 months, from 2004 to 2006. Cases were followed-up for 6-30 months. Women with breast cancer were subgrouped according to the tumor-node-metastasis (TNM) classification for breast cancer. Two subgroups of patients were considered: the M^- subgroup without evidence of metastasis (n=60), and M^+ subgroup with evidence of metastasis at the end of follow-up (n=60). Five ml of venous blood from each subject was drawn into a vacutainer tube containing EDTA and stored at 4°C for further studies and -80°C for long-term studies. Genomic DNA was extracted according to the salting out method (Miller *et al*., 1988) with some modifications. Besides using saturated NaCl, Chloroform was also used to increase DNA quality and quantity (Davis *et al*., 1994). Genetic polymorphism of the *MMP*-3 gene was detected using PCR followed by digestion with restriction enzymes. The promoter sequences of the *MMP*-3 gene were obtained from the Eukaryotic Promoter Database (EPD). According to available published primers, reverse and forward primers were selected and slightly modified. In the case of the 5A allele a substitution mutation from A to G in the second nucleotide close to the 3′ end of the reverse primer (showed "g" at the primer sequences; Figure 2) at the polymorphism region was made to create a *Tth111*I (*Psy*I) recognition site (Figure 2). PCR was carried out in a 25 µl volume containing 100 ng of DNA template, 2.5 µl of 10 X PCR buffer, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase, 200 nM dNTPs and 20 pM forward and reverses primers. The PCR cycling conditions were 5 min at 94°C followed by 30 sec at 94ºC (denaturation), 30 sec at 65°C, 60 sec at 72°C (extension), repeated for 30 cycles followed by a final step at 72°C for 10 min. Detection of the allelotype was carried out by digestion of PCR products

Reverse Primer: 5´-GGTTCTCCATTCCTTTGATGGGGGGAAAgA-3´
Forward primer: 5´-CTTCCTGGAATTCACATCACTGCCACCACT-3´

Figure 2. The allele 5A has only a recognition site for restriction endonuclease Tth111I (3´-CAGN-5´).

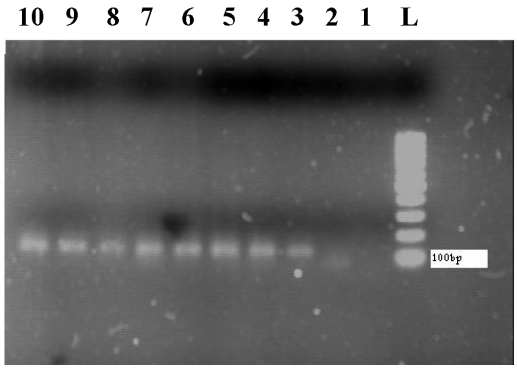

Figure 3. Agarose gel electrophoresis of the *MMP*-3 promoter PCR products. Lanes 1 and 2 negative control; Lanes 3 and 4, PCR products of healthy control; Lanes 5, 6 and 7, PCR products of metastasis- free group; Lanes 8, 9 and 10, PCR products of metastasis group. Lane L: Gene Ruler T^M 100bp DNA Ladder.

with the *Tth111*I restriction enzyme (*Psy*I). Digestion of PCR products was performed according to a standard protocol. Electrophoresis was performed using a 2-3% (w/v) agarose gel stained with ethidium bromide. After digestion and electrophoresis, the 5A allele was expected to be represent by two bands of 97 and 32 bp and the 6A allele by a band of 129 bp. Statistical analyses were performed using the SPSS 12.0 software package. Hardy-Weinberg analysis was also carried out to compare the observed and expected genotype frequencies using the χ^2 test. Odd Ratios (OR; approximate relative risk) were calculated as an index of the association of the *MMP*-3 promoter genotypes with breast cancer invasion and metastasis. For each OR, two-tailed probability values and 95% confidence intervals (CIs) were calculated. $P<0.05$ was assumed to be statistically significant.

There was a statistical correlation between 5A allele in the M^+ subgroup versus controls (P=0.074, OR=2.9, 95%CI, 0.94-8.9). There was no statistical difference between the controls and M^- subgroup with respect to the frequency of the 5A allele. (P=0.043, OR=0.77, 95% CI, 0.23-2.5). Homozygotes for the 5A allele were more prevalent among the M^+ patients than the controls (P=0.074). The difference between the M^+ and M^- subjects was not statistically significant (Table 2). As expected, the PCR products were 129 bp. Figure 3 shows the band after electrophoresis on the 2% (w/v) agarose gel stained with ethidium bromide. The PCR products for the 5A and 6A alleles are of the same size for all samples, but these alleles may show up different ly after RFLP. The 6A alleles do not have the *Tth111*I recognition site; therefore, they show only one band of approximately 129 bp on the gel. But the 5A alleles have the *Tth111*I recognition site, as represented by one band of approximately 97 bp on the 3% (w/v) agarose gel (the 32 bp band could not be detected by the agarose gel electrophoresis, Fig. 4). The *MMP*-3 genotyping was successfully determined in 120 patients and 60 controls. The distribution of *MMP*-3 alleles in controls and patients were consistent according to the Hardy-Weinberg principle ($\chi^2 = 4.4$, P =0.011). From the 120 participated patients in this study, 30 subjects were homozygous for the 6A allele, 28 subjects were homozygous for the 5A allele and 62 subjects were heterozygous. The 5A and 6A allele frequencies in the controls were 0.45 and 0.55, respectively (Table 1). In breast cancer patients, the *MMP*-3 allelic variation was significantly different compared

Table 1. Distribution of *MMP*-3 promoter polymorphism.

MMP-3	patients (n= 120)		Control (n = 60)	
5A/5A	28	(23.3)	8	(13.3)
5A/6A	62	(51.6)	38	(63.3)
6A/6A	30	(25.0)	14	(23.3)
5A frequency		(0.49)		(0.45)
6A frequency		(0.51)		(0.55)

P= 0.061, OR= 1.63, 95%CI, 0.59-4.4.
Parenthesis show % of samples.

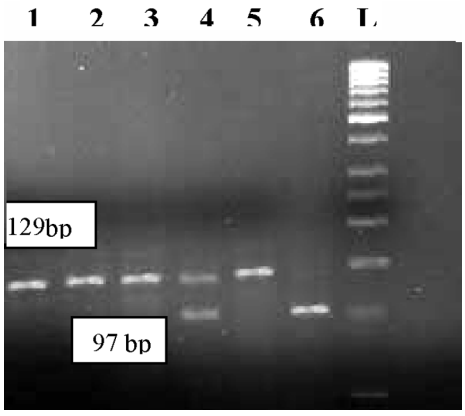

Figure 4. Agarose gel electrophoresis of the *MMP*-3 promoter PCR products and digested PCR fragments. Lane 1: PCR products, undigested; Lane 2: Digested PCR products with *Tth111*I (6A/6A homozygous genotype); Lane 3: PCR products, undigested; Lane 4: PCR products with digested *Tth111*I (6A/5A heterozygous genotype); Lane 5: PCR products, undigested; Lane 6: PCR products with digested *Tth111*I (5A/5A homozygous genotype). Lane L: Gene Ruler TM 50bp DNA Ladder.

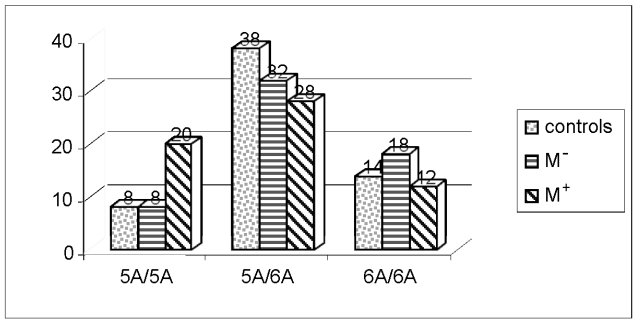

Figure 5. Distribution of *MMP*-3 promoter genotypes.

with that of the controls (P = 0.061, OR= 1.63, 95% CI, 0.59-4.4). Figure 5 shows distribution of various genotypes in patients and controls. No statistical correlation was found among the TNM stages at the time of breast cancer diagnosis (data not shown). According to our results the *MMP*-3 5A/6A polymorphism does not influence breast cancer susceptibility but there is a statistical correlation between tumor cell progression and metastatic activity among patients carrying the 5A allele.

Studies on the *MMP*-3 promoter polymorphism and its correlation with breast cancer have shown different results. Hughes and colleagues (2007) showed a significant and independent association of the C/T genotype for *MMP*-9 and the 1G/2G genotype of *MMP*-1 with the lymph node-positive disease of breast cancer. But he could not find any association between *MMP*-3 polymorphism and breast cancer. However, some studies have reported that the presence of the 5A allele is associated with susceptibility and severity of breast cancer. But in other cancers like colorectal cancer, the 6A/6A genotype in patients has been observed to occur more frequently among controls, however, the difference is not statistically significant (Zhang *et al*., 2004; Ghilardi *et al*., 2002). It is conceivable that the higher transcriptional activity associated with the 5A allele may enhance tumor invasiveness (Blons *et al*., 2004). Radisky and colleagues (2005) havereported that the 5A allele correlates with breast cancer metastatic activity. The results of this study do not show a statistically significant difference between the breast cancer patients without metastasis and the healthy controls. However, they show a close association between the 5A allele and metastasis of breast cancer. The 5A allele is substantially more frequent in the M^+ subgroup than both the control group and M^- subgroup (Table 2). Observed differences between reported risks are probably due to the differences in genetic background, environment and sample size of the studied population.

Acknowledgments

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Table 2. *MMP*-3 promoter polymorphism distribution in patients (M^+/M^-)* and controls.

	M +	M -	Controls
5A/5A + 5A/6A	48	40	46
5A/5A	20	8	8
6A/6A	12	18	14
5A frequency	0.56	0.40	0.45
6A frequency	0.44	0.60	0.55

(Patients/ controls) 5A/5A: 6A/6A, OR = 1.63, 95%CI, 0.59- 4.4, P = 0.061; (Patients/ controls) 5A/5A +5A/6A, 6A/6A, OR = 0.89, 95%CI, 0.43-1.84, P=0.047; M^+/ controls, OR= 2.9, 95%CI, 0.94- 8.9, P= 0.074; M^+/ M^-, OR= 3.75, 95%CI, 1.25- 11.24, P= 0.078; * M^+ : Patients with metastatic activity; * M^- : Patients without metastatic activity.

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