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Research Article

Epigenetics and Apo-E Promoter Methylation: Association of Apo-E Promoter Methylation in Pathogenesis of Atherosclerosis and Cardiovascular Diseases

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Abstract

Cardiovascular diseases are the leading cause of death worldwide. Epigenetics and environmental factors act as a major risk factor for atherosclerosis and cardiovascular diseases. In our present study, we detected aberrant promoter methylation of Apo-E genes involved in atherosclerosis and cardiovascular diseases to investigate their possible role in major metabolic pathways that result in pathogenesis of CVDs. The possible outcome of this research will help to better understand CVDs. In this present study, we analyzed 290 blood samples consisted of 200 patients and 90 respective controls from IDPs migrated from different regions of FATA K.P.K Pakistan to districts Bannu and LakkiMArwat. DNA was extract from their blood for epigenetic study. DNA methylation was analyzed by Bisulfate modification. Bisulphite treated samples were then amplified with methylated and unmethylated specific primers in a PCR reaction and result was visualized in gel electrophoresis. Our results indicate that epigenetic status of Apo-E promoter methylation may provide new tools to control atherosclerosis-related cardiovascular diseases.

Keywords: *Cardiovascular diseases; Epigenetics; Apo-E genes; Pathogenesis; Methylation*

Introduction

Atherosclerosis is one of cardiovascular diseases. The name comes from Greek words *athero* (meaning gruel or paste) and *sclerosis* (hardness). Atherosclerosis is caused due to hardening and narrowing of coronary arteries of heart by formation of plaques. The main causative factors for atherosclerosis development are lipoproteins, which play a key role in development of atherosclerotic and cardiovascular disease (CVD) in humans [1].

Lipoprotein consists of esterifies and un-esterified cholesterol, phospholipids, triglycerides and protein. Protein components of lipoprotein are known as apo-lipoproteins (Apo) or apo-proteins. The different apo-lipoproteins serve as cofactors for enzymes and ligands for receptors [1].

There are two type of lipoproteins; High Density Lipoproteins (HDL) and Low Density Lipoproteins (LDL), respectively. High levels of LDL are associated with coronary atherosclerosis, whereas high levels of HDL appear to protect against cardiovascular diseases. The levels of lipoproteins in plasma are determined by apo-lipoproteins present on their surface. Apo-lipoproteins (Apo) function as ligands for various receptors and determine metabolic fates of lipoprotein. This gives rise to hypothesis that mutations in genes coding for any of apo-lipoproteins may result in impaired clearance of lipoproteins. Genetic variation could thus be a major determinant of inter-individual variation in susceptibility to coronary artery disease (CAD) [1].

Apo-lipoproteins E (Apo E) are a class of apol-ipoproteins found in Intermediate-density lipoprotein (IDLs) and Chylomicron, which binds to a specific receptor on liver cells and peripheral cells. It is essential for normal catabolism of triglyceride-rich lipo-protein constituents [2]. Apo-lipoproteins E (Apo E) are normally present in plasma at 5 mg/dl [3]. It is a 299 amino acid peptide [2]. The Apo E gene is located from base pair 45,409,038 to base pair 45,412,649 on long (q) arm of chromosome 19 at position 13.2. It is 3.7 kb in length [3]. The Apo E gene consists of four exons and three introns, totaling 3597 base pairs [4].

Apo E plays a key role in transport of lipoproteins, and it is involved in numerous processes in arterial wall [3]. Apo-lipoproteins E is a major component of a specific type of lipo-protein called very low-density lipoproteins (VLDLs). VLDLs remove excess cholesterol from blood and carry it to liver for processing. Apo-lipoproteins E also appear to be involved in repair response to tissue injury [3]. In your present study, we analyzed blood samples of cardiovascular IDPs patients from migrated from different regions of FATA K.P.K Pakistan to districts Bannu and LakkiMArwat.

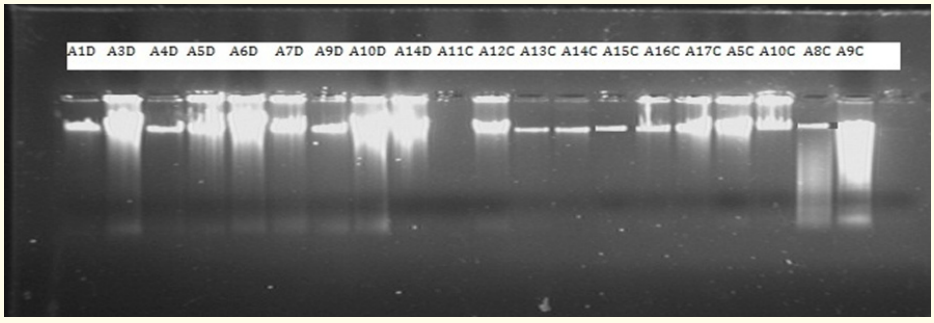

Figure 1: *Gel electrophoresis of the ethidium bromide stained 1% agarose gel, AC represents control samples whereas AD represents disease samples.*

Materials and Methods

Sample collection

Blood samples were collected in BD Vacutainer EDTA tubes from D.H.Q Hospital Bannu, T.H.Q Hospital Serai Naurang and D.H. Q Hospital LakkiMarwat. Only blood of (atherosclerosis) cardiovascular patients was collected with consent of concerned Ethical Committee. In this work, 290 samples were studied in which 200 were disease patients and 90 was respective control. 5cc peripheral blood was injected from study population and collected in EDTA tubes then stored at 4°C. Complete clinical history of patients and respective control individuals was recorded for epidemiological analysis.

DNA Extraction

The DNA was extracted from blood samples of study population through Phenol chloroform extraction protocol (Organic Method) by using solution A, solution B, solution C (24:1), Solution D, Solution C+D protinase K, 20% SDS, CH_3COONA (3M,Ph 6), isopropanol, 70% ethanol, T.E buffer/PCR water. The solutions are shown in Table 1. 0.5 ml of blood was taken in 1.5 ml eppendorf tube. To this 0.75 ml of solution A was added and inverted it and then placed it for 10 to 15-20 mins. After this it was centrifuged for 3 min at 13000 rpm. The supernatant was discarded and 400 µl of sol A was added to it. This mixture was centrifuged for 3 mins and discarded the supernatant and 400 µl of sol B was added in it. To this mixture 12 µl of 20% SDS and 25 µl of Protinase K was added. Brocken the pellet and place it

at 37°C overnight. On next morning 0.5 ml of sol C+D was added to the overnight mixture and then centrifuged it for 10 mins at 13000 rpm. To this solution C was added in new layer and shacked well. It was centrifuged for 10 mins at 13000 rpm. The supernatant was discarded. 55 µl of CH_3COONa and 500 µl of isopropanol were added to the pallet and inverted the tubes for several times to precipitate DNA. After this, it was centrifuged for 4 mins and discarded the supernatant. The DNA was washed by 70% ethanol (400 µl) and centrifuged for 3 mins at 13000 rpm. Discarded the supernatant and air dry the tubes were placed in inverted form. 30 µl of PCR water was added for further analysis.

Solution A	Solution B	Solution C	Solution D (24:1)
0.32 M Sucrose	10 mM Tris pH 7.5	Buffered phenol	Chloroform (24 ml)
10 mM Tris pH 7.5	400 mM NaCl		
5 mM $MgCl_2$	2 mM EDTA pH 8.0		Isoamy Alcohol (1 ml)
1% Triton X-100			

Table 1: *Composition of Solution Used In DNA Extraction.*

Agarose Gel Electrophoresis

The extracted DNA samples were analyzed by gel electrophoresis using a 1% agarose in 100 ml TE buffer (tris base, boric acid and EDTA (pH 8.0) and stained with 5 µ lethidium bromide. The gel was run for 35-40 minutes. The results was visualized and analyzed by GEL documentation.

Primer Design

1000 bp upstream region of Apo E is selected for primer design as shown in Table 2. For designing primers methylation PCRs METHprimer software was used. The DNA sequence was taken as input and searched the sequence for potential CpG islands. Primers were picked around predicted CpG islands and or around regions specified by users. Results of primer selection are delivered through a web browser in text and in graphic view.

Primers	Types	Primer Sequence	Tm value °C	GC%
Apo EML	Methylated	TTAGGTTTTATTTGTAAAGTTTCGA	62	64.34
Apo EMR	Methylated	AAAAAACTAAAAACGATAACTCACG	64	65.98
Apo EUL	Unmethylated	AAAATTTAGGTTTTATTTGTAAAGTTTTG	68	65.29
Apo EUR	Unmethylated	AAAAACTAAAAACAATAACTCACACC	66	66.58

Table 2: *Lists of Primers Used.*

Bisulfite Treatment

For bisulfite treatment 4 µg of DNA was added to a sterile 1.5 ml eppendorf tube and made final volume up to 24 µl by addition of PCR water. To this 5 µl of 3M NaOH was added to this and incubated for 15 min at 37°C. To incubated mixture, 30 µl of hydroquinone solution and 530 µl of $NaHSO_3$ solution were added. By slowly addition an over layer of mineral oil (100 µl) was made and incubated for overnight at 45°C. The overnight incubated mixture was centrifuged to ensure DNA in bottom and then it was taken leaving mineral oil behind. The above reaction mixture was div0ided into two separate tubes equally avoiding mineral oil.

On next morning, reaction mixture was taken and processed using Silica Bead DNA Extraction Kit (Ferments) by using Silica Bead DNA Extraction Kit protocol. It was then stored at -20°C for desulfonation. For desulfonation 5 µl of 3M NaOH was added to 40 µl of DNA and incubated for 15 min at 37°C. To this 20µl of 5M ammonium acetate solution and 20 µl of ice cold isopropanol was added. Kept it

at -20°C for 1 hour and centrifuged for 3 mins at 14000 rpm. The supernatant discarded and washed the pellet by adding 1 ml of 70% ethanol. Centrifuged it for 3 mins for 14000 rpm and discarded the supernatant. The pellet dried at room temperature and dissolved it in 45 µl 0.1X PCR water. The DNA was stored at -20°C until use. Polymerase Chain Reaction (PCR). The PCR reaction was performing in BIOTER XP cycler.

Gel Electrophoresis

The PCR products were analyzed by gel electrophoresis using a 2% agarose in 100 ml TE buffer (tris base, boric acid and EDTA at pH 8.0) and stained with 5 µl ethidium bromide. Gel was visualized and analyzed by UV Gel Documentation System (BioRAD, UK).

Statistical Analysis

Statistical analysis includes different characteristics contribution in atherosclerosis i.e. hypertension, diabetes, smoking, cholesterol, blood pressure, life style, over weight and family history with respect to their age. Our statistical analysis shows that mean age of our patients was 50. Different percentages were also calculated during analysis regarding the area involved in atherosclerosis disease.

Results

Main aim of this study was to detect rule of mutation in promoter region of Apo E genes among atherosclerosis patients among Pakistani population. The present study included a total of 290 samples consisted of 200 patients and 90 respective controls. For this purpose blood sampling of cardiovascular patients and respective control individuals was done with consent of patients and ethical committee of D.H.Q hospital Bannu, D.H.Q Hospital LakkiMarwat and T.H.Q Hospital Serai Naurang. The patients were IDPs migrated from FATA regions. This study was carried out between 2013-2014 supported by ZAHRA TRUST AND FREE MEDICAL CLINICS (Serai Naurang, K.P.K, Pakistan).

Optimization of Meth Primers

This study was conducted to detect methylation in promoter region of Apo E genes of patients of Pakistani origin. Polymerase chain reaction was used to amplify specific exonic and intronic regions of Apo E gene through primers. 2% agarose gel was then used for visualization of amplified DNA products through PCR. PCR products were further visualized via UV Gel Documentation System (BioRAD, UK), for Bisulfite modification study, through Product size and banding pattern analysis of PCR products.

For optimization of METH primers PCR was done on controls samples at following temperatures 58°C, 60°C and 62°C shown in Table 3. For optimization primers were used at three different concentrations (1.5 µl, 1 µl and 2 µl)and two different quantity of DNA was used (2 µl and 3 µl). In few of samples NP 40 was add to avoid primer and template DNA hybridization. Best result was obtained at 60°C with 1 µl primers concentration and 2 µl DNA quantities. Figure 2; shows some PCR results, representing amplified PCR products of methylated primer at specific band size i.e.100 bp.

Steps	Temperature	Time	Cycle
Initial Denaturation	95°C	4 min	1
Denaturation	94°C	45 sec	30-35
Annealing	58-60°C	45 sec	
Extension	72°C	1 min	
Final Extension	72°C	10 min	1

Table 3: *Conditions of PCR.*

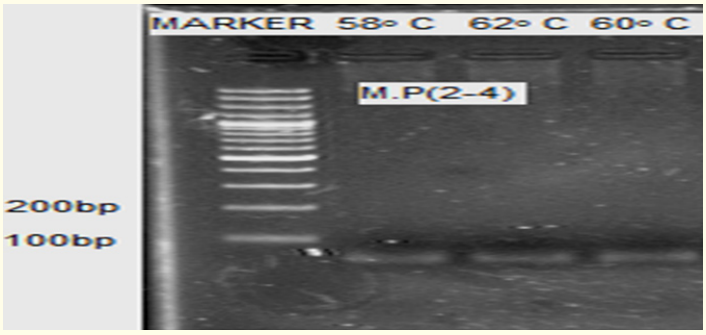

Figure 2: *Amplified products of methylated primer, PCR product of controls sample.*

Bisulfite Modification Analysis

Bisulphite treated samples were amplified with methylated and unmethylated specific primers in a PCR reaction at 60°C. Result of bisulfite treated PCR product were analyzed on 2% agarose gel as shown in figure 3.

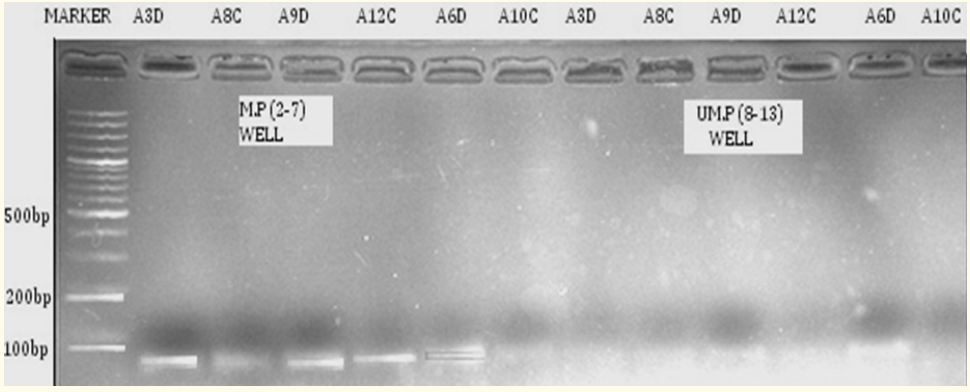

Figure 3: *Gel Electrophoresis with 8% ethidium bromide, methylation and unmethylation related to disease and control samples.*

Discussion

The contribution of epigenetic mechanisms to cardiovascular diseases (CVD) remains unclear. Hypo-methylation of genomic DNA is present in human atherosclerotic lesions and methylation changes also occur at promoter level of several genes involved in pathogenesis of atherosclerosis, such as extracellular superoxide dismutase, estrogen receptor-α, endothelial nitric oxide synthase and 15- lipoxygenase. So far, no clear data is available about histone modification marks in atherosclerotic lesions [5,6]. It remains unclear whether epigenetic changes are causally related to pathogenetic features, like clonal proliferation of lesion smooth muscle cells, lipid accumulation and modulation of immune responses in lesions, or whether they merely represent a consequence of ongoing pathological process [7].

Disease		Control	
Methylated	Incomplete Methylation	Methylated	Incomplete Methylation
77%	23%	65%	35%

Table 4: *Percentage of Methylation in Promoter Apo E Vary In Diseased and Control Samples.*

Atherosclerosis is a chronic disease of large and medium sized arteries that is characterized by accumulation of cholesterol in arterial wall together with proliferation of arterial smooth muscle cells (SMC) and accumulation of extracellular matrix components, which lead to occlusion of blood vessels, myocardial infarction, peripheral vascular disease, amputations, aneurysms and stroke [5,8,9]. Chronic inflammatory response with infiltration of monocytes macrophages and T-cells and endothelial dysfunction are also prominent features of atherogenesis [10].

Increasing evidence points to dietary lipids and their derivate as dynamic modulators of pro- or anti-inflammatory gene expression pathways via their ability to interact with nuclear receptors that are central to the regulation of numerous biological functions, including lipid metabolism, inflammatory mediator production, and vascular homeostasis [8,9]. The biological effects of these receptors are result of a finely tuned equilibrium between gene activation and repression, resulting from their ability to switch between chromatin remodeling; co- repressor and co-activator partners. We proposed that atherosclerosis might have a strong epigenetic basis.

Deficiency of folate, vitamin B_6, and B_{12}, all of which are necessary to maintain an adequate level of available methyl groups, might be physiological causative links for cardiovascular diseases (CVD) and atherosclerosis by inducing aberrant DNA methylation. The role of nutrition in CVD and atherosclerosis has already been developed in number of studies [5,6,8,9]; how these dietary effects link to disease phenotype at physiologic level and how they cause abnormal gene expression of factors involved in lipid metabolic pathway remain elusive. In our study we predict the role of methylation in promoter region of Apo E gene.

Our results show that Apo E promoter region in cardiovascular patients got hyper-methylated in 77% of cases studied whereas in 23% cases we detect hemi-methylation as shown in table 4. This highlights fact that in these individuals promoter methylation was not complete and some of the CpG sites remained unprotected. This might also be related to level of disease severity or other dietary factors. This result indicates that Apo E promoter methylation can be an important event in development of atherosclerosis and cardiovascular diseases. In normal individuals 65% promoter methylation and 35% hemi-methylation was found. These interesting results tell that promoter methylation of Apo E gene is an important phenomenon for normal gene expression as have already been mentioned by many researchers. Our results need a more rigorous examination keeping in mind shortcomings of study. Moreover, bisulphite conversion and specificity of primers are very important for efficient detection of promoter methylation. Whether promoter methylation is a causative agent for atherosclerosis or merely a consequence of disease pathogenesis remain elusive and further investigation in this direction might help to fully understand the problem.

Conclusion

In conclusion our study has demonstrated that Apo E promoter methylation can be an important event in pathogenesis of atherosclerosis and cardiovascular diseases. These interesting results tell that promoter methylation of Apo E gene is an important phenomenon for normal gene expression as have already been mentioned by many researchers. Our results need a more rigorous examination keeping in mind shortcomings of study. Whether promoter methylation is a causative agent for atherosclerosis or merely a consequence of disease pathogenesis remain elusive and further investigation in this direction might help to fully understand the problem.

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