ORIGINAL ARTICLE

The *stromelysin*-1 5A/5A genotype enhances colorectal cancer cell invasion in Iranian population

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Background: Matrix metalloproteinases comprise a family of enzyme degrade components of extra cellular matrix. There are single nucleotide polymorphisms in the promoter regions of several genes with ability to influence cancer susceptibility. The aim of this study was to analyze association between *MMP*3 promoter polymorphisms and colorectal cancer occurrence and progression. **Materials and Methods:** In this case-control study 120 colorectal cancer patients and 100 controls were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) on the genomic deoxyribonucleic acid (DNA). The patients group was divided into different subgroups: a subgroup without metastatic activity (M^-) and a subgroup that had developed metastasis (M^+). **Results:** There was a significant difference in frequency of the *MMP*-3 genotype between cases and controls (χ^2 = 16.17; P = 0.0003). The 5A homozygote in patients and controls was significantly different. The frequency of the 5A allele among affected patients (67.91%) was significantly higher than among the healthy controls (49%; χ^2 = 16.17, P = 0.00005). At the time of diagnosis, individual who was carrying the 5A allele was more represented in the M^+ subgroup than in M^- subgroup (χ^2 = 7.49; P = 0.006, OR = 3.86; 95% CI, 1.43–10.33). The difference between M^- and controls did not observe statistically significant (χ^2 = 0.009; P = 0.92). **Conclusions:** Our results suggest that the presence of 5A polymorphism at the *MMP*-3 promoter region may favor the growth and the metastasis process in colorectal cancer patients and could be looked at as a risk factor for a worse prognosis.

Key words: Invasion, MMP-3, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), single nucleotide polymorphism (SNP)

INTRODUCTION

Variant genes are involving in extra cellular matrix (ECM) turnover, including matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). They have been considered as strong candidates for a genetic susceptibility factor to different diseases and cancer. Degradation of ECM is essential in many physiological processes, for example, during development, growth, and repair of tissue.[1-3] Tumor cell invasion, metastasis and angiogenesis require controlled degradation of ECM. Increased expression of MMP enzymes is associated with invasion and metastasis of different malignances.[4] MMPs comprise a family of enzymes that are able to degrade components of ECM.[5] At least 26 human MMPs have been identified, which are classified according to their substrate specificity and structural similarities.[2] These subgroups follow: Collagenases, gelatinases, stromelysins, matrilysins, and transmembrane MMPs.[4,5]

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Colorectal cancer (CRC) is the most common malignancy of the gastrointestinal tract. It still carries a relatively poor prognosis despite recent improvement in early diagnosis, surgical techniques, and chemotherapy. About 40% of patients who undergo operative resection will die within 5 years due to recurrent disease or metastasis.[6-8]

Expression of the most MMPs is normally low in tissues and they are induced when remodeling of ECM is required.[9] *MMP*s expression in tumor cells is regulated primarily at the transcriptional level, but there is also evidence of modulation of mRNA stability in response to growth factors and cytokines.[9-11] There is much evidence that MMPs play essential roles in migration, tumor growth, and development.[5,12,13]

The gene coding for *MMP*-3 is located on the long arm of chromosome-11[14] and consists of 10 exons, although

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Received: 07-07-2011; **Revised:** 16-08-2012; **Accepted:** 11-09-2012

there may be other shorter splice forms in addition to its full length transcript.[15] MMP-3, also known as stromelysin-1, is up-regulated in a variety of tumors and it has been shown to influence tumor initiation and neoplastic risk.[11,15] MMP-3 is involved in the turnover of the ECM component. This enzyme degrades proteoglycans, collagen III, IV, V, and IX, laminin, fibronectin, gelatin, and elastin. Stromelysin-1 activates other members of the MMP family, such as MMP-1 and MMP-9.[15-17] A large number of polymorphisms have been reported in this gene, but the 5A/6A polymorphism in the promoter region has been most extensively investigated as this polymorphism increases the activity of the *MMP*-3 promoter.[16,18,19] The adenine insertion/deletion polymorphism (5A/6A) at position -1171 of the *MMP*-3 gene promoter influences transcription factor binding and *MMP*-3 promoter activity.[11,16,18]. *In vitro* assays of promoter activity showed that the 5A allele had 2- to 4-fold higher promoter activity than the 6A allele.[10,20] It has been reported that there is strong coloration between the *MMP*-3 polymorphism with breast, lung, and ovarian cancers.[21-23] There are some reports about correlation between 5A/5A polymorphism allele with progression and invasion activity of tumor cells, for example, in the breast cancer.[10,11,23]

The aim of this study was to determine whether 5A/6A polymorphism may be associated with initiation and development or/and progression and invasion of tumor cells of CRC in Iranian population.

MATERIALS AND METHODS

Subjects

This case-control study includes 120 cancer patients and 100 healthy controls. Median age of cases was 53 years (age range, 32–74 years) and controls were age-matched (± 3 years). This research is a retrospective case-control study. Cases were collected from a consecutive incident series with pathologically confirmation using colonoscopy and surgery; including 100 gender, age, and smoking status-matched healthy subjects without any diagnosis or history of cancer and any acute disease, were collected from Omid and Imam Khomeini hospitals, based in Isfahan and Tehran, respectively between 2009 and 2011. All the samples were collected from the patients with their permission. Detailed questionnaires, including clinical and family history, were initially collected. Patients were placed in two categories: with detectable metastasis position category (M^+) and without detectable metastasis position category (M^-). CRC patients consisted of 60 nonmetastasis patients (Stages 1, 2, and 3) and 60 metastasis patients (Stage 4). Healthy control subjects were basically collected from central laboratories in the hospitals (tumor-free volunteer). In the cases of smoking habit, complete information about the former and present smoking habits, the numbers of cigarettes/day and the time of starting and quitting were inquired from each subject. The definition of smoker was considered as described by other related publications. Nonsmokers are classified as people smoking less than 5 cigarettes/day; individuals who formerly or currently smoked 5 or more cigarettes/day for at least 2 years were defined as smokers.[11]

DNA extraction

Five milliliters of venous blood from each subject was drawn into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) and stored at 4°C for short study and -80°C for long study. Genomic deoxyribonucleic acid (DNA) was extracted using salting out method as published by Miller *et al*., with some modifications.[24]

MMP-3 SNP genotyping

The *MMP*-3 genotype was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The PCR primers were designed in previous study and checked by OLIGO 7 primer analysis software. A mutation from A to G at the second nucleotide close to the 3'end of the forward primer was made to create a *Psy*I recognition site in the case of the 5A allele (shown "g" in the forward primer sequences). Primers sequences are shown in Table 1. PCR reaction was performed in a 25 µl volume containing 100 ng of DNA template (genomic DNA), 2.5 µl of 10× PCR buffer, 200 mM $MgCl_2$, 1 unit of taq DNA polymerase (Cinnagen, Iran), 0.2 mM dNTPs, and 200 pM forward and reverse primers. The PCR cycling conditions were 3 min at 94°C followed by 30 cycles including: 30 s at 94°C, 30 s at 65°C, 30 s at 72°C, and final step at 72°C for 10 min to complete extension of all PCR fragments. A 5 µl PCR product was digested overnight at 37°C in a 15 µl reaction containing 5 units of *Psy*I enzyme (fermentase- Germany). After digestion, the digested fragments were subjected to electrophoresis on a 3% agarose gel stained with ethidium bromide. As expected, the 5A alleles would be represented by DNA bands of 97 and 32 bp (the small one could not be detected on the agarose gel electrophoresis) and the 6A alleles by a DNA band of 129 bp. For a negative control distilled water was used instead of DNA in the reaction system for each PCR.

Statistical analysis

Statistical analyses were performed using the SPSS 12 Software package. Hardy–Weinberg analyses were

Table 1: Forward and reverse primers sequences, the substitution mutation a nucleotide to G nucleotide was shown as "g" in the forward primer close to 3′ end

F	5′-GGTTCTCCATTCCTTTGATGGGGGGAAAgA-3′
R	5′-CTTCCTGGAATTCACATCACTGCCACCACT-3′

F = Forward primer; R = Reverse primer

performed to compare the observed and expected genotype frequency using the χ^2 test. The odd ratio (OR) and 95% confidence interval (95% CI) were calculated using an unconditional logistic regression model and adjusted for age and sex. They were also calculated as an index of the association of genotype and risk of CRC initiation and metastatic activity. All statistical tests were two-sided, and a *P* value of less than 0.05 was considered significant.

RESULTS

At the time of CRC diagnosis, patients were aged between 32 and 74 years, with a mean of 53 years, and controls were age-matched (±3 years). The *MMP*-3 genotype was successfully determined in 120 patients and 100 healthy controls [Figure 1]. There were no significant difference in the frequency of the *MMP*-3 genotype according to gender and age.

Of the 120 patients participate in this study, 11 subjects (9.17%) were homozygous for the 6A allele, 54 subjects (45%) were homozygous for the 5A allele, and 55 subjects (45.83%) were heterozygous. The frequency of allele 5A was 49% in controls and 67.91% in cases, but the frequency of allele 6A was 51% in controls and 32.09% in cases [Table 2]. As shown in the Table 2, the *MMP*-3 genotype and allelic distribution between CRC patients and healthy control were significantly different ($\chi^2 = 16.17$, $P = 0.0003$; $\chi^2 = 16.17$, $P = 0.00005$, respectively).

The 5A homozygote genotype was more frequent among patients than the healthy control groups [OR = 5.31, 95% CI, 2.28-12.38; $P = 0.000$; Table 3]. Therefore, 5A/5A genotype was used as reference and at this case 5A/6A genotype (individual who was carrying the 5A allele) showed significantly influence on the risk of CRC (OR = 2.04, 95% CI, 1.10–3.76).

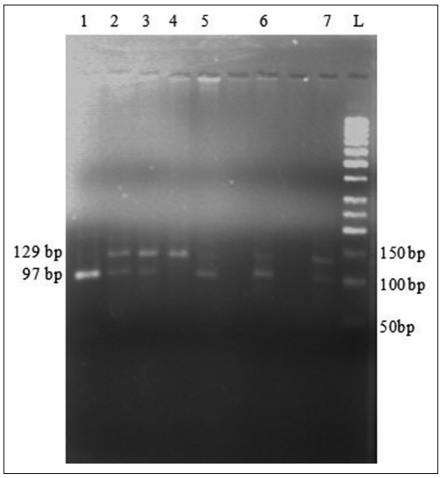

Figure 1: *Stromelysin-1* genotyping using PCR-RFLP method. 1 and 5, 5A/5A genotype. 2, 3, 6, and 7, 6A/5A genotype. 4, 6A/6A genotype. L, 50 bp DNA Ladder

By dividing patients into metastatic activity subgroup and nonmetastatic activity subgroup, as shown in Table 4, individual who was carrying the 5A allele was more frequent in metastasis subgroup than the healthy controls (OR = 3.86, 95% CI, 1.43-10.33; $P = 0.006$). However, no statistical differences were observed in nonmetastasis subgroup versus healthy controls ($\chi^2 = 0.009$, $P = 0.92$). Therefore, it was found that individual carrying the 5A allele with OR of 3.86 (95% CI, 1.43-10.33) compared with those who were 6A/6A genotype expose on median increased risk for metastatic activity of CRC.

According to our results (data not shown), it is demonstrated that 5A/5A genotype and even genotypes with at least one 5A allele, could have important effect on the age of onset of CRC initiation (62.33 vs 64.0, $P = 0.0001$). However, 5A homozygote individuals are spatially at risk for early onset of metastasis spread of CRC (69.42 vs 67.21, $P = 0.0011$).

Table 2: *Stromelysin-1* genotype and allelic frequencies at the patients and healthy controls

Groups	Control (%), *n* = 100	Patient (%), *n* = 120	*P*[a] value
Genotype			
5A/5A	24 (24)	54 (45)	
5A/6A	50 (50)	55 (45.83)	0.0003
6A/6A	26 (26)	11 (9.17)	
Allelotype			
5A	98 (49)	163 (67.91)	0.00005
6A	102 (51)	77 (32.09)	

The 5A/5A genotype and 5A allele frequencies are more frequent among patients versus healthy subjects (OR=5.318; 95% CI=2.285–12.358 and OR=2.203; 95% CI=1.496–3.245, respectively). [a]*Stromelysin*-1 genotype and allelic distribution are significantly different in CRC patients compared with healthy controls (χ^2=16.17, *P*=0.0003 and χ^2=16.17, *P*=0.00005, respectively)

Table 3: Association analysis of *stromelysin*-1 SNP with CRC patients compared with healthy controls

MMP-3	Genotype			OR (95% CI)	*P*[c] value
	6A/6A	5A/5A	5A/5A + 5A/6A		
Control	26 (26)	24 (24)	74 (74)	a: 5.31 (2.28-12.35)	0.000
Patient	11 (9.17)	54 (45)	109 (90.83)	b: 3.48 (1.63-7.38)	0.001

[a]the age adjusted OR of the 5A/5A genotype against the 6A/6A genotype, [b]the age adjusted OR of the 5A/5A + 5A/6A genotype against the 6A/6A genotype. [c]*P* value is calculated from χ^2 test. Parenthesis show % of samples

Table 4: Association analysis of *stromelysin-1* SNP with metastasis spread of CRC

MMP-3	Genotype		*P*[a] value
	6A/6A	5A/5A + 5A/6A	
Healthy control	26 (26)	74 (74)	
Metastasis	5 (8.33)	55 (91.67)	0.006
Nonmetastasis	16 (26.6)	44 (73.4)	0.92

Individual who is carrying the 5A allele (5A/5A +5A/6A) presents a stronger correlation with metastatic activity (OR=3.86; 95% CI=1.43–10.33). [a]*P* value is calculated from χ^2 test

DISCUSSION

Many studies show association of *MMP* polymorphism with initiation of cancer and especially with invasion risk and metastatic activity of different types of cancers such as breast, lung, and colorectal.[10,11,21] Our previous studies showed the relationship of *MMP*-1 polymorphism with invasion risk of CRC[8] and breast cancer in Iranian population.[13] We also demonstrated a strong association of *MMP*-3 polymorphism with tumor cell progression and metastasis of breast cancer in Iranian population.[12] The most of cancer deaths are resulting from tumor spread and metastasis, the process that is accompanied by degradation of components of the basal membrane and the ECM.[5] It has been demonstrated that those individuals carrying the 5A allele (5A/5A and 5A/6A genotypes) of *MMP*-3 associate with tumor cell progression and metastatic activity.[25] It has been suggested that the higher transcriptional activity of 5A allele may enhances tumor invasiveness.[10,26] Our present statistical analyses did not show strong association between the 5A allele and M^- CRC group by comparison M^- versus healthy control group at the time of diagnosis (OR = 0.96, 95% CI, 0.47–1.98). Our present results suggest that MMP-3 plays significant roles in spread and cell progression of CRC metastatic activity. Therefore, over expression of *MMP*-3 due to 5A promoter polymorphism may be a facilitating factor for CRC invasion and metastasis rather than initiation of CRC. The 5A allelic and 5A/5A genotypic frequencies in patient were 67.91% and 45%, respectively. The results showed that 5A/5A genotype and those individuals carrying the 5A allele were frequent in M^+ group and therefore, could be correlated to CRC invasion and metastasis ($\chi^2 = 7.49$, $P = 0.006$).

Present study demonstrated a weak association between *MMP*-3 promoter polymorphism and the initiation of CRC. But it demonstrated an important statistical association between those individual carrying the 5A allele and tumor cell progression and metastasis of CRC. Our results show that 5A allele may be a moderate risk factor for invasion/ progression and metastatic activity of CRC. Therefore, *MMP*-3 genotype determination could be a moderate genetic marker to identify prone for development of tumor cell progression and invasion of CRC. However, it is conceivable that detection of cases with high potential for development of invasion and metastatic activity and then prevention of their development are important to enhance the survival rate of CRC patients and to treat the cancer.

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How to cite this article: Bashi MM, Hojati Z, Hajihoseiny S, Hemmati S. The *stromelysin*-1 5A/5A genotype enhances colorectal cancer cell invasion in Iranian population. J Res Med Sci 2012;17:962-66.

Source of Support: Nil, **Conflict of Interest:** None declared.