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ASSOCIATION OF *COL4A1* (RS605143 AND RS565470) GENE POLYMORPHISM WITH CORONARY ARTERY DISEASES (CAD)

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ABSTRACT
CAD is one of the biggest cause of death around the world, with the number of people affected continually increasing. Increasing Incidence of CAD is a major concern not only in India but across the world. Genetic Polymorphism of many genes is associated with increased risk of various diseases in a particular ethnic group. This case control study was carried out to investigate the association of *COL4A1* gene polymorphism with CAD. The study includes 25 angiographically proven CAD cases and 25 controls. Blood was collected in EDTA tube. Genomic DNA was extracted using phenol-chloroform extraction method (Sambrook J, 1989). Polymerase Chain Reaction(PCR) was done on extracted DNA. PCR product was digested with 5 U of restriction enzyme (Fermentas, UK). Statistical analysis was performed using SPSS 20.0 version. For SNP-1 rs605143 Frequency of *COL4A1* AG, GG, AA genotype in CAD samples were 52%, 40%, 8% and in Control samples were 64%, 32%, 4% respectively. Frequencies of *COL4A1* A and G alleles were 34% and 66% in CAD samples and 36% and 64% in control Samples. For SNP-2 rs 565470 Frequency of *COL4A1* CT, CC, TT genotype in CAD samples were 60%, 12%, 28% and in Control samples were 52%, 16%, 32% respectively. Frequencies of *COL4A1* C and T alleles were 42% and 58% in CAD samples and 42% and 58% in control Samples. Polymorphism of *COL4A1* gene (rs605143, rs565470) was not associated with the risk of CAD (Statistically). For rs605143, frequency of A allele was significantly higher in Control subjects than in CAD patients which indicates that A allele of rs605143 is a protective factor for CAD while the frequency of G allele was significantly higher in CAD cases than controls suggesting it to be a risk factor for CAD. For rs565470, no significant difference was found between the allelic ratio of the case and the control samples.

KEYWORDS: Coronary Artery Disease, *COL4A1*, Gene Polymorphism.

INTRODUCTION
Coronary artery disease (CAD) is the leading cause of death worldwide. CAD involves building up of plaque (Atheroma) inside the coronary arteries causing obstruction of blood flow to the heart. It's a wide spectrum encompassing Stable Angina, Unstable angina, Myocardial Infarction, Heart Failure, Arrhythmia, Sudden Death.[1] It's a major cause of death worldwide, caused by interaction of multiple endogenous and exogenous factors; heritable factors accounting for 40%-60%.[2,3] It's also the foremost reason of death in patients with chronic kidney disease (CKD). Patients with minor CKD tend to die of CAD rather than develop kidney failure.[4]

Collagens are set of proteins in which every third amino acid is glycine (Gly-Xaa-Yaa motif).[5,6] There are at least 25 different types of collagens. The most abundant four found in mammals are Type I, II, III and IV collagens. Type IV collagen is the major component of vascular basement membrane, could provide physical barrier to both soluble molecules and migrating cells.[6,7,8] *COL4A1* gene is found on the telomeric end of 13q position 34 (13q34), having 52 exons. It encodes for α1 chain of collagen type IV.[9] Numerous polymorphs of *COL4A1*, like rs3742207, rs605143, rs565470 have been identified which are significantly associated with prevalence of various diseases. A Genome-Wide Association Scan (GWAS) study conducted by Kirill et al. reported that *COL4A1* gene is associated with arterial stiffness.[10] Hence the present study was conducted to investigate the association of *COL4A1* gene polymorphism with CAD and its role in increasing the susceptibility to CAD.

Review of Literature
CAD is defined as atherosclerosis (AS) of the coronary arteries (blood vessels supplying the cardiac muscle) which is a decades-long process which results in progressive narrowing of coronary artery blood vessel walls, by lipid-laden plaques that ultimately lead to symptoms of ischemia, a condition where in blood vessel narrowing, or atherothrombotic blockage causes a

deficiency of oxygen and nutrient supply to the myocardium (heart muscle). The development of CAD is silent, and is asymptomatic for many years or throughout an individual's life. It usually presents clinically as one or more of these four syndromes: 1) angina pectoris; 2) myocardial infarction; 3) chronic CAD with heart failure and 4) sudden cardiac death.[11]

Epidemiology of coronary artery disease

Coronary artery disease (CAD) is the single largest disease responsible for the death of men and women around the globe. Globally, CVD led to 17.5 million deaths in 2012.[12] About more than 75% of these deaths occurred in developing countries. In comparison to developed countries, where death from CHD is rapidly declining, it is rising in developing countries.[13] This rise is due to industrialization, urbanization, and related lifestyle changes and is called epidemiological transition.[14]

The office of the Registrar General of India (RGI) has constantly reported data on cardiovascular death(CVD) rates in India.[15] These data have been mentioned as circulatory system deaths in the Medical Certification of Cause of Deaths reports, and in 1980s and 1990s it was seen that CVD led to 15%-20% of deaths in the country.[16] An increasing trend in CVD mortality has been seen, with 20.6% deaths in 1990, 21.4% in 1995, 24.3% in 2000, 27.5% in 2005, and 29.0% in 2013.[15] In India, more than 10.5 million deaths occur annually, and it was documented that CVD led to 20.3% of these deaths in men and 16.9% of all deaths in women.[17] According to 2010-2013 RGI data,[18] death from CVD increased to 23% of total and 32% of adult deaths in years 2010-2013.

Development and progression of Atherosclerotic plaque

Being an inflammatory process each stage of its development - initiation, progression and thrombosis, are controlled to a large extent by inflammatory cellular and molecular mediators of the immune response. AS initiates when the arterial endothelium is activated at focal areas such as, blood vessel openings and branch points by a combination of turbulent flow, laminar shear stress haemodynamic disturbances and other risk factors including: dyslipidaemia, hypertension, hyperglycaemia end-products, homocysteine, bacterial products, viruses, cigarette smoke toxins, hypoxia, pro-inflammatory cytokines and complement leading to endothelial cell expression of adhesion molecules such as, intracellular adhesion molecule 1(ICAM-1), vascular adhesion molecule 1(VCAM-1), E- and P- selectin and chemokines interleukin 8 (IL-8) and monocyte chemotactic protein 1 (MCP-1), which initiates the inflammatory innate immune response and leads to rolling, recruitment and tight adhesion followed by transmigration of blood leukocytes into the sub-endothelial intimal space via diapedesis.[19]

Collagen

These are a set of proteins consisting of a unique sequence in which every third amino acid is a glycine (Gly-Xaa-Yaa motif).[5,6] There are 25 different kinds of collagens out of which mostly a Type I, II, III and IV collagens are present. Type IV collagen is the core element in the basement membrane and is expressed in all tissues including the renal glomerular, vasculature and ocular structures.[6,7,8]

Type I collagen, being the most abundant protein in the body of humans, plays an important role as a primary structural protein in connective tissues because it provides mechanical support for the body [20]. Therefore, Type I collagen has been widely used as scaffolding material in biomedical applications, specifically for tissue regeneration [20-24]. As of now, many commercially available collagen-based products are extracted from mammalian animal sources such as cows, pigs and sheep.[25,26]

The *COL4A1* and *COL4A2* genes are present next to each other in the head-to-head orientation on chromosome 13q34 and also share common transcriptional regulatory sequences.[27–33] These two genes encode the collagen IV protein α1 and α2 chains, respectively.[27-33] Collagen IV being the major constituent of the basement membrane and is therefore essential for its integrity and functionality.[34] The basement membrane underlies the endothelium and surrounds smooth muscle cells (SMCs) in the walls of blood vessels.[35] The basement membrane serves as an extracellular scaffold and also regulates cell behaviour.[34,36] Abnormalities of vascular endothelial cells (ECs) and SMCs play a very crucial role in the pathogenesis of atherosclerosis, the vascular pathology underlying CHD.[37]

Different types of Single Nucleotide Polymorphism (SNP)

Approximately 99.5% of the human genome sequence is absolutely similar amongst all humans, thus the remaining 0.5% (~16 million bp) is responsible for all human differences, and this includes disease susceptibility. SNPs; di-, tri-, and tetra-nucleotide repeats called 22 microsatellites; large variants called copy number variants (CNVs) of varying length, mostly >0.5kb caused by deletions, insertions or duplications; and short nucleotide substitutions evenly distributed throughout the genome, make up the genetic variations.

It has been postulated that the genetic component responsible for complex disease is down to multiple low risk genetic variants, of which some are common, and others rare. The most frequent (~80%) non-repetitive sequence variants are SNPs, and these have advantages over previous genetic markers used in genetic analysis (i.e. microsatellite tandem repeats). Firstly, SNPs are biallelic, so their frequencies can be easily estimated in any population. Secondly, SNPs are very stable genetic markers compared to tandem repeat markers, whose high

mutation rate can affect genetic analysis in different populations. Thirdly, technologies have been developed that can genotype SNPs simply, accurately and rapidly via automated methods. There are estimated to be on average one SNP for every 1000 base pairs (bp), when any two chromosomes are compared, and that the human genome has approximately 3 million common (>5%) SNPs per individual[38]; and perhaps as many as 17 million SNPs present in the general population.[39] SNPs are distributed evenly throughout the genome, so that every gene will be covered by several SNPs, which allows indirect detection of candidate genetic variants through the LD between a SNP marker of complex disease, and a functional variant of a gene, this is known as the proximity hypothesis.[40] In recent years, numerous polymorphs of *COL4A1* has been identified which are significantly associated with prevalence of various diseases like rs3742207, rs605143, rs565470 etc. SNP rs3742207 is a common nonsynonymous coding polymorphism which is located in exon 45 of the *COl4A1* gene. The polymorph involves a substitution of adenine by cytosine resulting in an amino acid change from Glycine to Histidine at position 1334 (A→C (Gln1334His)), which is located in a central region of the protein that consists of multiple triple-helix re*peat domains. This SNP causes upregulation of the gene and also have a strong replicated association with arterial stiffness. The SNP rs3742207 was recently found to be linked with the prevalence of myocardial infarction.[41-43]

Dilare Adi et al (2013) carried out a study on 1095 subjects of Uygur population of China. (727 men, 368 women) including 471 CAD patients and 624 controls and concluded that rs605143 was found to be related with CAD by in a dominate model and also the rs565470 was also found to be related with CAD in a recessive model for total and men.[44] Tarasov et al (2009) conducted a genome-wide association study in two populations of 4221 and 1828 individuals and identified a SNP rs3742207 in the *COL4A1* gene that was significantly associated with Pulse wave velocity and arterial stiffness.[10] Adam et al (2015) concluded through Epistasis analyses that the SMAD3-dependent regulation of *COL4A1*/*COL4A2* may be of functional significance for CAD pathogenesis.[45] Tom Van et al (2010) indicate that mutations in *COL4A1* result in a complex vascular phenotype encompassing defects in maintenance of vascular tone, endothelial cell function and blood pressure regulation in Mice.[46] Wei Yang et al(2016) conducted a Genome-wide association study and found that rs4773144 of *COL4A1* gene association of G allele with higher rates of Myocadial Infarction, associating with thinner plaque cap and lower cap/intima ratio.[47]

AIMS AND OBJECTIVE

1. To investigate the association of *COL4A1* (rs605143 and rs565470) with CAD.
2. To study the different allele and genotype frequencies of *COL4A1* (rs605143 and rs565470) and compare them between cases and controls.
3. To detect the role of genetic polymorphism in *COL4A1* (rs605143 and rs565470) genes in increasing the susceptibility to CAD.

MATERIAL AND METHODS

Ethical approval

This study was approved by the Institutional Ethics Committee of our medical College. Each participant gave written informed consent and explicitly provided permission for DNA analyses as well as collection of relevant clinical data.

Study type and setting

A Hospital Research based study was conducted at Department of Biochemistry, of our College.

Study Group

Subjects: A total of 50 individuals were included in the study and were categorised into two groups

- Cases: 25 Angiographically proven CAD Patients.
- Control Group: 25 age/ sex, ethnicity matched healthy controls.

1. Inclusion Criteria

- Angiographically Proven CAD Patients visiting Cardiology Department of our college

2. Exclusion Criteria

- Acute Myocardial Infarction patients
- Patients with Myocarditis
- Cardiogenic shock
- Severe Hepatic or renal Dysfunction
- Unstable Angina caused by other etiological mechanisms (e.g. coronary focal spasm, coronary artery dissection);
- secondary Unstable Angina related to precipitating factors, such as anemia, fever, tachycardia, hypotension, etc
- Leucopenia, leukemia, thrombocytopenia, or ongoing inflammatory and malignant diseases.
- Failure to obtain consent

Samples

The blood samples of 25 angiographically proven CAD cases and 25 healthy controls was collected from the Cardiology Unit of Department of Medicine of our Medical College. All the samples were stored at -20°C. Data was collected from each patient on clinical variables like lipid profile, blood sugar level etc. including age, blood pressure, height, weight, body mass index, cigarette smoking, alcohol consumption and family history etc.

DNA extraction

Five milliliters of peripheral blood was collected from all the subjects in 0.5M EDTA tubes. Genomic DNA was isolated from whole blood using the standard phenol-chloroform extraction method (Sambrook J, 1989). The quality and quantity of isolated DNA was determined by

nanodrop spectrophotometer and finally stored at -20°C for further analysis.

Analysis of Polymorphism

Genotyping of *COL4A1* (rs605143 & rs565470) was performed using PCR-RFLP technique.

***COL4A1* Polymorphism**

PCR was performed in a total volume of 20 µL including 0.3 pmol of each primer, 0.2 mmol/L of each dNTP, 0.1 U Taq DNA polymerase, 56 mmol/L KCl, 11 mmol/L Tris-HCl (pH 8.3), and 2 mmol/L MgCl2. The PCR protocol was as follows: an initial denaturation step at 95°C for 5 min; 30 cycles of 95°C for 30s, 60°C for 35 s and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. The PCR products was digested with 5 U of restriction enzyme (Fermentas, UK) in a total volume of 25 µl. Bands were visualized by ethidium bromide staining after electrophoresis on a 3% high-resolution agarose gel.

Table 1: Primer sequences of each SNP.

SNPs	Polymerase chain reaction primers	Annealing temperature	Restriction enzyme
rs605143	Sense 5′AAAGCCATTGCTACCTCA3′	60°C	DraI
	Antisense 5′CTGCTCCTGGTGACTCTG3′		
rs565470	Sense 5′GAATGCGATAAGGACAGGG3	60°C	BanII
	Antisense 5′AGGAAAGGGAGGCACAAAA3′		

OBSERVATION AND RESULTS

Agarose Gel Picture

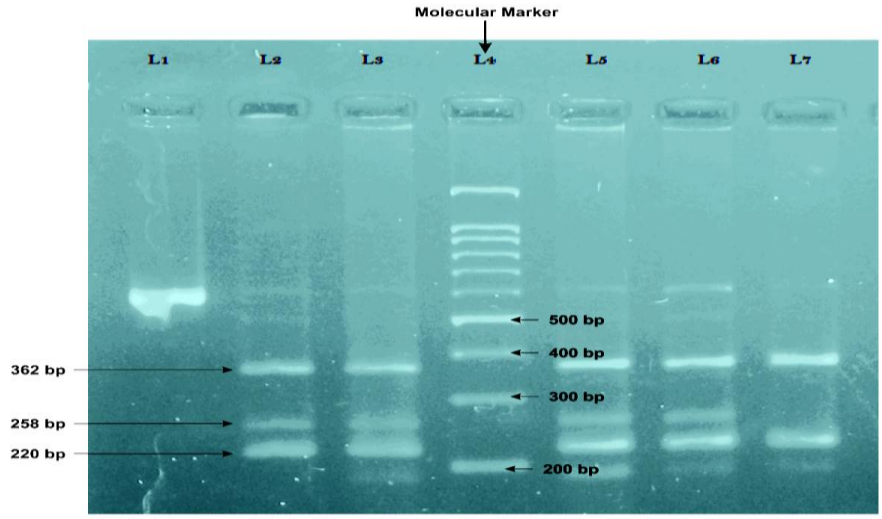

Figure 1: This is a 3% Agarose gel picture of Dra I digested products of *COL4A1* (rs605143) gene. The GG genotype shows two bands of 362bp and 220bp (L7). AG genotype shows three bands of 362bp, 258bp, 220bp (L2, L3, L5, L6). AA genotype shows three bands of 258bp, 220bp and 104bp. L1 shows undigested product and L4 is the molecular marker.

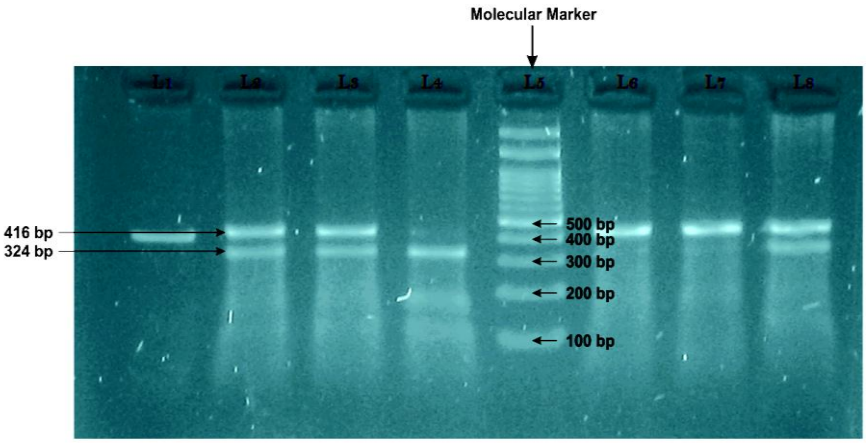

Figure 2: This is a 3% Agarose gel picture of Ban II digested products of *COL4A1* (rs565470) gene. The TT genotype shows one band of 416 bp (L6, L7); the CT genotype shows two bands of 416bp and 324bp (L2, L3, L8); the CC genotype shows one band of 324bp(L4); L1 is undigested product; L5 is molecular marker.

RESULTS

1. *COL4A1* (rs605143)

Frequencies of *COL4A1* AG, GG, AA genotype in CAD samples were 52%, 40%, 8% and in Control samples were 64%, 32%, 4% respectively. Frequency of *COL4A1* A and G alleles were 34% and 66% in CAD samples and 36% and 64% in control Samples. Odd Ratio (OR) of AG was found to be 0.609 (95% Confidence Interval {CI} is 0.20 – 1.89, $\chi 2$ is 0.739, p value is 0.39)**,** Odd Ratio (OR) of GG was found to be 1.417 (95% Confidence Interval {CI} is 0.44 – 4.52, $\chi 2$ is 0.347, p value is 0.556)and Odd Ratio (OR) of AA was found to be 2.087 (95% Confidence Interval {CI} is 0.18-24.62, $\chi 2$ is 0.355 , p value is 0.551).

Table 1: Genotype & allele frequencies of *COL4A1*(rs605143) gene polymorphism in CAD cases & controls.

		Control (25)		CAD cases (25)						
Col 4A1		**N**	**Frequency %**	**N**	**Frequency %**	**OR**	**95% CI**	**$\chi 2$**	**P values**	**Power**
Genotype	AG	16	64	13	52	0.609	0.20-1.89	0.739	0.39	0.553
	GG	8	32	10	40	1.417	0.44-4.52	0.347	0.556	0.667
	AA	1	4	2	8	2.087	0.18-24.62	0.355	0.551	0.725
Allele	A	18	36	17	34	0.916	0.40-2.08	0.044	0.834	0.583
	G	32	64	33	66	1.092	0.48-2.48	0.044	0.834	0.583

2. *COL4A1* (rs 565470)

Frequencies of *COL4A1* CT, CC, TT genotype in CAD samples were 60%, 12%, 28% and in Control samples were 52%, 16%, 32% respectively. Frequency of *COL4A1* C and T alleles are 42% and 58% in CAD samples and 42% and 58% in control Samples. Odd Ratio (OR) of CT was found to be 1.380 (95% Confidence Interval {CI} is 0.45-4.25, $\chi 2$ is 0.325, p value is 0.569), Odd Ratio (OR) of CC was found to be .716 (95% Confidence Interval {CI} is 0.14-3.59, $\chi 2$ is 0.166, p value is 0.684) and Odd Ratio (OR) of TT was found to be 0.826 (95% Confidence Interval {CI} is 0.25-2.78, $\chi 2$ is 0.095 , p value is 0.758).

Table 2: Genotype & allele frequencies of *COL4A1*(rs565470) gene polymorphism in CAD cases & controls.

		Control (25)		CAD cases (25)						
Col 4A1		**N**	**Frequency %**	**N**	**Frequency %**	**OR**	**95% CI**	**$\chi 2$**	**P values**	**Power**
Genotype	CT	13	52	15	60	1.380	0.45-4.25	0.325	0.569	0.662
	CC	4	16	3	12	0.716	0.14-3.59	0.166	0.684	0.341
	TT	8	32	7	28	0.826	0.25-2.78	0.095	0.758	0.500
Allele	C	21	42	21	42	1.000	0.45-2.21	0	1.000	0.500
	T	29	58	29	58	1.000	0.45-2.21	0	1.000	0.500

DISCUSSION

It was found that variation in *COL4A1* gene is associated with CAD in a Uygur population of China.[44] Collagen type lV is an important component of Basement membrane which has 6 different types of α-chains (α1-α6), all of the six chains of collagen IV has three domains: A short 7S domain at the N-terminal; along with collagenous domain which occupies the mid section of the molecule, and contains the classic Gly-Xaa-Yaa repeated amino acid sequence and a non-collagenous domain (NC1) which is positioned at the C-terminal. The six different collagen type IV alpha chains (α1- α6), form three sets of triple helical molecules called protomers, which are as follows α1.α1.α2, α3.α4.α4, α5.α5.α6[48,49-51], out of them α1.α1.α2 is very essential for the protein. α1 chain of type IV collagen is encoded by *COL4A1* gene, as new gene identified in the CARDIOGRAM Consortium.[48] *COL4A1* gene mutation was initially reported in a mouse which was a heterozygous that results in in-frame deletion of exon 40, this mutation results in abnormal synthesis of the protein which cannot be properly secreted outside the cell, and leaving mouse prone to brain hemorrhage at their birth.[52] The first report of a pathologic *COL4A1* mutation in humans was given in 2005, Gould and co-workers showed that mutation in *COL4A1* was related to congenital autosomal dominant porencephaly.[52] Many other researchers have reported that *COL4A1* gene mutation have resulted in human hemorrhagic stroke.[53, 54] Research about the polymorphism of *COL4A1* gene and cardiovascular diseases was initially reported by Yamada Y et al.[55], they showed that the A→C (Gln1334His) polymorphism (rs3742207) of *COL4A1* is associated with prevalence of MI in Japanese population, with C allele having a protecting action against this condition. A genome-wide association study (GWAS) for vascular stiffness measures showed a strong replicated association of SNP (rs3742207) in *COL4A1* with arterial stiffness, this study suggesting previously unrecognized cell-matrix interactions may exert a chief role in regulating arterial stiffness, but the regulatory mechanism of arterial stiffness is not yet clear; further work is needed to explain these mechanisms.[56]

In our study we found that polymorphism of *COL4A1* was not associated with the risk of CAD although A & G

allele of *COL4A1* rs(6055143) were found to be associated with CAD. There was no significant difference in the distribution of genotype of rs605143 and rs565470 between CAD patients and control subjects. For rs605143, compared with the Case samples, frequency of A allele is higher in Control subjects than in CAD patients. This result indicated that A allele of rs605143 is a protective factor for CAD in patients while the frequency of G allele was significantly higher in CAD cases than controls.(Table 1)

For rs565470, on comparing CAD cases with controls no difference was found between the allelic and genotype ratio showing that rs565470 gene polymorphism of *COL4A1* gene was not associated with CAD risk. (Table 2)

CONCLUSION

Polymorphism of *COL4A1* gene (rs605143, rs565470) were not associated with the risk of CAD. In SNP rs 605143 A allele was found to be a protective factor for CAD while G allele was found to be a risk allele for CAD. However additional studies will be needed to be undertaken with an increased sample size in order to clarify the underlying molecular mechanism which associates polymorphism of *COL4A1* gene with CAD and to validate our results.

SUMMARY

CAD is one of the biggest cause of death around the world, with the number of people affected continually increasing. Increasing Incidence of CAD is a major concern not only in India but across the world. Genetic Polymorphism of many genes is associated with increased risk of various diseases in a particular ethinic group, therefore this case control study was carried out to investigate the association of *COL4A1* gene polymorphism with CAD. The study includes 25 angiographically proven CAD cases and 25 controls. Blood was collected in EDTA tube. Genomic DNA was extracted using phenol-chloroform extraction method (Sambrook J, 1989). Polymerase Chain Reaction(PCR) was done on extracted DNA. PCR product was digested with 5 U of restriction enzyme (Fermentas, UK). For SNP-1 rs605143 Frequency of *COL4A1* AG, GG, AA genotype in CAD samples were 52%, 40%, 8% and in Control samples were 64%, 32%, 4% respectively. Frequency of *COL4A1* A and G alleles were 34% and 66% in CAD samples and 36% and 64% in control Samples. For rs605143, frequency of A allele was significantly higher in Control subjects than in CAD patients which indicats that A allele of rs605143 is a protective factor for CAD while the frequency of G allele was significantly higher in CAD cases than controls suggesting it to be a risk factor for CAD. For SNP-2 rs 565470 Frequency of *COL4A1* CT, CC, TT genotype in CAD samples were 60%, 12%, 28% and in Control samples were 52%, 16%, 32% respectively. Frequency of *COL4A1* C and T alleles were 42% and 58% in CAD samples and 42% and 58% in control Samples. For rs565470, no significant difference was found between the allelic ratio of the case and the control samples. Findings of this study conclude that *COL4A1* gene polymorphisms were not associated with the risk of CAD. However further investigation with a larger sample size may be required to validate this study.

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