MMP9 Promoter Polymorphism (-1562 C/T) Does not Affect the Serum Levels of Soluble MICB and MICA in Breast Cancer

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ABSTRACT

Background: The role of Matrix Metalloproteinase 9 (*MMP9*) in tumor invasion and progression is prominent. A single nucleotide polymorphism (SNP) in the promoter region of *MMP9* (-1562 C/T) increases the transcription and expression of this gene. On the other hand, MHC class I chain-related protein A and B (MICA/B) in soluble forms may impair tumor immunogenicity by reducing Natural Killer Group 2D (NKG2D) densities on NK cells and *MMP9* enzyme activity has a prominent role in shedding of MICA/B. **Objectives:** To investigate the association between *MMP9* (-1562 C/T) polymorphism and serum MICA/B level in breast cancer patients. **Methods:** In this case-control study, 105 patients with breast cancer and 100 healthy age-matched women were selected from Yazd hospitals, Iran. The polymorphism of *MMP9* (-1562 C/T) was determined by PCR-RFLP. Concentration of MICB and MICA in the sera of breast cancer patients and healthy women were measured using ELISA method. **Results:** The frequency of CC, CT and TT genotypes and T allele of the *MMP9* (-1562 C/T) did not show significant differences between breast cancer patients and healthy donors ($p>0.05$). On the other hand, the mean serum levels of MICB and MICA were significantly elevated in patients compared with healthy individuals ($p<0.05$). In patients with *MMP9*CC genotype, the mean serum MICB concentration was significantly higher than those patients with CT polymorphism ($p<0.05$). Although the mean of blood MICA concentration in patients with the CT genotype was higher than those patients with CC genotype, the difference was not statistically significant. **Conclusion:** The T allele of the *MMP9* (-1562 C/T) does not show a correlation with serum levels of MICA and MICB in breast cancer patients.

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Keywords: Breast Cancer, Matrix Metalloproteinase 9, MHC Class I-Related Chain A

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INTRODUCTION

Breast cancer consists of a heterogeneous group of malignancies derived from the ductal epithelium. It is the leading cause of cancer related death and the most common malignancy in women worldwide. Breast cancer incidence rates are increasing in all regions of Iran especially in rural areas (1). In recent years, matrix metalloproteinases (MMPs), especially *MMP9* and *MMP2* are considered as effective agents in early development and progress of breast cancer cells (2).

MMPs are a family of enzymes that degrade extracellular matrix and have been implicated in invasion and metastasis of tumor cells. Twenty-six MMPs have been identified in human so far. Pro-MMP is synthesized by many cells including fibroblasts, leukocytes, monocytes/macrophages and tumor stromal cells. According to previous studies, *MMP9* and *MMP2* degrade gelatin and type IV collagen, the main proteins in the basement membrane (3). Degradation of the basement membrane helps tumor cells to spread and metastasize (4). *MMP9* gene has a functional polymorphism that correlate with susceptibility, invasiveness or recurrence of various cancer types (5). The T allele functional genetic polymorphism of*MMP9* C/T (rs3918244) in the promoter region of the gene, is accompanied by higher levels of gene transcription (5,6). The impact of *MMP9* -1562 C/T polymorphism in different cancer is controversial. Zhang *et al.* suggested that the polymorphism might decrease both the colorectal and lung cancer risk (7). The polymorphism is associated with susceptibility in the oral sub-mucous fibrosis and head and neck squamous cell carcinomas according to other studies (8). The*MMP9* -1562 promoter polymorphism has also been associated with gastric cancer risk in females and bladder cancer susceptibility (9,10).

The major histocompatibility complex class I chain related (MIC) gene family consists of five members among which, MICA and MICB are the most important members in the immunologic responses. MICA and MICB are NKG2D ligands, which have been proposed to mediate tumor regression. MICs have prominent role in stressed-antigen presentation (11). Production of MICs is restricted to normal cells with limited surface expression, but under pathological conditions, such as cancer, infection, hypoxia, heat shock and oxidative stress, their surface expression is elevated. Tumor cells reduce NKG2D ligand surface levels by shedding MICs in a soluble form which could lead to down regulation of NKG2D surface expression (12). Other novel findings revealed that increased expression of MICA/MICB by *trans* retinoic acid in breast cancer cells could be an effective way in preventing tumor immune escape (13).

Many studies showed elevated MICA and MICB in sera of patients with cancers (14,15). Proteolytic enzymes such as ADAM10, ADAM17, *MMP9* and Erp5 may have a role in shedding of membrane MICA and MICA in tumor cells but, according to new investigations *MMP9* has the strongest enzymatic activity which was found in SiHa cervical cancer cells, associated with increased soluble MICA/B levels in the cervical carcinoma (16). According to other studies *MMP9* can degrade MICA on cancer cell surface (17). The results of other studies suggest that the *MMP2*-1306 C/T and *MMP9*-1562 C/T polymorphisms are significantly associated with bladder cancer susceptibility (18). Due to the high number of single nucleotide polymorphisms (SNPs) in *MMP9* gene and its important function during carcinogenesis, a deeper understanding of the functional consequences of these SNPs is essential (19). Although, many studies have done on *MMP9* -1562 C/T polymorphism in different cancers, effect of the *MMP9*

genotypes on serum MICA and MICB concentration in the sera of breast cancer patients, is not elucidated. Therefore, the present study investigates the *MMP9* polymorphism and its correlation with the concentrations of MICA/B in the sera of breast cancer patients in Iranian women.

MATERIALS AND METHODS

Subjects. In this case-control study, 105 patients with breast cancer and 100 healthy women were selected. The patients (45 ± 8 years) and healthy donors (43 ± 10) were age-matched. All patients had histologically-confirmed ductal breast carcinoma. All the patients were new cases and they had not received any treatment, surgery, chemotherapy and radiotherapy at the time of sampling. The patients, who were selected from Yazd hospitals in Iran, were in stages III of malignant breast cancer. The healthy women who enrolled in the study did not have diabetes, dominant infections, febrile diseases and they also had not received any immunosuppressive drugs at least 1 month before blood sampling. According to the interview with healthy women, there was no history of breast cancer in their family and they were directed to mammography center at Shahid Sadoughi Hospital, Yazd, Iran. The healthy women who had normal breast mammography were also enrolled. The patients and healthy individuals who had a history of heart diseases and metabolic disorders like diabetes were excluded of the study. This study was approved by the Institutional Review Board of Shahid Sadoughi Medical Sciences University (number: P17-179251).

The peripheral blood (7 ml) was taken after obtaining consent from the patients and healthy donors. For collection of sera, 4 ml of the blood were used and EDTA was added to the rest of blood) for extraction of genomic DNA.

ELISA Method. Sera were harvested by centrifugation at 1000 × g and were stored at -70°C. MICA and MICB concentrations (Pg/ml) were measured in the sera of patients and healthy donors by ELISA method (Abcam ,Cambridge, USA). The ELISA protocol was carried out according to the manufacturer'sinstructions.

Genotyping of the *MMP9* Promoter Polymorphism. Genomic DNA was extracted from the blood samples by Accuprep™ Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). The extracted DNA was quantified and qualified by spectrophotometer and agarose gel electrophoresis, respectively. Agarose gel (0.8%) with DNA green viewer was used to confirm the presence of genomic DNA. The *MMP9* genotype was determined by the PCR-RFLP method. The PCR primers (Pishgam, Tehran, Iran) for amplifying the *MMP9* gene are shown in Table 1.

Table 1. The primers were used for PCR of *MMP9* gene in patients and healthy donors.

Forward Primer	5'-ACGGTGCTTGACACAGTAAATCT-3'
Reverese primer	5'-GGCAGGGTCTATATTCACCTTCT-3'

PCR was performed in 25 µl total volume containing 1 µl DNA template, 15 µl master mix (Cinnagene, Karaj, Iran), 8.5 µl ddH2O and 0.25 µl of each the forward and reverse primer. The thermal PCR cycling conditions were 5 min at 94^{o}C followed by 30 cycles of 60s at 94^{o}C, 60s at 53^{o}C and 60s at 72^{o}C with a final step at 72^{o}C for 5 min to allow for the complete extension of all PCR reactions. Hin1II (N1aIII) restriction enzyme (Fermentas, NY, USA) was used to digest *MMP9*: The PCR product was digested at 37^{o} C for 1 hour in a 10µl volume containing 1U of Hin1II and 2µl reaction buffer and 7µl ddH2O. After digestion, the products were load on 0.8% agarose gel stained with DNA green viewer. Electrophoresis patterns were including: 290 and 355 bp bands for the homozygote CC individuals, the 135, 220 and 290 bp bands for the homozygote TT individuals and the 135, 220, 290 and 355bp bands for heterozygous CT individuals.

Statistical Analysis. Data analysis was carried out by SPSS version 18 (SPSS, Inc., Chicago, USA). To evaluate differences in the distributions of genotypes and alleles of *MMP9* between the cases and controls the $\chi 2$ analysis was used. The associations between *MMP9* genotypes and risk of breast cancer were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs).

RESULTS

Matching on age between the breast cancer patients and healthy donors was confirmed by the χ^2test (p=0.611). The mean age was 45 ± 8 years for the patients and 43 ± 10 years for healthy donors. Total DNA extraction was run on 0.8 % agarose gel (Figure 1). For electrophoresis of PCR-RFLP products, a 2% gel agarose was used (Figure 2).

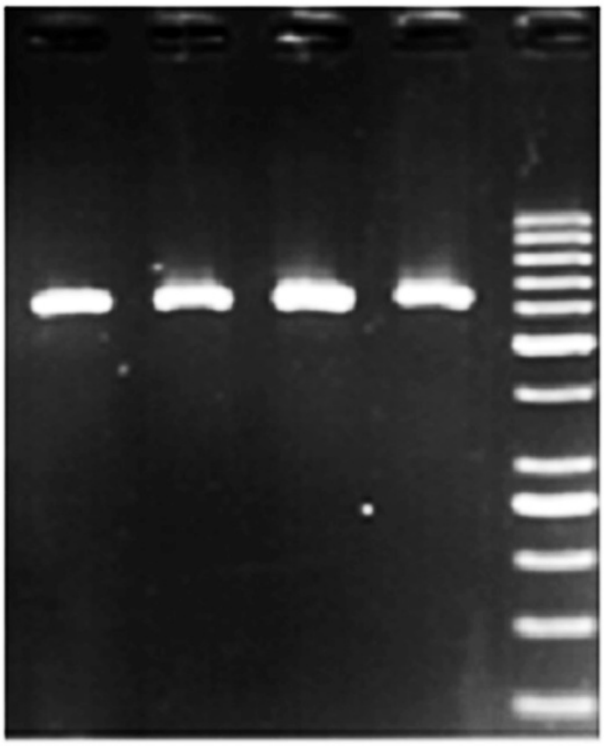

Figure 1. *MMP9* PCR product was loaded on 0.8% agarose gel. *MMP9* bands with 652bpwereseparated. The lanes from right hand to left show DNA ladder, 2 breast cancer patients and 2 healthy subjects of *MMP9* gene PCR products respectively.

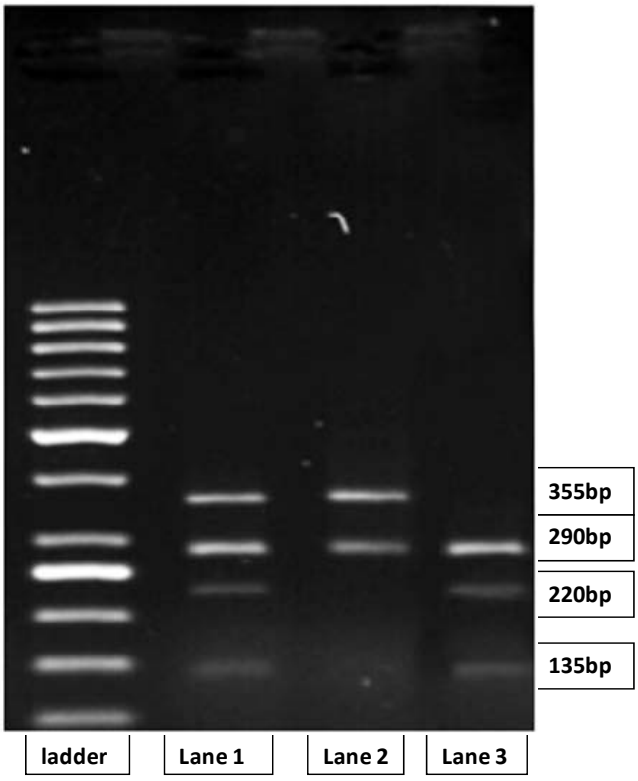

Figure 2. Amplified *MMP9* gene digested by Hin1II enzyme for 1 hours and run on the 2% agarose gel. Three molecular patterns were generated by the enzyme depending on *MMP9* -1562 C/T polymorphism type. CT, CC and TT genotypes are shown in lane 1, 2 and 3 respectively. The homozygote CC produced 290 and 355 bp bands, the TT genotype was represented by 135, 220 and 290bp bands and the heterozygous CT generated 135, 220, 290, 355bp bands.

The distribution of genotype frequencies of *MMP9* -1562 C/T promoter polymorphism in breast cancer patients were calculated (Table 2).

Table 2. Frequency of *MMP9* (-1562 C/T) genotypes in breast cancer patients and healthy donors.

Genotypes And Alleles	Healthy Subjects (N=100)	Breast Cancer Patients (N=105)	Odds Ratio (95% CI)	*P*
CC	72 (72%)	67 (63.80%)	1.45 (0.78-2.75)	0.209
CT	27(27%)	34 (32.38%)	1.35 (071-2.59)	0.326
TT	1 (1%)	4 (3.80%)	0.26 (0.01-2.48)	0.192
C	171 (85.5%)	168 (80%)	0.88 (0.39-1.18)	0.141
T	29 (14.5%)	42 (20%)	0.60 (0.02-1.21)	0.067

Statistical analysis showed no significant differences in the frequencies of TT, CC, and CT genotypes between patient and healthy donors (p>0.05). Allele frequency of *MMP9*gene was also determined. According to our analysis, the allele frequencies were similar in the patients and healthy subjects (Table 2).
Mean of MICA concentrations in the patients and healthy donors were 3302 ± 2515 vs. 91 ± 93 (Pg/ml), respectively. Statistical analysis showed significant a difference between two groups. The means of MICB serum concentration was also significantly increased in breast cancer (24.8 ± 60) compared with the healthy donors (2 ± 0.7) (p=0.002) (Table 3).

Table 3. MICB and MICA concentration in serum of breast cancer patients and healthy subjects.

	Breast Cancer Patients (N-105) Mean ± SD	Healthy Subjects (N=100) Mean ± SD	P
MICA (pg/ml)	3302 ± 2515	93 ± 91	0. 001
MICB (pg/ml)	24.8 ± 60	2 ± 0.7	0.002

Based on the previous reports that showed *MMP9* polymorphism has effect on the expression of the gene and enzymatic activity of the protein, we investigated the relationship between *MMP9* genotypes and MICA and MICB concentrations in the two groups.

Table 4. The MICA and MICB serum concentrations in breast cancer patients with the *MMP9* (-1562 C/T) CT and CC genotype.

	CT genotype	CC genotype	P
MICA (34 patients)	2951 ± 2308	1930 ± 1282	0.211
MICB (67 patients)	22.3 ± 13.63	75.4 ± 33.2	0.036

Our analysis showed that in the breast cancer patients with CC polymorphism the mean of MICB concentration was significantly increased compared with the patients with CT polymorphism (33.2 ± 75 vs. 13.6 ± 22.3, p=0.036). The MICA concentration did not show any significant difference between the two groups. It seems that CC genotype of *MMP9* (-1562 C/T) polymorphism may have a role in the elevation of MICB in the patients (Table 4).

DISCUSSION

The *MMP9* gene promoter (-1562 C/T) polymorphism affects the transcription of the gene, by which a C to T substitution results in the loss of binding of a nuclear protein to this region of the *MMP9* gene and an increase in the transcriptional activity (20). Individuals with T allele and CT genotype have higher risk of metastasis of lung and breast cancer than those with CC genotype (4). Recent studies showed that *MMP9* enzyme could digest stressed cellular ligands such as MICA and produce soluble MICA on osteosarcoma tumor cell surfaces (21). MICA and MICB are ligands for the activating immunoreceptor NKG2D (natural killer group 2, member. The NKG2D receptor is critical in activating NK cells. It is completely approved that some cancerous cells, by shedding of the NKG2D ligands, decrease NKG2D receptors on immune cells surface and thereby facilitate tumor immune escaping (22). Present study investigated the distribution of *MMP9*-1562 C/T single nucleotide polymorphism and concentration of MICA and MICB in Iranian breast cancer patients in order to evaluate the possible correlation between the allele type and soluble MICA/B concentration.

According to our results, the frequency of *MMP9* (-1562 C/T) polymorphism in the breast cancer patient and healthy subject were similar. Of the women who took part in this study, 72%, had CC *MMP9*-1562 C/T genotype and 28% showed CT + TT genotype. In a study in chinese population, the frequency of CT+TT was 14.4%. In another study in Brazil, the frequency of CC genotype in the brazilin population was about 76% and the frequency of CT genotype was 24% , which is consistent with our results (23). In our study, 38% of the patients showed CT+TT genotype, however, the differences in the distribution of CT or TT genotypes in breast cancer patients did not reach the significant level. High frequency of CT+TT genotype in Iranian women may be an important risk factor for developing breast cancer.

In the present study, elevated MICA and MICB in the sera of patients is compatible with other studies, especially with the notion that most of the patients who had taken part in our study were in stage III breast cancer. To elucidate the effect of *MMP9* genotype on MICA and MICB, concentration of the proteins were assessed in patients sera with different *MMP9* genotype. Our results showed that serum MICB concentration in the patients with CT *MMP9* genotype was 13.63 ± 22.3 pg/ml, whereas, in the patients with CC genotype it was 33.2 ± 75.4 pg/ml. This finding is not consistent with the hypothesis that elevated MICB is dependent on T alleles of *MMP9* gene. It is obvious that shedding of cell surface proteins like MICB does not totally depend on *MMP9* enzyme and other mechanisms may be involved in the shedding of MICB in breast cancer patient. In case of MICA, our study showed that patients with CT *MMP9* genotypes have elevated MICA (3302 ± 2515) in their sera in comparison with patients with CC genotype (1930 ± 1282). Statistical analysis did not confirm the significant differences between the two groups, but it is consistent with other studies showing that shedding of MICA may be related to *MMP9* activity. According to new findings, there are different genotypes of MICA, such as 4-, 6- and 9-GCT repeats in MICA gene (24). So different structures of MICA may be related to the extent of protein shedding. Although mean of circulating MICA concentration in the breast cancer patients with the CT genotype was higher than that in CC genotype patients, statistical analysis did not confirm significant difference between the two groups. Therefore, there is no obvious relation between sera MICA concentration and the MMP9 SNP.

In conclusion, high expression of *MMP9*, due to CT+TT genotypes, is reported in different cancers (4). Genotypes (TT+CT) could be considered as a susceptibility factor in developing cancers such breast cancer (24). Although the serum levels of MICA/B are elevated in breast cancer patients, compared to those of healthy donors, there is no obvious correlation between the MMP9 -1562 C/T SNP and the concentrations of circulating MICA/B in the breast cancer patients, since the relation between CT and MICA and MICB values is not statistically significant. Taken together, we suggest that investigation of other mechanisms in addition to MMP9 enzyme in the shedding of MICA and MICB in breast cancer patients is helpful.

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