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ASSOCIATION OF MATRIX METALLOPROTEINASE 2 (MMP2) WITH ATHEROSCLEROSIS AMONG POPULATION OF LAHORE PAKISTAN

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Contribution

NM, AY conceived the idea, did study design, data analysis, manuscript writing .HH, MF, AB did data collection and analysis. NM did the final review. All authors contributed equally to the submitted manuscript.

All authors declare no conflict of interest.

ABSTRACT

Objective: To determine the role of single nucleotide polymorphisms (SNPs) in matrixmetalloproteinase 2 gene and their outcomes in development of atherosclerosis.

Methodology: In this case-control study two SNPs in MMP 2 gene rs 243865 (BfaI) and rs243866 (NlaIII) were studied in subjects with atherosclerotic disease from Punjab Institute of Cardiology, Lahore, Pakistan and healthy controls. Genomic DNA was isolated from each blood sample and targeted sequence was amplified by PCR. Restriction Fragment Length Polymorphism Analysis (RFLP-PCR) and direct sequencing were used for genotyping.

Results: Total 200 subjects with 100 patients and 100 healthy controls. The data showed that male gender, higher BMI, smoking, positive family history, middle age and other clinical risk factors were significantly associated with the atherosclerosis. As a result of genotyping it was observed that allele and genotype frequency of rs243865 (BfaI) polymorphism differed significantly between atherosclerotic patients and controls. Significant association of A allele of rs243866 (NlaIII) polymorphism was found to be associated with atherosclerosis. However, there was no difference in genotype frequency between atherosclerotic patients and controls. Haplotype analysis indicated that CA, CG and TA haplotypes can be significantly connected with the atherosclerosis (p < 0.01). Whereas, haplotype TG frequency was higher in control individuals so it may act as a protective factor against the atherosclerosis development.

Conclusion: MMP2 gene polymorphism can be associated with development of atherosclerosis in Pakistani population.

Key Words: Atherosclerosis, MMP2, Single nucleotide polymorphism, Risk factors, PCR-RFLP.

INTRODUCTION

Atherosclerosis is an inflammatory process that starts from the initiation to the advancement of plaque which is made up of lipid molecules, calcium, cholesterol and other such materials that are found in blood.[1,2] Atherosclerosis plaque is classified into stable and vulnerable plaques depending upon the collagen and smooth muscle cells content of blood vessels and thickness of their cap. Collagen and smooth muscle cells makes up the stable atherosclerotic plaque. On the other hand, a large lipid core having a thin fibrous cap covering with dense inflammatory infiltrates (monocytes/macrophages, T lymphocytes, etc) characterizes the vulnerable plaque that is more prone to get ruptured and cause atherosclerosis.[3] Extracellular matrix remodeling is important for plaque development and instability.

Matrix metalloproteinase 2 gene has a position at the chromosome 16 at q31-21. MMP2 also gelatinase-A is a fundamental enzyme of the communication between MMPs and other proteases. It is capable of cleaving collagen. MMP2 is shown to be involved in all the stages of atherosclerosis, from the initiation of lesion formation to the plaque rupture. Modification in gene expression by binding sites occurs if MMPs have polymorphism in the corresponding promoters. This result in the lower transcriptional activity, thus these polymorphisms have dual role in disease.[4] It is suggested that -1306 C → T and -1575G → A transition might down regulate the expression of MMP2 gene. In this study, we investigated that if the SNPs are making our population at risk of atherosclerosis.

METHODOLOGY

This case control study was conducted at Punjab Institute of Cardiology, Lahore from 1st January to 31st July 2017. Atherosclerosis patients were contacted for their consent to participate in the study. Patients were clinically diagnosed with the disease by the physician according to WHO criteria. The participants were divided into two groups, Group I was considered control (healthy and with negative family history of atherosclerosis) and group II consisted of cases of atherosclerosis. The ethical approval for this study was obtained from Advance Studies Research Board, GC University, Lahore, Pakistan and Punjab Institute of Cardiology, Lahore, Pakistan. The participants of the study were investigated for environmental and demographic data like age, BMI and disease management etc.

Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) analysis was done by collecting blood sample with 5cc syringes in EDTA coated tubes and extraction of genomic DNA was performed by organic extraction method as described by Sambrook.[5] The presence of DNA in extracted blood samples was confirmed by the gel electrophoresis and Nanodrop. Two SNPs of MMP2 for atherosclerosis were selected on the basis of their role in causing coronary ischemic heart disease using NCBI. The sequence of primers and reaction conditions for the amplification of these SNPs is given in table 1.

In order to determine the selected mutations in the MMP2 region, all PCR products were subjected to restriction fragment length polymorphism analysis (RFLP) and sequencing. The sequencing results were visualized in BioEdit software and analyzed for the presence of SNPs using the BLAST and ClustalW2 tools.

Demographic data was presented using percentage and to evaluate the significance, t-test was applied. All data for polymorphism studies was passed from the Hardy- Weinberg equilibrium. SHEsis6 – a web-based platform for analyses of linkage disequilibrium, haplotype construction, and genetic associations at polymorphism loci-was used to carry out the generic data of SNP's. Haplotypes were evaluated7 to study their association with atherosclerosis. Chi-square test was used to calculate the gene and allele frequencies.

RESULTS

Total of 200 participants with 100 in each group that is case and control were included. According to the demographic data, it was observed that males (70%) were at higher risk of disease development as compared to females (30). Middle age (59.2± 10.2; p< 0.01) and elevated BMI (23.38 ± 3.42; p< 0.01) as well as smoking, positive family history and other clinical risk factors like hypertension, higher cholesterol level, and diabetes are significantly associated with the atherosclerosis.

The genetic polymorphism association of atherosclerosis is summarized in table 2.

The allelic and genomic frequencies are summarized in the table 3.

Table 1: Primer sequences, Annealing Temperatures and RFLP Enzymes for rs243865 and rs243865.

SNP	Primer sequence	Annealing temperature	RFLP enzyme
rs243865 -735 C > T	F:CTTCCTAGGCTGGTCCTTACTGA R: AGCTCTTCAGGTCTCAG	64°C	Bfal
rs243866 -1575G > A	F: GTCTGAAGCCCACTGAGACC R:CTAGGAAGGGGGCAGATAGG	62°C	NlaIII

Table 2: Single Site Analysis of Genetic Variants in Control and Atherosclerosis Patients (n=200)

Gene	SNP ID	Allele I (P/C)	Allele II (P/C)	*HWE P	Fisher P values
MMP2	rs 243865 C > T	C 55/0	T 49/100	1.0	0.001*
	rs 243866 G > A	G 99/100	A 7/0	1.0	0.004*

P: Patients; C: control; *HWE- Hardy Weinberg Equilibrium (HWE > 0.05); Fisher P-value with * are significant

Table 3: Allelic and Genotypic Frequencies in Case and Controls

SNP ID.	Case/Control					P-Value
	Maf	MAF	MM	mm	Mm	
rs243865	0.529/0.00	0.471/1.00	0.077/0.00 (CC)	0.019/1.00 (TT)	0.904/0.00 (CT)	0.001*
rs243866	0.066/0.00	0.934/1.00	0.057/0.00 (AA)	0.925/1.00 (GG)	0.019/0.00 (AG)	0.095738

MAF = Major Allele Frequency; maf = minor allele frequency; MM= Homozygous dominant; mm= Homozygous recessive; Mm; Heterozygous; *= Significant ρ value.

Hardy-Weinberg equilibrium was estimated for control samples and is included in this study. Out of two SNP's, rs243866 was found to be associated with atherosclerosis on allelic level in Pakistani population but not on genomic level.

Haplotype analysis was performed for selected SNPs (table 4).

Table 4: Haplotype Analysis for MMP2 Gene (Chromosome 16)

Haplotype Bfal-NlaIII	Case (Frequency)	Control (Frequency)	P-Value
T G**	32.64(0.481)	1.000	0.0001
C A*	4.64(0.061)	0.000	0.0001
C G*	36.36(0.478)	0.000	0.0001
T A	2.36(0.031)	0.000	0.052054

** represents the protective Haplotype against Atherosclerosis, *represents the susceptible haplotypes to Atherosclerosis

It was observed that haplotype CA, TA and TG of MMP2 gene (rs243865 and 243866) were strongly associated with atherosclerosis (p < 0.01). However, the frequency of CG was higher in controls than atherosclerotic patients so it was protective against atherosclerosis. Linkage equilibrium between rs243865 and rs243866 in both cases and controls showed that they are not significantly associated with the atherosclerosis as shown in Figure 1.

Figure 1: Linkage Disequilibrium of rs243865 and rs243866 in Atherosclerotic Patients (a) and Controls (b).
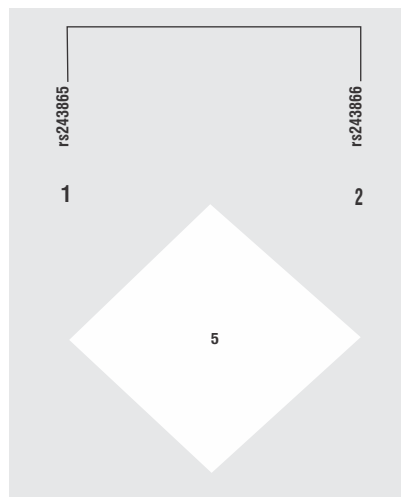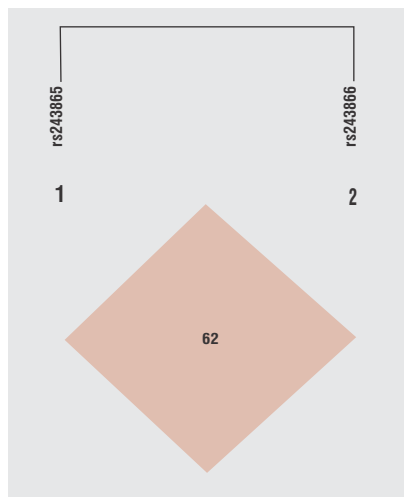
DISCUSSION

MMP-2 gene possesses polymorphisms that have been identified and their association with the atherosclerosis has been reported.[8] In MMP-2 gene, polymorphisms at -1575 G>A and -1306 C>T tend to upset the adherence of estrogen receptor and the Sp1 transcription factor respectively and thus alter MMP2 gene function.[9,10] As MMP2 SNPs rs243865 and rs243866 have vital role in the rupturing of the plaque that eventually cause atherosclerosis, the present study was designed in order to identify MMP2 SNPs common in Pakistani population.

The absolute trends of mortality by cardiovascular disease are getting higher since 1990. Growth and aging of the population as well as the changes in lifestyles and food system in low and middle income countries cause increase mortality from atherosclerosis.[11] A community based prevalence study was carried out in Karachi, Pakistan and this was the first study that demonstrated alarming prevalence of cardiovascular disease in Pakistan.[12]

Cardiovascular disease progresses 7.5 years to 10 years earlier in men than women; however, it is the most important reason of death in women with the age more than 65 years. There is a mis perception that females are secured against the cardiovascular disease because according to current data by the National Health and Nutrition Examination Surveys, it is indicated that in the past two decades, the prevalence of myocardial infarction has amplified in midlife (35 to 54 years) women, while declining in similarly aged

men.[13] As one gets older, enough plaque has been built up inside the arteries that represent the increased risk of atherosclerosis. Atherosclerosis development due to age factor also depends on the genetic and life style factors. This danger usually increases after age of 55 in men while in women; the risk intensifies after the age of 45. In our study, both males and female patients of older age had more disease prevalence. In a perspective study in Italy, degree of coronary atherosclerosis and cardiac events due to impact of body mass index in a cohort of patients at risk of coronary artery disease was examined. This study concluded that higher prevalence of atherosclerosis was not significantly associated with increased BMI but there was a great deal of association of hypertension, hypercholesterolemia and diabetes mellitus with greater magnitude of coronary atherosclerosis.[14]

Along with high cholesterol, increased blood pressure and diabetes, smoking is also considered to cause the substantial increase in the risk of atherosclerosis. Tobacco and its products accelerate the atherosclerosis in coordination with the other risk factors. Thrombosis rapidly occurs when plasma fibrinogen levels get elevated by smoking.[15,16] It has also been demonstrated that the chance of myocardial infarction got decrease after cessation of smoking.[17] A Canadian Coronary Atherosclerosis Intervention Trail (CCAIT) study was done in 1996 in which it was demonstrated that atherosclerosis tend to progress more rapidly in smokers than the nonsmokers.[18] The pattern of coronary atherosclerosis was seen in smokers than nonsmokers in Pakistani population and concluded that smokers tend to have significantly high multi-vessel disease and even have coronary heart disease in the absence of other risk factors.[19] In current study, 58% atherosclerotic males and 13% atherosclerotic females were smokers as compared to 20% male and 5% female controls. Parental history with cardiovascular disease is one of the key factors that are responsible for atherosclerosis. It is not only an independent risk factor but also has a synergistic effect with other cardiovascular risk factor.[20]

MMP2 gene processes various components of extra cellular matrix and cell surface proteins. If its expression get altered, then it can be implicated in various pathological processes. Promoter polymorphisms of MMP2 gene have significantly been associated with the atherosclerosis.[21] Our study demonstrated that single nucleotide polymorphism at rs243865 (1306C>T) was associated with the coronary heart disease. The C allele and CT genotype of rs243865 (BfaI polymorphism) were significantly associated with the atherosclerosis. In contrast to present study, a study was conducted in China and found no association of polymorphism -1306C>T of MMP-2 gene with systolic heart failure prognosis.[22] A clinical study was conducted in which it was concluded that although there was no significant association between 1306C>T and atherosclerosis, the more active C allele was frequently present in the cases than in controls.[23] Similar to current findings, Volcik determined the association of MMP2 genetic variation with the measures of fibrous cap thickness and concluded that C-1306T variant genotypes (CT+TT) were significantly associated with higher cap thickness measures, but not with wall thickness or lipid core.[24] Also C transition to T at 1306 was functional and that C allele was involved in higher promoter activity than T allele.[25]

A significant allelic association at rs243866 (-1575G>A) polymorphic site (NlaIII) was observed in our study whereas no significant difference was observed at genotypic level. In contrast to current finding, a study conducted in Mexican population demonstrated an increase frequency of A allele and AA genotype in

patients that suffered myocardial infarction than control ones. According to the dominant model, individuals which have AA or AG genotype had a 1.65 fold increase risk of having atherosclerosis.[25] Haplotype analysis in the current studies indicates that CA, CG and TA haplotypes are significantly associated with the atherosclerosis (p< 0.01). However, haplotype TG frequency is higher in control individuals than the atherosclerotic patients so it acts as a protective factor against the atherosclerosis. Current study demonstrated that rs243865 and rs243866 had linkage disequilibrium with the onset of atherosclerosis.

CONCLUSION

Pakistan is a multicultural country with certain traditions one of which is intra cast or cousin marriages. Due to narrowing of gene pool with fewer genetic recombinations, genetic disorders were frequently transferring from one generation to next generation. It was concluded that male gender, BMI, high cholesterol level, high blood pressure, Angina pectoris, myocardial infarction, diabetes mellitus, smoking and family history were significantly associated with disease onset. On the other hand, polymorphism at rs243865 was significantly associated with disease onset in studied population, whereas rs243866 was significantly associated with disease onset at allelic level but not at genotypic level. However, a large population size must be needed to evaluate the significance of both polymorphisms with the disease.

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