Proceedings S.Z.M.C. Vol: 36(3): pp. 01-12, 2022. PSZMC-845-36-3-2022

Family Based Genetic Association Study of *TLR4* Gene with Myocardial Infarction in Pakistan

[1]Riffat Iqbal, [1]Muhammad Shehzad, [2]Shaista Aslam, [3]Faiza Gul Durrani, [4]Kiran Aftab, [1]Iram Liaqat

[1]*Department of Zoology, Government College University, Lahore.*
[2]*Department of Biological Sciences, National University of Medical Sciences, Rawalpindi.*
[3]*Director, Science Shines Ltd. London, U.K.*
[4]*Department of Zoology, University of Gujrat.*

ABSTRACT

Introduction: TLR4-dependent pro-inflammatory signaling has been involved in the initiation, progression, and plaque destabilization phases of atherosclerosis which is the leading cause of MI.
Aims & Objectives: To determine primarily the association of *TLR4 gene* with myocardial infarction through family clustering analysis as well as a case control association study. Secondarily to evaluate the environmental factors responsible for onset of MI (myocardial infarction) in Pakistani population.
Place and duration of study: Current study was conducted at the Department of Zoology Government College University Lahore between 2019 and 2020.
Material & Methods: Demographic information and blood samples were collected from various families with at least one or two family members affected with MI. DNA was isolated and targeted sequence was amplified by primer specific PCR reaction. Genotyping was achieved by Sanger sequencing.
Results: The family clustering study found TT genotype of rs4986791 to be significantly associated with MI while other SNP (Asp299Gly; rs4986790) did not show any association with MI. In the case control association study, allele T was seen as a risk allele with significant association ($p<0.05$) of variants 4986791 to MI. Moreover, allele of rs 4986790 was found to be protective allele for participants. Smoking, air pollution, BMI, diabetes, positive family history and hypertension were found strongly responsible for development of MI.
Conclusion: Results of this study indicate that SNP 1196C/T (Thr399Ile; rs4986791) in TLR4 gene is strongly associated with MI and other SNP rs4986790 was not associated with MI onset in a Pakistani family.

Key words: Myocardial infarction, Family clustering, case control association study, TLR (Toll Like Receptor) 4 gene

INTRODUCTION

Myocardial infarction (MI) results in severe damage to the myocardium due to prolonged ischemia when coronary arteries are obstructed and blood supply to heart becomes compromised. This blockade is usually triggered by a thrombus in a coronary artery or the buildup of plaque in the artery walls.[1] In Asian countries including Pakistan, the individuals with age range of 40-45 years are more susceptible to MI than individuals with age of 60 years.[2] Various epidemiological studies have demonstrated that risk factors predominantly hypertension, smoking, alcohol consumption, obesity, diabetes, age and air pollution are involved in increase of MI .[3,4] In recent times, many investigations also have provided proof that genetic polymorphisms play a key role in MI development.[5] Polymorphisms in-genes encoding APOA5, ApoB, ApoE, PAI1, SLC6A4, IL6, PON1, F13A1, TNF, KIF6, COL3A1, CX3CR1, LPA, PAI1, NOS3, LPL, PLAT, CDKN2A/B, COMT, HMOX1, LGALS2, HNRPUL1, GP6, PPARG, CCR2, CCR5, CASR, PSRC1, ANRIL, NUTF2, CETP, AGT, ACE, LDLR, LTAgene, TLR4, MMP-3, MMP-9 and other genes have been linked with high hazard of atherosclerosis, CAD, and MI in certain populations.[6,7,8,9] TLR4 is well-known as an facilitator of adaptive and innate immunity.[10] Additionally it is an important mediator of MI due to its underlying role in inflammation.[11]

TLR4-dependent pro-inflammatory signalling has been involved in the initiation, advancement, and plaque de-stabilization phases of atherosclerosis which is the leading cause of MI.[12] By utilizing SNPs as molecular markers, some attempts were made to show relationship of TLR4 gene with

atherosclerosis and related consequences such as MI.[13,14] It was reported that early-onset MI showed familial clustering.[15] Family background of MI was studied to assess the inheritability of CHD and the effect of environmental factors on the onset of disease[16,17] being an indicator of increased cardio vascular risk in healthy persons.[18] Evidence therefore pointed towards the genetic and familial predisposition of MI in the Pakistani population as well. The reason behind this might have been the greater number of consanguineous marriages in Pakistani population. We assumed that if the allele transfer from a common ancestor was the risk allele, it would increase the risk of disease onset. It has also been observed that if parents share a common ancestor, they have higher chance of getting disease. Once they passed their common allele to their offspring, their children would be at greater risk of inheriting MI. The core goal of this study was to check the association of TLR4 gene with MI through family clustering analysis as well as case control association study and to evaluate the environmental factors responsible for emergence of MI in Pakistani population.

MATERIAL AND METHODS

Ethical approval: The current study received ethical approval from Board of Studies Department of Zoology and Ethical Committee vide (No. GCU-IIB-1085) Govt. College University Lahore. Information related to MI was asked in a Proforma duly signed by each Patient/ Guardian of the family for sampling purpose. The participant's age ranged from 12 to 75 years.

Sampling: Our populations of interest were five families living in Pakistan (Lahore, Khanewal, Punjab, Pakistan) with at least one or two members who had suffered an MI and received medical treatment. Family members of MI patients who were seriously ill or bedridden and with history of any other medical complication were excluded. Total five families consisting of 38 individuals were recruited in current study. Among them 19 were females (2 diseased) and 19 were male (4 diseased) that participated in this study. One to two generation pedigree was sketched to show the family history of MI and sanguinity among parents. Consanguinity was defined as marriage with first cousin relative. Most of the study participants were not aware about their grandparents' status of MI and these were excluded from study. Inclusion criteria were patients with MI as principal diagnosis and admission via CCU and emergency wards. A questionnaire was designed to collect data. The blood samples were collected from all participants of this study. The information of all contributors included family history, medical history, weight height, age, gender, and clinical findings for MI such as BMI were noted on Proforma. Summarized methodologies are shown in Fig-1.

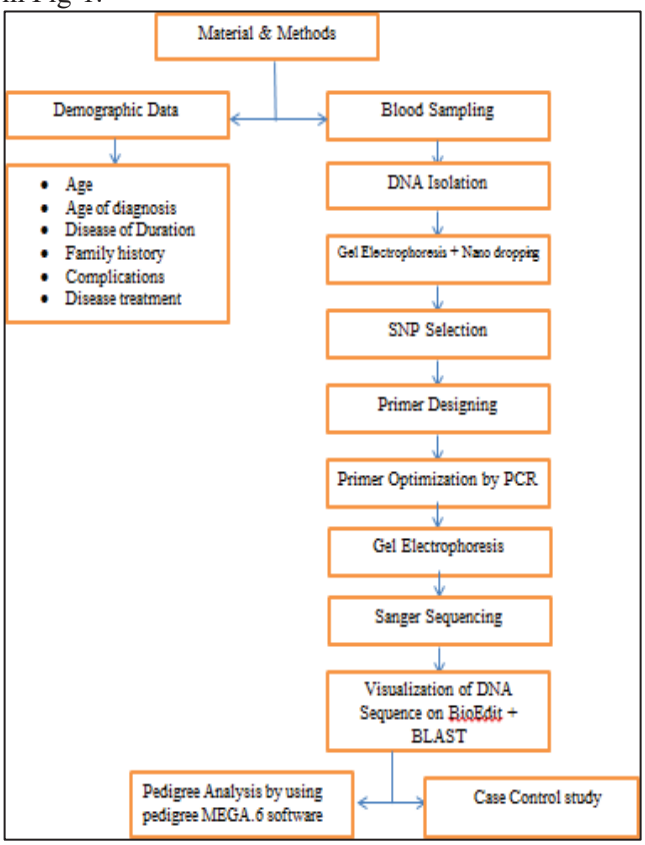

Fig-1: Schematic representation of research methodology

DNA Extraction and Nano-dropping:

DNA isolation from each sample was done by Chloroform-Isoamyl alcohol (24:1) method that consists of two days protocol. For this purpose, Blood (500µl) and TAE buffer (500µl) was taken in an eppendorf and mixed. Each of these eppendorfs were left for 10-15 minutes at room temperature and were centrifuged for 10 min at 13500 rpm at 4˚C. The Supernatant was disposed and the pellet was broken by vertexing. TE buffer 1000µl was added, and vortexed, it was centrifuged for 10 minutes at 13500rpm at 4˚C. This step was repeated 4-5 times, until the color of pellet became light pink/cream white. The pellet was broken by vortexing and 25µl of 10% SDS, 35µl of Sodium acetate solution and 5-10µl of proteinase K was added in each sample. The mixture was then re-vortexed and incubated at 37˚C for 24 hours. After incubation, 165µl of isoamyl alcohol and chloroform mixture (24:1) were added and centrifuged for 10 minutes at 13500rpm.Three layers were formed in which the upper clear layer

was taken in another eppendorf and 700 µl of iso propanol was added and vortexed. The eppendorfs were left for 15-20 min at room temperature and centrifuged for 10 min at 13500rpm. After centrifugation supernatant was discarded and 1000µl of 70% ethanol was added to it.

The eppendorfs were centrifuged, and supernatant was discarded. DNA pellet were dried for at least 30-60 min until the ethanol smell disappeared. 50µl DEPC H20 was added to the dried out pellets of DNA, and then incubated in water bath for 5 minutes. The pellets were cooled and finally stored at -20°C or 80°C.

After isolation, the extracted genomic DNA was checked by loading samples on 0.8% Agarose gel. For this purpose, 0.8g of agarose was dissolved in 100ml of 1XTAE buffer. The mixture was heated and 5-7µl of ethidium bromide was added. After this, the whole mixture was decanted in casting tray of gel. Sample was prepared by mixing 3µl with DNA sample in wells. After loading, the gel was run at 110V for forty to forty-five minutes. The gel was visualized on Trans illuminator (Model UVGL-25). Nano-dropping was done for quantification (concentration) and qualification (purity) of all the DNA samples.

Designing of primers and PCR: Selection of SNPs was based on the reported genetics associations with MI from Hap-Map Data Bank and National Center for Biotechnology Information (NCBI) (http:// www.ncbi.nlm.nih.gov) The SNPs (rs4986790, rs4986791) were selected from the gene which is involved in the initiation, progression, and plaque destabilization stages of atherosclerosis, the underlying cause of myocardial infarction (MI). For Polymerase chain reaction (PCR), all primers were rechecked with sequence taken from NCBI website https://www.ncbi.nlm.nih.gov/-for their validity.

Primers were optimized by PCR in which different ranges of temperature (From 54.5 to 57°C) were set for both SNPs according to primer literature. After PCR results, it was observed that annealing temperature of rs4986790 was 55°C while 55.5°C for rs4986791.

All primers (forward and reverse) along with SNP number (rs4986790, rs4986791), product size, and annealing temperature were shown in supplementary Table-S1. The PCR product was consisted of 12.5µl of reaction mixture having 1.5µl genomic DNA, 5µl master mix, 0.75µl of each primer, and 4.5µl of DEPC water. The conditions for PCR were set as: initial denaturation (94ºC for five mins), then thirty-five cycles with succeeding temperature. Profile: denaturation (94ºC for 30 secs), annealing (rs4986790, rs4986791: 55ºC and 55.5ºC respectively for forty-five seconds), extension (72ºC for 45 secs), and final elongation for 10 min. Agarose gel (1.2%) was used for confirming PCR products. The gel was run on 110V for 40-45 mins, and then it was observed on bench top UV Trans-illuminator. Each PCR products were purified by manual method. For this purpose, 20µl of each sample was mixed with 80µl of 100% chilled ethanol. The eppendorfs were placed in dark for 40-45 minutes and centrifuged for 25 minutes at 13500rpm. Supernatant was cast-off and pellets were dried. DEPC water (2µl) was added in each eppendorf. The PCR products were sequenced after purification at Lab Genetic. The sequences were visualized on Bio-Edit software and were Blast on NCBI and UCSC to determine the mutation.

rs4986790

AGAATTTAGAAATGAAGGAAACTTGGAAAA
GTTTGACAAATCTGCTCTAGAGGGCCTGTGC
AATTTGACCATTGAAGAATTCCGATTAGCAT
ACTTAGACTACTACCTCGAT**D**TATTATTGAC
TTATTTAATTTTTGACAAATGTTTCTTCATTT
TCCCTGGTGAGTGTGACTATTGAAAGGGTA
AAAGACTTTTCTTATAATTTCGG

rs4986791

ACAAAGGTGGGAATGCTTTTTCAGAAGTTG
ATCTACCAAGCCTTGAGTTTCTAGATCTCAG
TAGAAATGGCTTGAGTTTCAAAGGTTGCTGT
TCTCAAAGTGATTTTGGGACAA**Y**CAGCCTA
AAGTATTTAATCTGAGCTTCAATGGTGTTAT
TACCATGAGTTCAAACTTCTTGGGCTTAGAA
CAACTAGA

SNP ID	Product Size	Tm (°C)	Primer Pairs
rs 4986790	96	55	F.P;TTGAAGAATTCC GATTAGCATACTTA G,R.P;TCACCAGGGA AAATGAAGAAACA T
rs 4986791	99	55.5	F.P;CAAAGGTTGCT GTTCTCAAAGTGAT R.P;CCCAAGAAGTT TGAACTCATGGTAA T

Table-S1: Primers along with SNP number, product size annealing temperature.

Statistical analysis:

Frequency of allele and genotype were evaluated by SHEsis software (http://analysis.bio-x.cn/ SHE sis Main.htm). Pedigree analysis was done by online Pedigree progeny software.

RESULTS

Blood samples of 5 MI families (Heart family 1 to Heart family 5) were collected from Khanewal and Lahore area, Punjab, Pakistan. Among 38

individuals of the MI disease families, there were 19 females (2-diseased) and 19 males (4-diseased) participated in this research. It was observed that a few members of selected families were with high BMI. The pro-band (diseased) mean age was 51.4 years while mean body mass index of the pro-band was 26.4 kg/m^2. The physical and clinical characteristics of the selected families are shown in supplementary Table-S2(a). Few members were having history of smoking and alcohol consumption as shown in supplementary Table-S2(b).

Sr. No	Code	Mas-culine	Age (years)	BMI (kg/m^2)	Results	Diagnosis
Family=HF1(Heart Family 1)						
1	F1_M1	Male	56	30.5	Obese	Sick
2	F1_M2	Female	53	39	Obese	Normal
3	F1_M3	Male	21	23.9	Normal weight	Normal
4	F1_M4	Male	14	23.2	Normal weight	Normal
5	F1_M5	Male	24	31.14	Obese	Normal
6	F1_M6	Female	31	28.9	Overweight	Normal
7	F1_M7	Male	37	31.4	Obese	Normal
8	F1_M8	Female	12	35.9	Obese	Normal
Family=HF2 (Heart Family 2)						
9	F2_M1	female	77	29.4	Overweight	Normal
10	F2_M2	Male	55	19	Normal weight	Sick
11	F2_M3	Male	53	25.9	Overweight	Sick
12	F2_M4	female	46	23.1	Normal weight	Normal
13	F2_M5	female	43	27.6	Overweight	Normal
14	F2_M6	female	13	21.5	Normal weight	Normal
15	F2_M7	Male	16	22	Normal weight	Normal
16	F2_M8	Male	35	24.2	Normal weight	Normal
17	F2_M9	female	37	23.7	Normal weight	Normal
18	F2_M10	female	25	21	Normal weight	Normal
19	F2_M11	female	23	19.4	Normal weight	Normal
Family=HF3(Heart Family 3)						
20	F3_M1	Male	61	30.3	Obese	Normal
21	F3_M2	female	47	26.2	Overweight	Sick
22	F3_M3	Male	26	28.4	Overweight	Normal
23	F3_M4	Male	18	33.2	Obese	Normal
24	F3_M5	Male	28	33	Obese	Normal
25	F3_M6	female	29	18.3	Normal weight	Normal
26	F3_M7	female	10	16.6	Under weight	Normal
27	F3_M8	female	8	13.5	Under weight	Normal
Family=HF4 (Heart Family 4)						
28	F4_M1	female	72	33.6	Obese	Normal
29	F4_M2	Male	45	25.7	Overweight	Normal
30	F4_M3	Male	35	30.4	Obese	Normal
31	F4_M4	female	41	33.6	Obese	Sick
32	F4_M5	Male	61	14.9	Under weight	Normal
33	F4_M6	female	18	21.5	Normal weight	Normal
Family=HF5 (Heart Family 5)						
34	F5_M1	Male	56	23.21	Normal weight	Sick
35	F5_M2	female	43	22.4	Normal weight	Normal
36	F5_M3	female	25	19.4	Normal weight	Normal
37	F5_M4	Male	36	21.53	Normal weight	Normal
38	F5_M5	Male	6	27.5	Overweight	Normal

Table-S2(a): The clinical physical characteristics of selected families of MI.

F=Family, M=member, Heart=H

Sr. No	Code	Environmental Factors			Other Factors		
		Smoker	Alcohol consum-ption	Pollu tion	Hyper-tension	Dia-betes	Hyper-lipidemia
1	F1MI	-	-	*	*	*	-
2	F2M2	*	*	*	*		-
3	F2M3	*	*	*	*	*	-
4	F3M2	-	-	*	-	*	*
5	F4M4	-	-	*	*	-	-
6	F5M1	*	-	*	*	*	*

Table-S2(b): Association of Risk factors with selected families of M1.

*=Association,
=No.association,
F=Family, M=member,
HF= Heart family

DNA Isolation

DNA isolation was done by manual method (phenol, chloroform method) and was affirmed by run on 0.8% agarose gel electrophoresis shownin Fig-2. The quality and quantity of each DNA samples was measured by Nano-dropping and its values were shown in supplementary Table-S3.

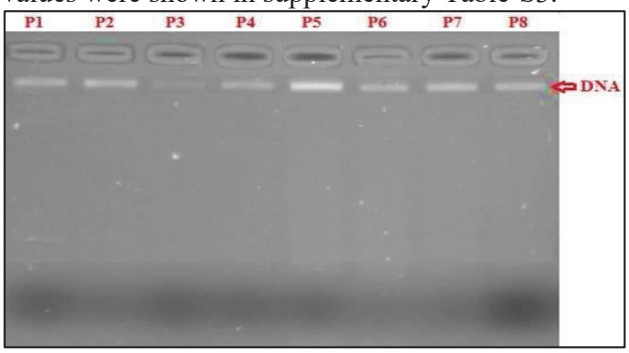

Fig-2: DNA isolated from blood sample of one selected family.

Sr. No	Code	Purity	Concentration ng/ul
1	F1M1	1.7	93.4
2	F1M2	1.75	75
3	F1M3	1.78	33.6
4	F1M4	1.8	175.4
5	F1M5	1.8	105
6	F1M6	1.8	527
7	F1M7	1.8	59.7
8	F1M8	1.75	169
9	F2M1	1.78	85.3
10	F2M2	1.8	1356.5
11	F2M3	1.7	165.8
12	F2M4	1.7	39.3
13	F2M5	1.75	89.00
14	F2M6	1.9	41.6
15	F2M7	1.7	104.3
16	F2M8	1.84	717.6
17	F2M9	1.87	84.2
18	F2M10	1.78	155.5
19	F2M11	1.8	109.5
20	F3M1	1.78	426.1
21	F3M2	1.75	59.8
22	F3M3	1.8	134
23	F3M4	1.85	105.6
24	F3M5	1.7	197
25	F3M6	1.8	88
26	F3M7	1.8	107
27	F3M8	1.8	201
28	F4M1	1.7	191.7
29	F4M2	1.82	84.1
30	F4M3	1.69	780.1
31	F4M4	1.81	245.6
32	F4M5	1.8	55.8
33	F4M6	1.77	166.8
34	F5M1	1.7	272.9
35	F5M2	1.85	72.4
36	F5M3	1.75	52.8
37	F5M4	1.8	1368.2
38	F5M5	1.78	253.5

Table-S3: DNA quantification and qualification of selected MI families

Polymerase Chain Reaction

After primer optimization, amplification of PCR was done for SNPs (rs4986790 and rs4986791)

Fig-3. The PCR product of 96 bp and 99 bp of both SNPs rs4986790 and rs4986791 were obtained by using gradient PCR. The products were affirmed by running on two percent agarose-gel, gel doc system was used to visualize it. PCR product of a one family with both SNPs (rs4986790 and rs4986791) is represented in Fig-4a & Fig-4b.

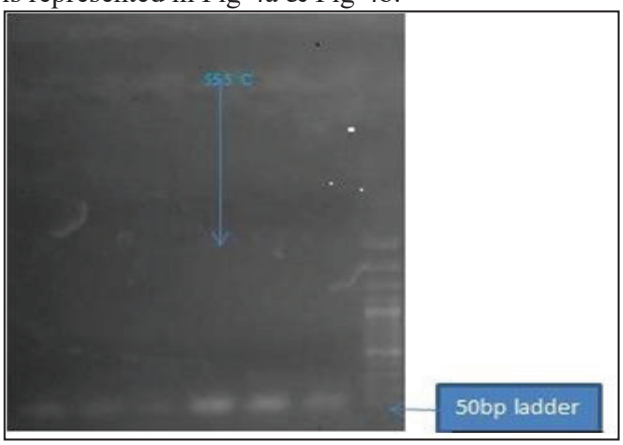

Fig-3: Primer Optimization of rs4986791 at 55.5˚C

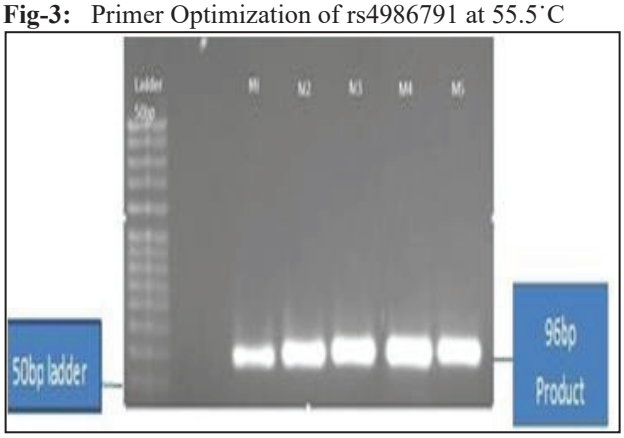

Fig-4(a): PCR product (96bp) of single family (Member M1-M5) with rs4986790 while in the first column 50bp Ladder used.

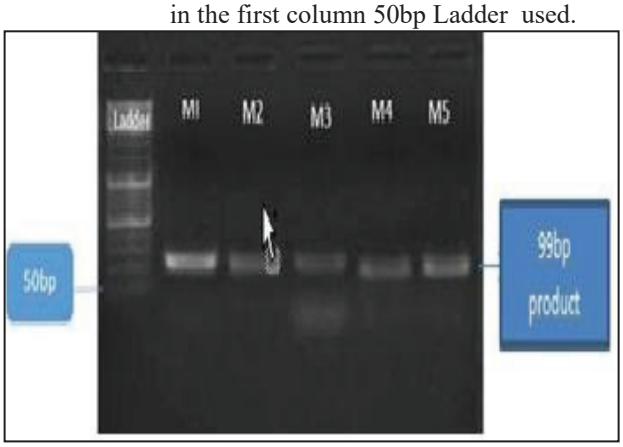

Fig-4(b): PCR product (99bp) of single family (Member M1-M5) with rs4986791 while in the first column 50bp Ladder used.

Sanger Sequencing

The selected SNPs (rs4986790 and rs4986791) were sequenced by Sanger sequencing. For Sanger sequencing following protocol was used. Each cycle of sequencing reaction consisted of initial denaturation at 95ºC for 2 mins followed by 35 cycles each of 10 sec at 95 ºC, annealing at 55ºC for 16 sec, extension at 60 for 1 min. The recipe of reaction mixture for PCR products sequencing are given in supplementary Table-S4. The reaction mix was rotated in centrifuge at 8000 rpm for ten seconds. Each sequencing reaction consists of 10 µl reaction mix.

As a result of sequencing, no mutation was found in SNP rs4986790 as shown in Fig-5. While it was found that both homozygous and heterozygous mutations were present at rs4986791, and their chromatographs were shown in Fig-6(a&b). The sequences were blast on NCBI. The results of sequences Blast results on NCBI for SNPs rs4986790 and rs4986791 are shown in Fig-7 and 8(a&b).

Reagent	Quantity
PCR product	3 µl
Forward primer (3.2 pmol)	1 µl
Big Dye	1 µl
Reaction Buffer (5X)	1 µl
DEPC water	4 µl
Total	10 µl

Table-S4: Recipe of Reaction Mixture for sequencing of PCR products.

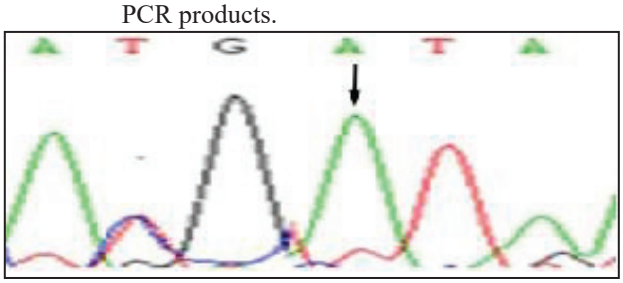

Fig-5: Chromatographic representation of SNPs rs4986790 of TLR4gene (Chromosome 9) showed normal sequence means no mutation was found.

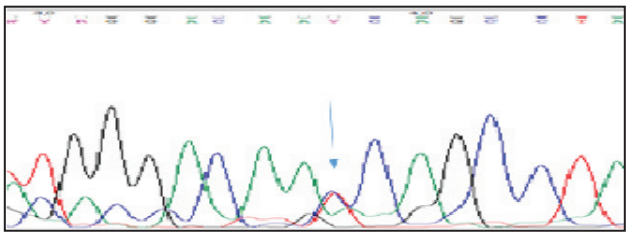

Fig-6(a): Chromatographic representation of SNPs rs4986791 of TLR4 gene (Chromosome 9) showed heterozygous mutation (CT) as compared to normal sequence at that point.

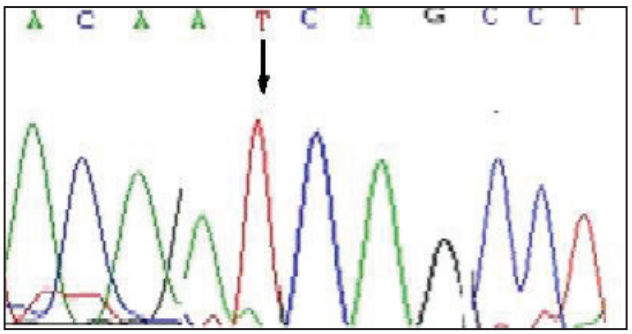

Fig-6(b): Chromatographic representation of SNPs rs4986791 of *TLR4* gene (Chromosome 9) showed homozygous mutation (T/T) as compared to normal sequence.

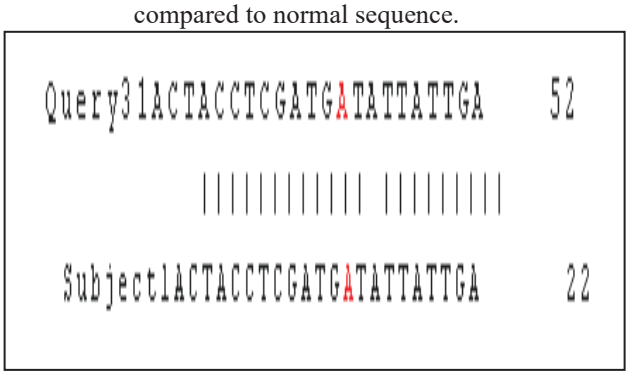

Fig-7: Blast representation of rs4986790 (HF1MI) in which subject have nucleotides (1-22 nucleotides) similarity with Query nucleotides.

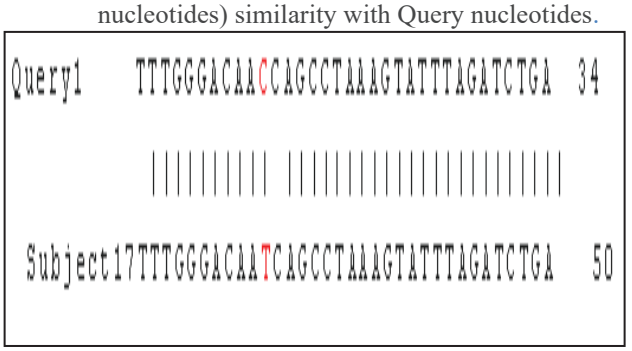

Fig-8(a): Blast representation of rs4986791 (HF1M4) in which subject have nucleotides (17-50 nucleotides) similarity with Query nucleotides.

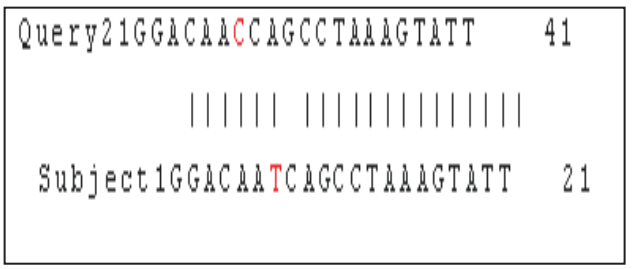

Fig-8(b): Blast representation of rs4986791 (HF1M1) in which subject have nucleotides (1-21 nucleotides) similarity with Query nucleotides.

Pedigree Analysis

One to two generation pedigree was sketched to show the family history of MI and consanguinity among parents. In family clustering study, TT genotype of rs4986791 was found to be significantly associated with MI while heterozygous CT of same SNP representing carrier genotype was found in few family members of pro band (patient). Other SNP (Asp299Gly; rs4986790) did not show any association with MI, all subjects were homozygous for normal AA genotype. We did not find any heterozygous condition with this SNP. All genotypic pedigrees were shown in Fig-9 (a, b, c, d & e).

In Heart family1 (F1) with rs4986791, father (MI patient) had homozygous genotype TT while mother showed normal genotype CC. However, one of the offspring was at higher risk of MI transmission as it contained genotype "CT" (Fig-4.b). In Heart family2 (F2) with rs4986791, pro band and his brother had homozygous genotype TT while mother showed abnormal genotype CC. However, one of the pro-band's offspring was at higher risk of MI transmission as it contained genotype "CT" (Fig-4). In other families, only pro-bands having homozygous genotype TT while all other members showed normal CC genotype. All genotypic pedigrees are shown in Fig-10 (a, b, c, d & e).

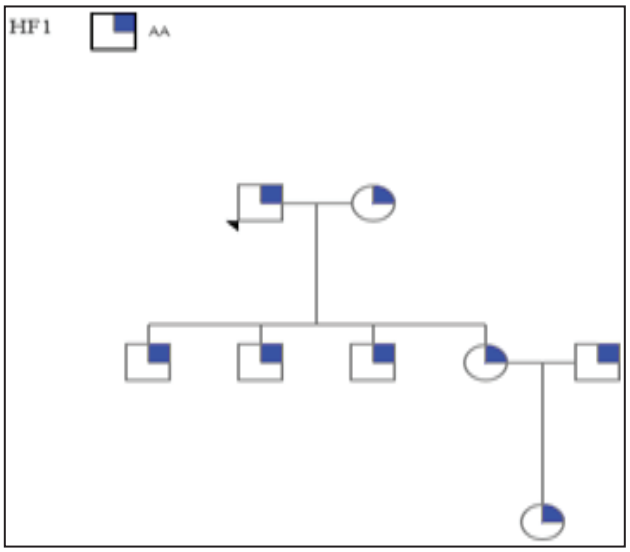

Fig-9(a): Pedigree of Heart family 1 (HF1) with SNP rs4986790 in which all members showed normal genotype.

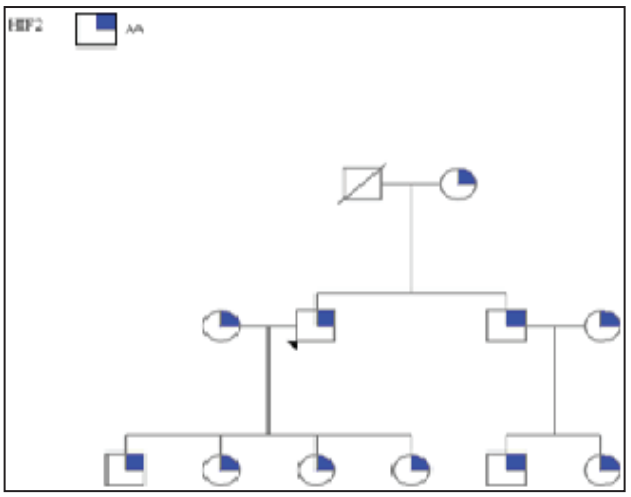

Fig-9(b): Pedigree of Heart family 2 (HF2) with SNP rs4986790 in which AA genotype showed normal condition.

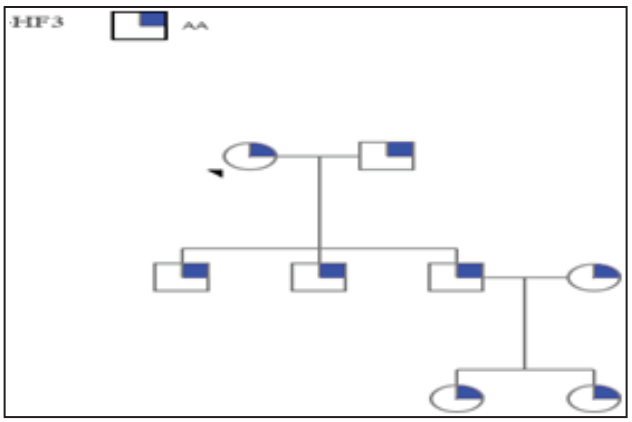

Fig-9(c): Pedigree of Heart family 3 (HF3) with SNP rs4986790 in which all members showed normal genotype.

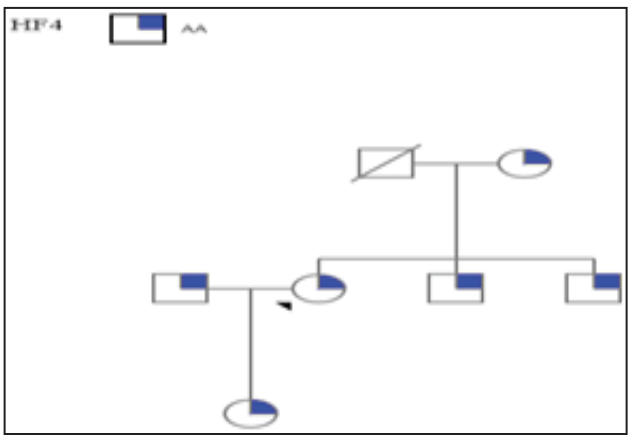

Fig-9(d): Pedigree of Heart family 4 (HF4) with SNP rs4986790 in which AA genotype showed normal condition.

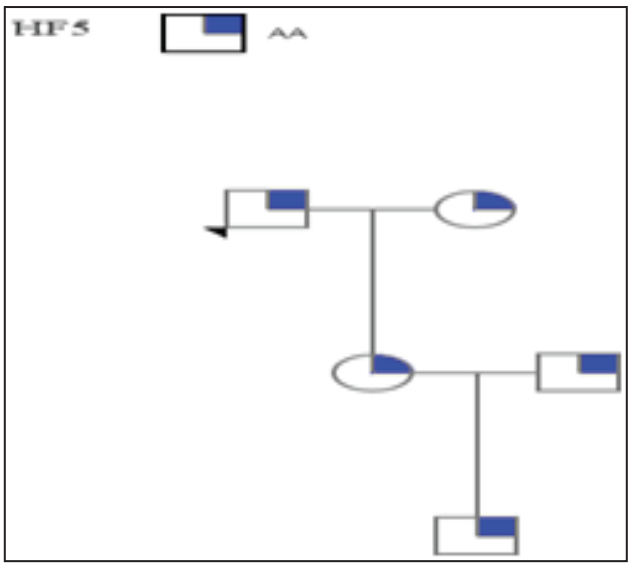

Fig-9(e): Pedigree of Heart family 5 (HF5) with SNP rs4986790 in which AA genotype showed normal condition.

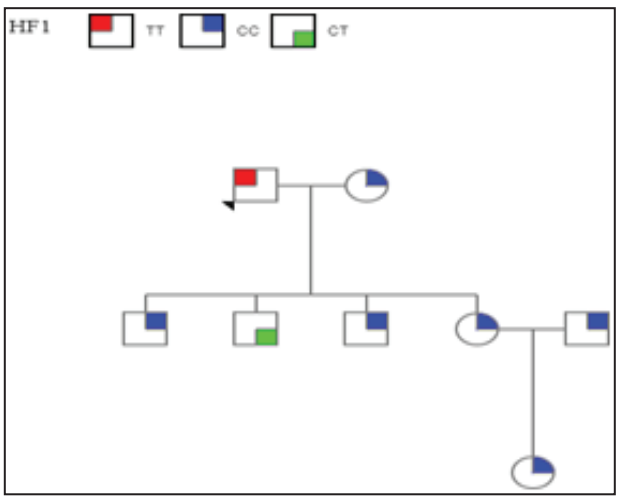

Fig-10(a): Pedigree of Heart family 1 (HF1) with SNP rs4986791 in which father have homozygous genotype TT while one of the offspring had genotype "CT

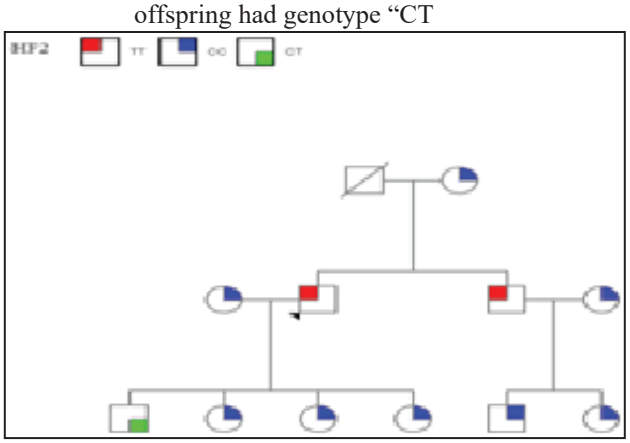

Fig-10(b): Pedigree of Heart family 2 (HF2) with SNP rs4986791 in which pro-band and his brother have homozygous genotype TT.

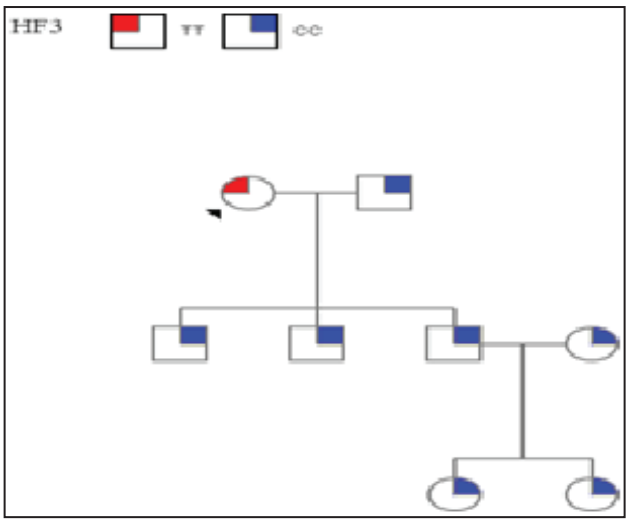

Fig-10(c): Pedigree of Heart family 3 (HF3) with SNP rs4986791 in which all members except pro-band showed normal genotype.

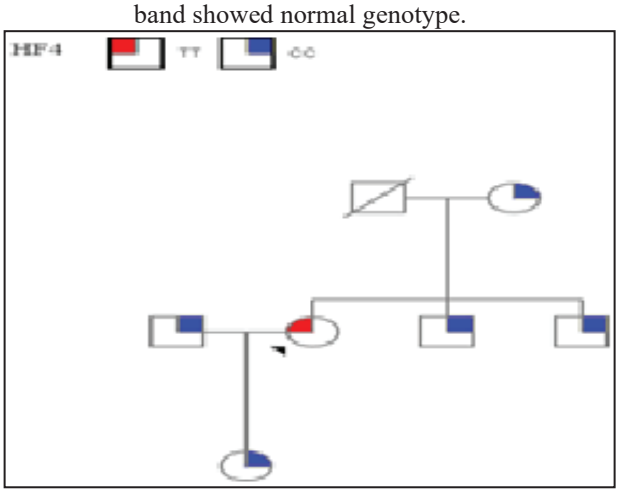

Fig-10(d): Pedigree of Heart family 4 (HF4) with SNP rs4986791 in which all members except pro-band showed normal genotype CC.

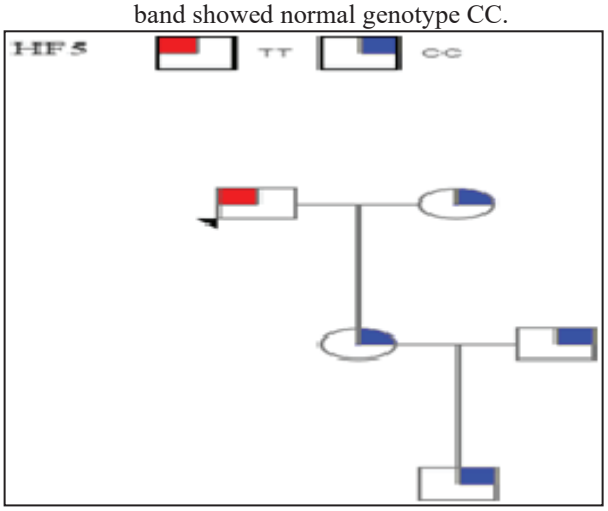

Fig-10(e): Pedigree of Heart family 5 (HF5) with SNP rs4986791 in which all members showed normal genotype CC except pro-band.

Genotypes of all families

The genotypes of selected families were mentioned in Table-1.

Coding	Genotype	
	rs4986790	**rs4986791**
family 1(F1)		
F1M1	A/A	T/T
F1M2	A/A	C/C
F1M3	A/A	C/C
F1M4	A/A	CT
F1M5	A/A	C/C
F1M6	A/A	C/C
F1M7	A/A	C/C
F1M8	A/A	C/C
family 2 (F2)		
F2M1	A/A	C/C
F2M2	A/A	T/T
F2M3	A/A	T/T
F2M4	A/A	C/C
F2M5	A/A	C/C
F2M6	A/A	C/C
F2M7	A/A	C/C
F2M8	A/A	C/T
F2M9	A/A	C/C
F2M10	A/A	C/C
F2M11	A/A	C/C
family 3(F3)		
F3M1	A/A	C/C
F3M2	A/A	T/T
F3M3	A/A	C/C
F3M4	A/A	C/C
F3M5	A/A	C/C
F3M6	A/A	C/C
F3M7	A/A	C/C
F3M8	A/A	C/C
family 4(F4)		
F4M1	A/A	C/C
F4M2	A/A	C/C
F4M3	A/A	C/C
F4M4	A/A	T/T
F4M5	A/A	C/C
F4M6	A/A	C/C
family 5 (F5)		
F5M1	A/A	T/T
F5M2	A/A	C/C
F5M3	A/A	C/C
F5M4	A/A	C/C
F5M5	A/A	C/C

Table-1: Genotypes of selected SNPs in selected families.

Case control association study

The case control association analysis was done for all MI patients and control. The allelic and genotypic frequency of all eight members were investigated and indicated in Table-2 and Table-3 respectively. TLR4SNP sequencing recognized TT and CT genotypes (rs4986791) in patients while CC was seen in control group. Furthermore, sequencing identified AA genotype (rs4986790) in both patients and controls. The SNPs obey the Hardy Weinberg equilibrium and Thr399Ile; rs4986791 was significantly linked with the MI onset in Pakistani population at genotypic along with allelic level ($p<0.05$) whereas Asp299Gly; rs4986790 was not found to be associated with MI. In the present study it was seen that T allele is risk allele of SNP rs4986791 and can be responsible for onset of MI as frequency of allele was higher in patients than control. Furthermore, allele A of rs4986790 was detected as protective allele for participants as frequency of allele was more in control groups. Fisher's test was used for the measurement of p-values.

Sr.#	SNPs	Allele type	Patients (frequency)	Control (frequency)	*P*-value
1	rs4986790	A	13(1.00)	65(1.00)	
2	rs4986791	C	0(0.00)	63(0.97)	0.00e*+00
		T	13(1.00)	3(0.031)	

Table- 2: Allelic frequency of controls and patients in selected families *e=2.71

Sr.#	SNPs	Geno type	Patients (frequency)	Control (frequency)	*Fisher P*-value
1	rs4986790	A/A	7(1.00)	33(1.00)	
2	rs4986791	C/C	0(0.00)	32(0.94)	6.02e*-009
		C/T	0(0.00)	2(0.06)	
		T/T	7(1.000)	0(0.00)	

Table-3: Genotypic frequency of controls and patients in selected families.* e-009=10*-009

DISCUSSION

The MI disease is defined by inheritance of numerous genetic variants acting in concert with environmental factors serving as triggers of the disease state.[19] The chances of getting MI in males are increased by the age of 55 years and in females after 65 years of age.[15] Recently, various investigations have provided the evidence that genetic polymorphisms play a fundamental role in

the development of MI.[5] *TLR4*-dependent pro-inflammatory signaling has been involved in the initiation, development, and plaque destabilization phases of atherosclerosis which is the leading cause of MI.[12] The current research was planned to determine the association of *TLR4* gene with MI in multiple families of Pakistani population.

Studies into MI risk factors have gained higher momentum in last few years due to high prevalence of heart attack in the Pakistani population. Current findings showed that high BMI and age were strongly connected with increased risk of MI. It was observed in our findings that higher BMI of pro-bands 26.39 (kg/m^2) within mean age of 51.4 years. Similar to the current findings, it was revealed that overweight and obesity are responsible for MI.[20] Our current results were also similar to findings indicated by Wilson etal [21] that obesity is one of the independent risk factors of coronary artery disease. A 10kg increase in body weight increases the coronary artery disease risk by12%.[22] Our findings also observed that the cases of MI were more in men than women. The age and gender were also important in the progression of MI. The MI incidence was higher in men than women.[23]

Current outcomes also demonstrated that hypertension and diabetes, history of smoking were associated with MI. Similar to current outcomes, it was revealed that chance for MI is one fold when systolic pressure is between 120 and 129-mmHg while twice more when the systolic blood pressure is more than 140mm Hg .[24] Similar to the present findings, diabetes was also an important risk factor for the progression of MI.[25] The risk of MI was higher in diabetic females as compared to diabetic male.[26] Our outcomes were also similar to studies which stated that increased duration of smoking correspondingly incremented the occurrence of MI.[27]

The present study revealed that positive family history acts as risk factor in the development of MI. Five families were included for geno typing of SNP rs4986790 and rs4986791. Heart family1 (HF1) showed pro-band with T/T homozygous mutation for wild type allele while one of siblings had heterozygous mutation for 4986791C/T. In heart family2 (HF2), pro-band and his brother had T/T homozygous mutation for wild type allele while one of pro-band's offspring showed heterozygous mutation for 4986791 C/T. In some of our selected families, all diseased members showed T/T homozygous mutation for wild type allele with rs4986791.While in other selected heart families had normal homozygous genotype AA with rs4986790. These findings were similar to a study in which first-degree relative of a patient with MI themselves had a substantial higher risk of MI.[28] The case control part of present study found that 1196C/T (Thr399Ile; rs4986791) on TLR4 gene was connected with MI onset in Pakistani population. We also found frequency of Tallele was more in patients than controls. After meta-investigation, Sheng-et al [29] proposed that rs4986791 is negatively linked with heart disease in Asians. Our results were also in contrast with a study done by Koch et al.[30] They claimed 1196C/T (Thr399Ile; rs4986791) polymorphism of TLR4 gene or haplotypes based on the polymorphism were not linked with MI. Current study also determined that 896A/G (Asp299Gly; rs4986790) on TLR4 gene was not associated with MI. We observed that all members (normal and diseased) had similar homozygous genotype AA. These findings were similar to another study in which no relationship of the 896A/G SNP with MI was detected in the white US men population.[31] In another study by Ameziane et.al.[13] showed that the Gly299 allele of the *TLR4* gene was associated with a decreased risk of acute coronary events in European white subjects, an effect that might involve decreased levels of inflammatory mediators. The frequency of SNP 896A ⁄ G variants in the TLR4 gene between the controls and patients did not differ (P =0.286). As a result of gene and allele frequencies it was concluded that TLR4 gene was not linked with MI in Croatian Patients.[32]

Limitations:

Current study was limited by the sample size, we recommend doing further a large-scale family association study for better understanding of the association of cousin marriages with onset of myocardial infarction at genetic level.

CONCLUSION

At the end, it can be concluded that high BMI, positive family history, smoking, hypertension, diabetes, and 1196C/T (Thr399Ile; rs4986791) on TLR4gene were strongly responsible for the onset of MI in different families of Pakistani population. There was no association found between rs4986790 and MI. By family clustering study for genetic analysis, those siblings that have risk genotype can save themselves in future from onset of disease or can delay the disease onset by changing their lifestyle. However other studies with greater sample size are needed to verify our results.

REFERENCES

1. Marvaki C, Argyriou G, Karkouli, G, Kossivas P, Mrvaki A, Pilatis N & Dimoula Y. The role of education on behavioral changes to modifiable risks factors after myocardial infarction. J. Health Sci 2007.
2. Jayaraj J C, Davatyan K, Subramanian SS, Priya J. Epidemiology of myocardial infarction. In Myocardial Infarction 2018 Nov 5 (pp. 9-19). London: Intech Open.
3. Claeys MJ, Rajagopalan S, Nawrot TS, Brook RD. Climate and environmental triggers of acute myocardial infarction. Eur. Heart J 2017; 38 (13):955-60.
4. Tabei SM, Senemar S, Saffari B, Ahmadi Z, Haqparast S. Non-modifiable factors of coronary artery stenosis in late onset patients with coronary artery disease in Southern Iranian population. J Cardiovasc Thorac Re2014; 6(1):51.
5. Wang YL, Sun L M, Zhang L, Xu H T, Dong Z, Wang L Q, Wang M L. Association between Apolipo protein E polymorphism and myocardial infarction risk: A systematic review and meta-analysis. FEBS Open Bio 2015; 5: 852-8.
6. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, Sato H, Sato H, Hori M, Nakamura Y, and Tanaka T. Functional SNPs in the lymphotoxin-α gene that are associated with susceptibility to myocardial infarction. Nat. Genet2002; 32(4):650-4.
7. Wang Q, Chen Q. Cardiovascular disease and congenital heart defects. e LS.2001.
8. Wang Q, Pyeritz RE. Molecular genetics of cardiovascular disease. Textbook of cardiovascular medicine 2000; 1:1-2.
9. Yamada Y, Ichihara S, Nishida T. Molecular genetics of myocardial infarction. Genom. Med 2008; 2(1-2):7-22.
10. Akira, S., Takeda, K., &Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. Nature immunology, 2(8), 675-680.
11. Libby, P., Ridker, P. M., & Maseri, A. (2002). Inflammation and atherosclerosis. Circulation, 105(9), 1135-1143.
12. Edfeldt, K., Swedenborg, J., Hansson, G. K., & Yan, Z. Q. (2002). Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation, 105(10), 1158-1161.
13. Ameziane, N., Beillat, T., Verpillat, P., Chollet Martin, S., Aumont ,M.C.,Seknadji,P.,& de Prost, D. (2003). Association of the Toll-like receptor 4 gene Asp299Gly polymorphism with acute coronary events. Arteriosclerosis, thrombosis, and vascular biology, 23(12), e61-e64.
14. Balistreri,C.R.,Candore,G.,ColonnaRomano,G.,Lio ,D.,Caruso,M.,Hoffmann,E.,... & Caruso, C. (2004). Role of Toll-like receptor 4 in acute myocardial infarction and longevity. Jama, 292(19), 2335-2340.
15. Cipriani V, Mannucci PM, Ardissino D, Ferrario M, Corsini G, Merlini PA, Notarangelo F, Lina D, Bernardinelli L. Familial aggregation of early-onset myocardial infarction. Eur. J. Intern. Med 2010; 21(6):511-5.
16. Colditz GA, Stampfer MJ, Willett WC, Rosner B, Speizer FE, Hennekens CH. A prospective study of parental history of myocardial infarction and coronary heart disease in women. Am J Epidemiol1986; 123(1):48-58.
17. Jousilahti P, Puska P, Vartiainen E, Pekkanen J, Tuomilehto J. Parental history of premature coronary heart disease: an independent risk factor of myocardial infarction. J Clin Epidemiol 1996; 49(5):497-503.
18. BanerjeeA, SilverLE, Heneghan C, Welch SJ, Bull LM, MehtaZ, Banning AP, Rothwell PM. Sex-specific familial clustering of myocardial infarction in patients with acute coronary syndromes. Circ. Cardiovasc. Genet 2009; 2 (2):98-105.
19. Guella I, Rimoldi V, Asselta R, Ardissino D, Francolini M, Martinelli N, Girelli D, PeyvandiF, TubaroM, Merlini PA, Mannucci PM. Association and function alanalyses of MEF2A as a susceptibility gene for premature myocardial infarction and coronary artery disease. Circ. Cardiovasc. Genet 2009; 2 (2):165-72.
20. Thomsen M, Nordestgaard BG. Myocardial infarction and ischemic heart disease in overweight and obesity with and without metabolic syndrome. JAMA Intern. Med 2014; 174(1):15-22.
21. Wilson P W, D' Agostino RB, Sullivan L, Parise H, Kannel WB. Over-weight and obesity as determinants of cardio-vascular risk: the Framingham experience. Arch Intern Med 2002; 162(16):1867-72.
22. Din-Dzietham R, Liu Y, Bielo MV, Shamsa F. High blood pressure trends in children and adolescents in national surveys, 1963 to 2002. Circulation2007;116(13):1488-96.
23. Millett ER, Peters SA, Woodward M. Sex differences in risk factors for myocardial infarction: cohort study of UK Biobank participants. Bmj2018; 363.
24. Escobar E. Hypertension and coronary heart disease. J. Hum. Hypertens2002;16 (1): S61- 3.
25. Gleissner CA, Galkina E, Nadler JL, Ley K. Mechanisms by which diabetes increases cardiovascular disease. Drug Discov. Today Dis. Mech2007; 4(3):131-40.
26. Juutilainen A, Kortelainen S, Lehto S, Rönnemaa T, Pyörälä K, Laakso M. Gender difference in the impact of type 2 diabetes on coronary heart disease risk. Diabetes care 2004; 27(12):2898-904.
27. Elkhader BA, Abdulla AA, Omer MA.

Correlation of smoking and myocardial infarction among sudanese male patients above 40 years of age. Pol J Radiol2016; 81:138.

28. Nielsen M, Andersson C,Gerds TA,Andersen PK,Jensen TB, Køber L,Gislason G,Torp-Pedersen C. Familial clustering of myocardial infarction in first-degree relatives: a nationwide study. Eur. Heart J2013; 34 (16):1198-203.

29. Sheng, J., & Xu, J. (2019). Association of coronary artery disease with toll-like receptor 4 geneticvariants: Ameta-analysis. Advances in clinical and experimental medicine: official organ Wroclaw Medical University, 28(5), 651-658.

30. Koch W, Hoppmann P, Pfeufer A, Schömig A, Kastrati A. Toll-like receptor 4 gene polymorphisms and myocardial infarction: no association in a Caucasian population. Eur. Heart J2006; 27(21):2524-9.

31. Zee RY, Hegener HH, Gould J, Ridker PM. Toll-like receptor 4 Asp299Gly gene polymorphism and risk of atherothrombosis. Stroke2005; 36(1):154-7.

32. Džumhur, A., Zibar, L., Wagner, J., Šimundić, T., Dembić, Z., & Barbić, J. (2012). Association Studies of Gene Polymorphisms in Toll-Like Receptors 2 and 4 in Croatian Patients with Acute Myocardial Infarction. Scandinavian Journal of Immunology, 75(5), 517-523.

The Authors:

Dr. Riffat Iqbal
Assistant Professor,
Department of Zoology,
Government College University, Lahore.

Muhammad Shehzad
Student,
Department of Zoology,
Government College University, Lahore.

Dr. Shaista Aslam
Assistant Professor,
Department of Biological Sciences,
NUMS, Rawalpindi, Pakistan.

Dr. Faiza Gul Durrani
Director,
Science Shines Ltd. London, U.K.

Dr. Kiran Aftab
Assistant Professor
Department of ZoologyUniversity of Gujarat, Pakistan.

Dr. Iram Liaqat
Associate Professor,
Department of Zoology,
Government College University, Lahore.

Corresponding Author:
Dr. Riffat Iqbal
Assistant Professor,
Department of Zoology,
Government College University, Lahore.
Email: riffatiqbal@gcu.edu.pk