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Association of MMP 9-1562 C/T Single Nucleotide Polymorphism with the Susceptibility to Lung Cancer Disease in South Iranian Population

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Authors' contributions

This work was carried out in collaboration between all authors. Author SH supervised the study, reviewed the protocol, wrote the manuscript for publication and edited the manuscript. Authors MJ and AP carried out the laboratory studies. Authors MM and AAK performed the statistical analysis and managed the literature searches. All authors approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The aim of this study was to investigate the association of MMP9 -1562 C /T single nucleotide polymorphism with lung cancer in south Iranian population. Matrix metalloproteinases (MMPs) are a family of highly conserved metal-dependent proteolytic enzymes that are able to degrade ECM components and regulate various cell behaviors. Among several candidate genes, MMP9 is one of the most important genes known to play a key role in relation to lung cancer initiation and progression. A common -1562(C/T) single nucleotide polymorphism in the promoter region of MMP9 was reported to have an association with lung cancer disease.
Study Design: A case - control study was carried out using 90 lung cancer patients and 100 healthy controls.

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Place and Duration of Study: Cellular and Molecular Gerash Research Center, Shiraz University of Medical sciences, shiraz, Iran, between 2010-2012.
Methodology: The association of MMP9 promoter single nucleotide polymorphism and lung cancer was examined in a PCR-RFLP method. Our results suggest an association between MMP9 single nucleotide polymorphism and lung cancer initiation and progression.
Conclusion: According to our results, the frequency of T allele in patient group (OR=4.6111, 95% CI, 1.9005 to 11.1879) and TT genotype in smoker group (OR=1.7726, 95% CI, 0.7947 to 3.9538) is higher compared to other groups.

Keywords: Matrix metalloproteinase; single nucleotide polymorphism; PCR-RFLP; lung cancer.

1. INTRODUCTION

The penetration and migration of cancer cells through the extracellular matrix (ECM) is included in the etiology of the tumorgenesis and metastasis processes [1]. Matrix metalloproteinases (MMPs) are a family of highly conserved metal-dependent proteolytic enzymes that are able to degrade ECM components and regulate various cell behaviors [1-4]. The MMP-9 gene (also known gelatinase B) is the only member of the metalloproteinase family that plays a role in degrading components of the basal membrane (BM) [1,5]. BM is the first vital barrier reached by tumor cells when they start invasion to other tissues. MMP9 plays several specific roles in progression and invasion of different types of cancers [6,7]. Though the regulation of MMP-9 expression is not completely known, there is evidence that its expression is primarily regulated at the transcription level, with the gene promoter responding to different growth factors and cytokines [8,9]. Furthermore, the promoter region of this gene shows variability. It is shown that a single nucleotide polymorphism (SNP) caused by cytosine (C) to thymine (T) base exchange at nucleotide position -1562 in the upstream of the MMP9 transcription initiation site (MMP9 -1562C/T) is associated with the transcription rate of this gene [10]. The C allele has been associated with low and the T allele has been associated with high promoter activity [11,12]. The upregulation of MMP9 in T allele carriers is associated with numerous cancers types including colon and breast cancers [9-12], so it was hypothesized that this polymorphism might act as a genetic marker in the development and progression of lung cancer. Finally, a case-control study was carried out to investigate the association of different MMP9 single nucleotide polymorphism with lung cancer disease in an Iranian population.

2. MATERIALS AND METHODS

2.1 Subjects

This case- control study included 100 healthy controls and 90 lung cancer patients. Both the patients with (stage III and IV) and without metastatic activity (stage I and II of disease) have been selected from Fars Cancer Research Institute as the cases of this study. Control subjects randomly selected among those visiting the hospital for regular health checkups. Information including their sex, age, smoking habit and family history was obtained from both groups. In case of smoking habit all information regarding the former and present smoking status, the number of cigarettes smoked per day and the time of starting and quitting of smoking were collected for each subject. Individuals who had formerly or currently smoked

five cigarettes/day for duration of at least 2 years were defined as smokers. The list of clinical and histological characteristics of lung cancer is represented in Table1. All individuals from patients and controls are agreed to take part in this study. Informed consent on the use of samples for analysis was obtained from all participants before entry and the study was approved by the local research ethics committee.

Table 1. Selected characteristics of MMP9 in lung cancer patients and healthy controls

Groups	Controls n=100	Patients n=90	%	p value
Sex				0.0
Male	35	60	66.7	
Female	65	20	22.2	
Miss*	-	10	11.1	
Available	100	80	88.9	
Smoking				0.0
Miss	-	58	64.4	
Available	100	32	35.6	
Smoker	29	29	32.3	
Non smoker	71	3	3.3	
Tumor type				0.0
Miss		4	4.4	
Available		86	95.6	
Small cell carcinoma		31	34.4	
Squamous cell carcinoma		33	36.7	
Adenocarcinoma		21	23.3	
Malignant		1	1.1	
Familial history				0.0
Miss	-	73	81.1	
Available	100	17	18.9	
Positive	13	1	1.1	
Negative	87	16	17.8	
Site				
Miss				0.36
Available		75	83.3	
Left		15	16.7	
Right		10	11.1	
		5	5.6	

** Miss (missing data): data not applicable, P value for χ2 test, 95%* accuracy

2.2 DNA Extraction

3 ml of venous blood from each subject was collected into an EDTA- containing vacutainer tube and stored at 4ºC for future analysis. Genomic DNA was extracted within 1 week since sampling using whole blood human DNA Isolation kit (Roche diagnostics, GmbH, Mannheim, Germany).

2.3 PCR-RFLP

To analyze the -1562C/T polymorphism, a part of MMP9 promoter sequence was amplified making use of MMP9- specific forward (5´-GCC TGG CAC ATA GTA GGC CC–3´) and reverse (5´-CTT CCT AGC CAG CCG GCA TC -3´) primers. Each PCR reaction was carried out in a total volume of 25 µl consisting of 100 ng of DNA template, 2.5 µl 10X PCR buffer, 0.4 µl MgCl2 (100 mM), 1U of Taq DNA polymerase (BIORON), 0.5 µl dNTPs (10mM) and 0.2 µl of forward and reverse primer (1mM). The PCR cycling condition was as follows: initial denaturation at 94ºC for 5 minutes followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 61ºC for 30 sec, extension at 72ºC for 30 sec and final extension at 72ºC for 5 minutes. For DNA digestion, 10 µl of the digestion reaction consisting of 5U of Sph1 (GCATG/C, BIORON), of 1X reaction buffer and 8 µl of PCR product was incubated overnight at 37ºC. While Sph1 enzyme is not effective on C allele, it generates two fragments of 202 bps and 258 bps acting on T allele. Therefore, for those with C allele Sph1 enzyme generates only fragments of 460 bps, while for those with T allele Sph1 produces two fragments of 202 bps and 258 bps. Digested fragments were separated on 2% agarose gel electrophoresis for duration of 60 minutes and subsequently visualized using a gel documentation unit (Fig. 1).

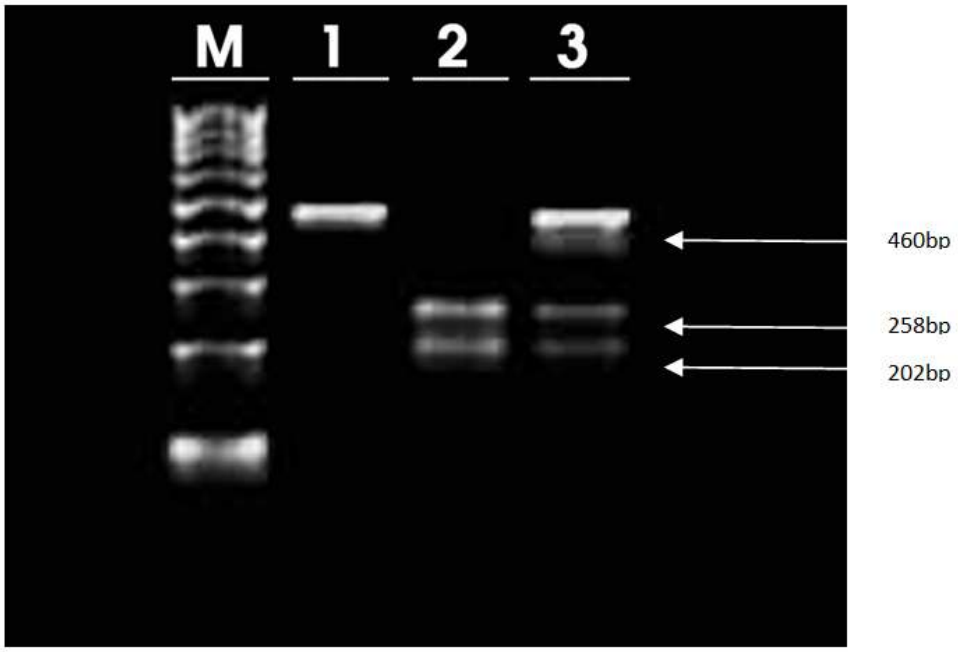

Fig. 1. 2% agarose gel electrophoresis of MMP-9 promoter PCR products. Lane 1. CC genotype, lane 2. TT genotype, lane3. TC genotype. M. 100bp ladder

2.4 Statistical Analysis

Statistical analysis was performed making use of SPSS12.0 software package. Hardy weinberg analysis was performed to compare the observed and expected genotype frequencies using $\chi 2$ test. Comparison of the MMP9 genotypes distribution in the study groups was carried out by means of two – sided contingency table in a $\chi 2$ test. The odds

ratios (OR) and 95% confidence intervals (CI) were calculated using an unconditional regression model and adjusted by age and sex. A probability level of 5% was considered as significant level.

3. RESULTS AND DISCUSSION

A total of 90 lung cancer patients and 100 healthy controls were considered as the cases of this study. Age distribution among the cases and controls was not statistically different. The mean age of the patients and controls were recorded as 64.9 years and 60.97 years, respectively. More than 66.7% of the patients and 35% of the controls were from men group (P= 0.0). Furthermore, more smokers were presented among the cases compared to the controls (35.5 versus 29%; P=0.0). The relevant characteristics of the study subjects are shown in the Table 1. Genotype and allelotype detection of MMP9 was carried out with PCR-RFLP (restriction fragments length polymorphism) technique and our results were validated by digestion repetitions and increased in number of case and controls. However the allelic frequencies of C and T alleles were reported 0.27 and 0.72 among the cases and 0.44 and 0.55 among the controls (Table 2). Comparing MMP9 genotype frequencies between the cases and controls showed that genotype distribution is significantly different between the cases and controls ($\chi 2$=13.00, P=0.001). Comparing the frequency of CC genotype between the patients and controls showed that CC genotype is a rare genotype among the patients (7.77% in patients vs. 27% in controls) so it is concluded that T allele may be the risk factor for lung cancer progression (OR=1.78, 95% CI, 1.0038 to 3.1831, Table 2). The proportion of smokers among patients was not significantly different from that of healthy controls (35.5 versus 29%; P=0.96). As the frequency of TT genotype in smokers, especially in men group was greater than other genotypes (53%), it is assumed that TT genotype may have increased the risk of developing lung cancer, compared to the CC genotype (OR=1.7726, 95% CI, 0.7947 to 3.9538). According to our results, the frequency of squamous cell carcinoma is greater than other type of cancers (36.7%).

Table 2. The genotype and allelotype frequencies of MMP9 among lung cancer patients and healthy controls

MMP9	Controls n= 100	Patients n=90 value	%	P
MMP9 genotype				
CC	28	7	7.8	
CT	33	35	38.9	0.001
TT	39	48	53.3	
MMP9 allelotype				
C	44.5	24.5	27.2	
T	55.5	75.5	72.8	

OR=1.78, 95% CI, 1.0038 to 3.1831.

Lung cancers, also known as bronchogenic carcinomas, are broadly classified into two types: small cell lung cancers (SCLC) and non-small cell lung cancers (NSCLC). This classification is based upon the microscopic appearance of the tumor cells themselves. These two types of cancers grow and spread in different ways and may have different treatment options, so a distinction between these two types is important. As a multistage

process disease, several genes and environmental factors affect cancer development [4,13-16]. The identification of patients at risk in the early stages is very important for the better inhibition of the cancer cells growth. NSCLC as the most common lung cancer, account for about 80% of all lung cancers. They are divided into three main types based upon the type of cells found in the tumor: adenocarcinoma, squamous cell carcinoma and large cell carcinoma. SCLC comprises about 20% of lung cancers and is the most aggressive and rapidly growing of all lung cancer types. SCLC is strongly related to cigarette smoking, with the prevalence of only 1% among nonsmokers. SCLC metastasizes rapidly to many sites within the body and is most often discovered after it has spread extensively.

But about the correlation between MMP9 and cancer, recent studies have been shown that MMP9 is a pro angiogenic factor and its expression induced in tumors especially second tumors. In tumors MMP-9 is produced by tumor-associated stromal cells and inflammatory cells, strongly implicating the host enzyme in tumor induced angiogenesis [17,18].

Several polymorphisms gene have been reported in the promoter of MMP9 gene but two of them are more functionally important than other [10]. These are a (CA)n microsatellite polymorphism at position from -90 and a single nucleotide polymorphism at position -1562 [10,19,20].

The more frequency of microsatellite polymorphism are (CA)14 allele and (CA)21, (CA)22 and (CA)23 alleles [20]. It has been shown that in oesophageal carcinoma cells, an MMP-9 promoter encompassing 14 CA repeats has only 50% of the transcriptional activity of an MMP-9 promoter containing 21 CA repeats [18]. Another polymorphism in MMP9 promoter is a single nucleotide polymorphism C to A substitution at position -1562 relations to transcription start site [10]. In vitro studies have shown that the C to T substitution results in the loss of binding of a nuclear protein to this region of the MMP-9 gene promoter, and an increase in transcriptional activity in macrophages [10]. A cohort study of Caucasian patients' detected that C-1562T polymorphism in MMP9 promoter is associated with severity of coronary atherosclerosis [10]. This association may be explained by enhanced ability of vascular smooth muscle cells to migrate and proliferate during atherogenesis in individuals carrying the T allele, because this allele have a higher transcriptional activity.

In the present study , we observed for first time that MMP9 -1562 C/T polymorphism have been correlated with lung cancer progression in south Iranian population especially Fars province. Our previous study did not show any correlation between MMP9 genotype and HNSCC initiation and progression [16]. However, the results of this study showed a strong association between TT genotype and lung cancer initiation and progression ($\chi 2 = 13.00$, P= 0.001). Our recent study on MMP9 -1562 C/T single nucleotide polymorphism showed a T allele-induced overexpression of MMP9 gene. Nowadays MMP9 overexpression and its proteolytic activity are known as potential diagnostic markers of several types of cancers [14, 21- 23]. In the present study, MMP9 genotyping was assessed in 90 lung cancer patients and 100 healthy controls. Investigating the correlation between MMP9 allele types and various factors affecting cancer initiation and progression showed that T allele can be a risk factor for lung cancer initiation and progression (OR= 4.6111, 95% CI, 1.9005 to 11.1879). The frequency of TT genotype was high in smoker lung cancer patients (OR =1.7726, 95% CI, 0.7947 to 3.9538). These results are consistent with the previous findings from other studies on different kinds of cancers including HNSCC [15]. It is assumed that the carcinogenic material in cigarette may be associated with MMP9 overexpression, target tissue cells transformation and increased risk of cancer [22-25]. As most of the smokers in Iranian population are from men group and smoking can affect lung cancer initiation and

progression, it is expected to have a higher ratio of lung cancer among the men group. Looking at our results on the association of TT genotype and gender type it is supposed that lower frequency of men among control group has affected the results of our study [25].

Distribution of MMP9 genotypes in all type of lung cancer were not much differentiated from each other. As we mentioned above that SCLC subtype of lung cancer were susceptible to cancer metastasis and malignancy, but more cases in our study are in NSCLC subtypes that more frequent than SCLC and cancer progression in this group is lighter.

4. CONCLUSION

Our result about association of T allele in MMP9 polymorphism with lung cancer initiation and progression were same as other studies results on other cancer, so we suppose this polymorphism could be as a biomarker for detection susceptibility to metastasis and malignancy. Nowadays researchers try to treatment cancer progression by blocking of MMPs enzymatic activity especially MMP9 that has important role in various process of cancer initiation and progression as tumor growth, angiogenesis and apoptosis. Therefore detection of MMP9 allelic form with high activity (T allele) in-1562 MMP9 promoter polymorphism could be important for cancer detection and treatment. Moreover additional clinical investigation should be done.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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