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Original Article

MMP3 -1171 5A/6A Promoter Polymorphism Affects Level of Serum Major Histocompatibility Complex Class Chain Related B in Breast Cancer Patients

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Background and Aims: Involvement of matrix metalloproteinases 3 (MMP3) in breast cancer tumor progression and metastasis has been revealed. MMP3 -1171 5A/6A and 5A/5A polymorphisms in the gene promoter increase expression of the enzyme. The possible relationship of these polymorphisms and serum levels of major histocompatibility complex class I chain-related protein A and B (MICA/B) in breast cancer patients was goal of the study.

Materials and Methods: In this case-control study, 105 breast cancer patients and 100 age-matched healthy women were selected. The MMP3-1171 5A/6A polymorphism was determined by nested and restriction fragment length polymorphism polymerase chain reaction. Concentration of MICB and MICA in serum of breast cancer patients has measured using enzyme-linked immunosorbent assay method**.**

Results: Our results showed that 5A/5A + 6A/5A genotypes in the breast cancer group (77.18%) were more frequent compared to healthy participants (64%). However, the frequency of MMP3 -1171 5A/6A genotypes in the breast cancer patients and healthy donors was similar. Additionally, in the patients with 5A/6A genotypes, the mean of MICB concentration was 51.3±16 pg/ml whereas; in the homozygote 6A/6A genotype, it was 4.7±2.6 pg/ml. Statistical analysis confirmed a significant difference between MMP3 -1171 5A/6A and serum MICB levels in the patients (p=0.02). Based on our results MMP3-1171 5A/6A polymorphism did not alter MICA concentration in the patients (p=0.15)

Conclusions: The MMP3 -1171 5A/6A genotype might be associated with shedding of MICB in breast cancer patients.

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Introduction

Breast cancer is the most common malignancy in women worldwide. Incidence rates of the devastating disease are increasing in many countries [1]. Matrix metalloproteinases (MMPs) especially MMP3, MMP2, MMP7 and MMP-9 are considered as an effective agent in early fetus development, progression and metastasis of breast cancer cells [2, 3]. Fibroblasts, leukocytes, monocytes/ macrophages and tumor stromal cells are able to produce MMPs. MMP3 is a zinc dependent proteolytic enzyme that converted to active form through autocleavage. The active form of the enzyme effectively digests the extracellular matrix (ECM) components. This function of MMP3 is vital in fetus development and organogenesis [4]. According to the new findings, the enzyme can degrade other proteins, including cytokines and chemokines [5]. Several studies have demonstrated that -1171 5A/6A single nucleotide polymorphism in promoter region of the MMP-3 gene substantially alters the expression and function of the enzyme [6]. The elevated enzymatic function of MMP3 might be the cause of cleaving and shedding some surface antigen presentation proteins such as class chain related gene family.

The major histocompatibility complex class chain-related (MIC) gene family consists of five members, which MICA and MICB are the most well-known that act as activator ligands for NKG2D receptors on NK and gamma delta T cells [7]. According to the new findings, shedding of MICs by tumor cells and virally infected cells is an effective way for escaping of the cell of natural killer surveillances [8]. Elevated MICA and MICB in serum in different cancers are reported [9, 10]. Proteolytic enzymes such as a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), ADAM17, MMP9 and Erp5 have obviously exerted in shedding of membrane MICA and MICA on tumor cells [11]. According to the new studies, the implication of MMP3 and its genetic variation in advanced gastrointestinal carcinoma, breast and lung carcinoma, ovarian cancer and other malignancies has been approved [12-17]. However, MMP3 might also have a role in shedding of MICA and MICB. Effects of MMP3 -1171 5A/6A polymorphism on serum MICA and MICB concentrations in breast cancer has not elucidated yet.

Present study for the first time investigated the MMP9 polymorphism and its relationship with MICA and MICB in the Iranian women population whom suffering from breast cancer.

Materials and Methods

The methodology of the present study was approved by the Ethics Committee of Shahid Sadoughi university of medical sciences . In this case-control study, 105 breast cancer patients and 100 healthy women were recruited from Oncology Yazd hospitals, Iran. The patients and healthy donors were age matched (Mean±SD Patients' age were 45±8 years vs. healthy donors' age 43±10). All the patients recognized as a new case. Blood collection was carried out before the patients received any related-medication, including surgery, chemotherapy and radiotherapy. Most of the patients were in stages III malignant

breast cancer. No patients and healthy donors show metabolic diseases and dominant infections such as the common cold at time of sampling. The subjects also did not receive any immunosuppressive drugs at least one month before taking blood samples. Additionally, the inclusion criteria for selecting of healthy women were normal breast mammography, no breast cancer history in their family, no history of metabolic disease, no dominant viral and bacterial infection. The peripheral blood (7 ml) was taken after obtaining consent of the patients and healthy donors. Four milliliter of blood was used for preparation of serum and the rest blood added to the tubes with Ethylene-di-amine-tetra-acetic acid for extracting of genomic DNA.

Enzyme-linked immunosorbent assay (ELISA) method

Harvested sera were kept at -70°C until ELISA assays. MICA and MICB concentrations were measured by the Human ELISA Kits (Abcam, Cambridge, USA) according to the written instructions.

Nested and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods

Genomic DNA was extracted from the blood samples by DNA extraction kit (Bioneer, Daejeon, Korea). The extracted DNA was quantified and qualified by spectrophotometer and agarose gel electrophoresis, respectively. Agarose gel (1%) with the DNA green viewer was used to confirm the presence of genomic DNA. Nested PCR methods were used and the primers designed by Primer Premier Version 5.0 statistical software (PREMIER, Biosoft International, CA), which illustrated in table 1. Nested PCR was performed in 25 µl total volume containing 100 ng DNA template, 12 µl Master Mix (PCR buffer, MgCL2, Tag DNA polymerase, dNTP: 200 nmol) and forward and reverse primer (0.05 µM). The thermal PCR cycling conditions were 5 min at 94ºC followed by 30 cycles of 60s at 94ºC, 60s at 53ºC and 60s at 72ºC with a final step at 72ºC for 5 min. to allow for the complete extension of all PCR reactions. The product of Nested PCR was a 435 bp band and using this product PCR-RFLP was carried out to detect the genetic polymorphism of -1171 5A/6A MMP3 genes. MMP3 PCR was done in total volume of 25 µl total volume containing 20 ng (2 µl) DNA template, 12.5 µl Master Mix (PCR buffer, MgCL2, Tag DNA polymerase, dNTP, 200 nmol) and 0.5 µM (2 µl) of MMP3 forward and reverse primer and 8 µl distilled water.

Table 1. The primers, which were used for Nested PCR and amplification of MMP3

Type of primer	Size	
NES-F MMP-3	453bp	5'-GGAAGTCGTCGAAGCTGTTTTA-3'
NES-R MMP-3		5'-GATTACAGACATGGGTCACGG-3'
MMP-3 F	129bp	5'-TTTCAATCAGGACAAGACGAAGTTT-3'
MMP-3-R		5'- CTCTCAAAGTGCTAGGATTACAGACAT-3'

The thermal cycle was the same with Nested PCR. Product of the PCR was 129 bp of DNA, which evaluated on 1% agarose gel. To RFLP-PCR, PdmI (XmnI) restriction enzyme was used. The MPP3 PCR product was digested at 37ºC for one hour in a 10 µl volume containing 1U of PdmI (XmnI) and 2 µl reaction buffer and 7 µl distilled water. After digestion, the products were load on 2% agarose gel stained with DNA green viewer. Electrophoresis patterns, including 129 bp for 6A/6A genotype, 97 bp for 5A/5A and 97, 129 bp for 5A/6A genotypes.

Statistical analysis

Data analysis was carried out by SPSS 18 version (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 and their 95% confidence intervals with logistic regression analyses.

Result

MMP3 -1171 5A/6A polymorphisms

The breast cancer patients and healthy women who took part in the study were in the same age (p=0.611). First, Nested PCR was carried out and 435 bp of DNA piece produced (Fig. 1). RFLP-PCR done on the 435 bp DNA to determine MMP3 -1171 5A/6A polymorphism. Three molecular patterns generated by the enzyme depending on type of MMP3 -1171 5A/6A polymorphism. As the results are shown in fig. 2, 129 and 97 bp bands show 5A/6A polymorphism, 97 bp bands approves 5A/5A genotype and 127 bp band corresponds with 6A/6A genotype. The distribution of genotype frequencies of MMP3 -1171 5A/6A promoter polymorphism in breast cancer patients and healthy donors are given in table 2. Statistical analysis showed no significant differences in the frequency of 5A/5A, 5A/6A and 6A/6A genotypes in the patient and healthy donors (p>0.05). According to our results, more than 77% of breast cancer patients have 5A/5A and 5A/6A genotypes that comparing to 64% of healthy donors it is significant. Allele frequency of MMP3 gene also showed in table 2. According to our analysis, the frequency of alleles in the patients and healthy participants was also similar.

MICA and MICB concentrations in serum of breast cancer patients with 5A/5A and 6A/6A polymorphisms

As it was mentioned earlier, one of the main aims of the present study was investigating the possible relationship between the type of MMP3 polymorphisms and MICA as well as the MICB concentrations in the breast cancer patients. Our results showed that in the breast cancer patients MICA concentrations have not affected by 6A/6A and 5A/6A polymorphism (p=0.15) (Table 3). Consistently, in the breast cancer the patients with 6A/6A and 5A/6A polymorphisms the mean of MICA concentration was 1457±819 and 1729±2501 pg/ml, respectively.

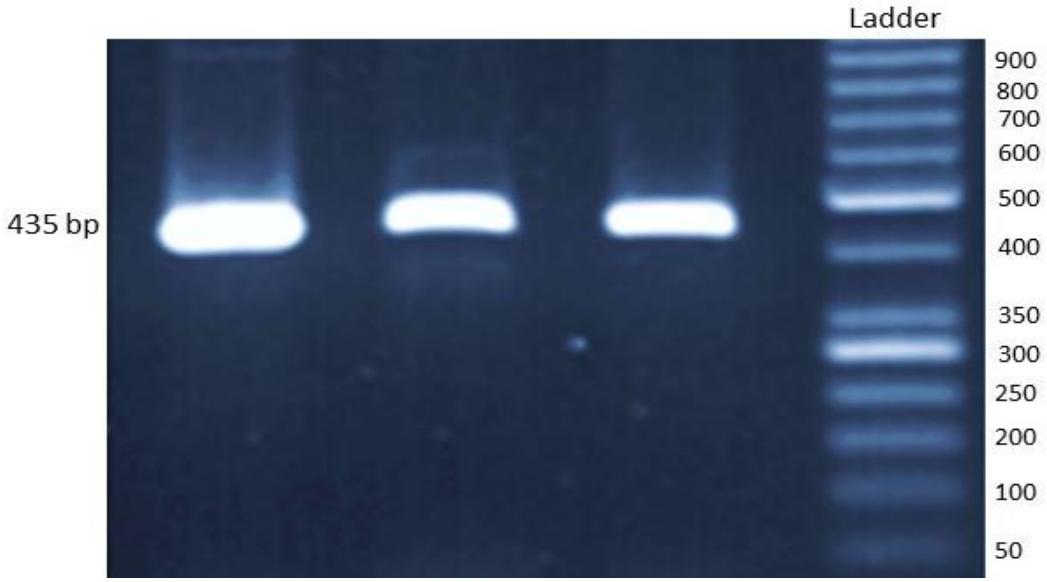

Fig. 1: Nested PCR used for amplification of 435 base pair piece containing MMP3 gene. The PCR product was run on 1% agarose gel

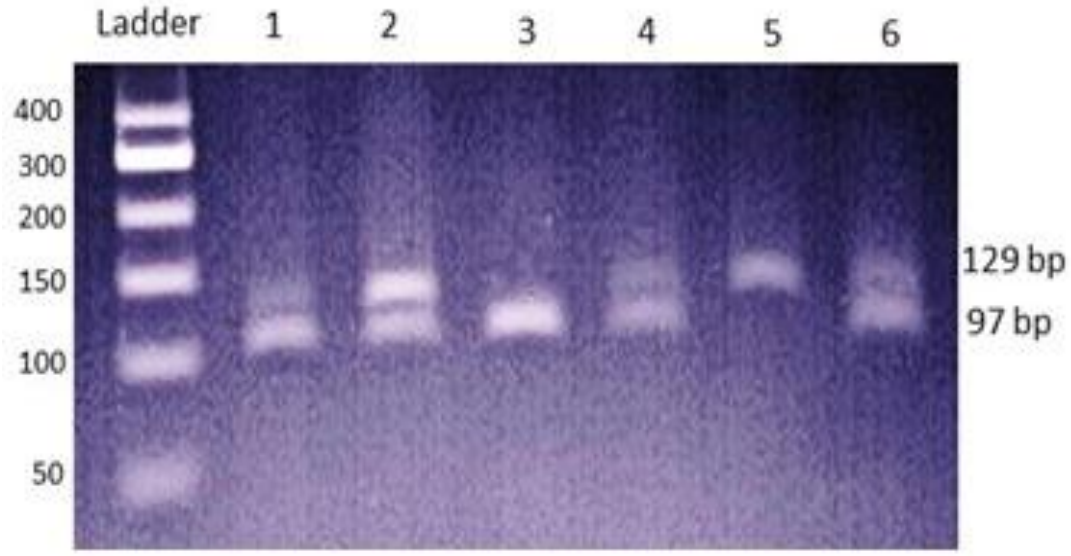

Fig. 2: Nested PCR products used for RFLP-PCR of MMP3 gene. Digestion of the gene carried out by PdmI (XmnI) restriction enzyme at 37° C for 1 hour. Digested products ran on 1% agarose gel. Lanes of 1,2,4,6 show 5A/6A polymorphisms of MMP3 -1171 5A/6A. Lane 3 shows 5A/5A polymorphism and lane 5 illustrates 6A/6A polymorphism.

Table 2. Frequency of MMP3 genotypes in breast cancer patients and healthy donors using PCR-RFLP method

Genotype	Healthy subjects (n=100)	Breast cancer patients (n=105)	Odds Ratio (95% CI)	p-value
5A/5A	2 (2%)	4 (3.88%)	1.96(0.06-0.027)	0.21
6A/5A	62(65%)	77(73.3%)	1.35 (0.07-2.59)	0.081
6A/6A	36 (33%)	24 (22.8%)	1.26(0.01-2.48)	0.192
5A	66(33%)	85(40.47%)	1.24(0.02-1.98)	0.11
6A	134(67%)	125 (59.52%)	1.45(0.04-1.78)	0.23

On the other hand, our results confirmed that the mean of concentration of MICB in the patients with 5A/6A genotype had significantly increased when compared with the patients with 6A/6A genotype. In the breast cancer patients with 6A/6A and 5A/6A polymorphisms, the mean of MICB concentrations were 4.7±2.6 and 51.3±16.6 pg/ml, respectively (Table 3).

Table 3. MICA/ MICB concentrations in serum of breast cancer patients with MMP3 -1117 5A/6A and 6A/6A polymorphisms

	Type of polymorphisms	Number	MICA (pg/ml)	MICB (pg/ml)
Breast cancer patients	6A/6A	19	810±1457*	4.7±2.6**
	5A/6A	21	2501±1729	51.3±16.6

Data presented as Mean±SD
*p= 0.15; **p=0.02

Discussion

According to the new findings, MMP3 -1171 5A/6A polymorphisms may be one of the important risk factors for the development and/or progression in breast, pulmonary and ovarian cancers [18]. Expression of MMP3 is influenced by the heterozygotes and homozygotes for the 5A allele (5A/5A and 5A/6A) genotypes. Heterozygote 5A allele increases promoter activity more than the 6A allele [12, 19]. Elevated MMP3 levels in different cancers are approved, but the exact mechanisms for functioning of the enzymes in progressing and developing of cancer is not elucidated yet [13]. Whether MMP3 -1171 5A/6A polymorphisms are able to increase serum MICA/MICB concentrations possibly by cleavage and shedding of surface MICA and MICB on cancerous cells? Our results showed that 5A/5A+6A/5A genotypes in the breast cancer group (77%) were more frequent comparing to the healthy participants (64%). Moreover, MICB concentration in the breast cancer patients with -1171 5A/6A polymorphism was significantly increased in comparison with -1171 6A/6A patients. Therefore, 5A allele might have a role in elevated MICB in the breast cancer.

MICA/B as important NKG2D ligands plays vital role in induction immune responses to tumor antigens [20]. Current knowledge of regulatory mechanisms of expression, shedding and recycling of the stressed proteins is incomplete. While majority of studies have confirmed diminished expression of the ligands on cancerous cells and increased serum concentration in the different cancers, some studies reported that MICA/B expression is restricted to only a few normal epithelial tissue [21, 22]. On the other hand, elevated serum levels MICA/B in breast cancer patients is correlated with stage of the disease and associated with the poor prognosis disease [21,

23]. So far, some mechanisms are reported for describing MICA/B expression and increased level of the proteins in the patients. Intracellular retention of MICA in human melanoma cell lines [24], short half-life of MICA/B on the cell surface [25], the release of MICA/B by exosomes [26], hypoxia, decrease in the membrane expression of MICA/B on some cell lines by hypoxia [27] are explained for the decrease of MICA/B on cancerous cells. Although, shedding of MICA/B and proteolytic cleavage of MICA/B is recognize the major mechanisms for evading tumor cells by NKG2D-expressing cytotoxic cells [17, 28, 29]. Consistent with other studies, elevated expression MICB due to MMP3 -1171 5A/6A genotype could be considered as a possible route for more shedding of MICB of tumor cells. Moreover, long-term exposure of soluble MICs molecule derived from tumor cells down regulated surface NKG2D expression on T cells [30]. Our results confirmed that soluble MICB was elevated in serum of breast cancer patients with MMP3 -1171 5A/6A. Therefore, it can be concluded that MMP3 -1171 5A/6A is associated with breast cancer. Additionally, based on other studies, with progressing of breast cancer, the level of MICA/B also increases in the serum of the patients [17, 31]. The patients who enrolled in our study were mostly in stage 3 of the disease; so, the stage of the disease could not affect on our results. Therefore, based on our results, the MICB as effective ligand in activating natural killer cells and gamma delta T cells is affected by MMP3 -1171 5A/6A that this issue can be considered in possible immunotherapy.

Conclusions

MMP3 -1171 5A/6A has affected on the expression of MMP3 positively and this polymorphism can be considered as one of the shedding factors of MICB in the breast cancer patients. Investigation of the possible ways in interfering or diminishing of expression of MMP3 seems to be an effective immune therapy in breast cancer. Whether the MMP3 polymorphism has effect on the prognosis of the breast cancer should be investigated. By recognition of the mechanisms in MICA and MICB shedding, we can open new approaches in the treatment of devastating cancers like breast cancer.

Conflict of Interest

The authors declare that they have no competing interests.

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