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A Genetic Variant (S4338N) in *Apolipoprotein B* Gene in Hypercholesterolemic Families from Pakistan

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ABSTRACT

Mortality due to coronary heart disease (CHD) has increased in recent decades and risk of CHD increases many folds in patients with hypercholesterolemia. Familial hypercholesterolemia (FH) is a genetic disorder associated with autosomal dominant mutations in any of 3 genes namely *LDLR*, *ApoB*, and *PCSK9*. We aimed to assess genetic variations in exon 29 of apolipoprotein B (*ApoB*) gene in FH patients from 10 affected families of Punjab origin. A guanine to adenine change (c.G13013A; rs1042034) was detected in 9 families, resulting in serine to asparagine change (p.Ser4338Asn, S4338N). The S4338N polymorphism was further screened in 95 CHD patients and 96 healthy individuals with normal cholesterol level to determine its relation with CHD. The distribution of S4338N polymorphism was found to be significantly different in CHD and control groups (P = 0.0008; RR (95% CI) = 0.685 (0.543, 0.864); Odds Ratio (95% CI) = 0.486 (0.319, 0.739), suggesting that S4338N polymorphism in *ApoB* gene may have minor contribution in early development of coronary heart disease and therefore can be considered as a genetic marker for disease risk assessment.

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AA and MEB conceived and designed the study. AA, ARA and MT performed the experiments. AA, WS and MI analyzed the data. AA, MEB and MI wrote the article.

Key words:
Familial hypercholesterolemia, *Apolipoprotein B*, autosomal dominant, polymorphism, risk assessment.

INTRODUCTION

Globally, cardiovascular diseases (CVDs) are the major causes of mortality and morbidity, recently and in coming decades (Mathers and Loncar, 2006). About 17.3 million mortalities occurred due to CVDs in 2008 which represents 30% of world mortality. An estimated 7.3 million deaths out of 17.3 million occurred due to coronary heart disease (CHD) (WHO, 2011a,b). Majority of the death incidences due to CVDs occur in developing countries and distributed equally in both the genders (WHO, 2011b). An estimation indicate that 23.3 million people will die to CVDs by 2030 (WHO, 2011b; Mathers and Loncar, 2006). The elevated level of cholesterol is a main contributing factor in premature CVD development (Wilson *et al.*, 1998). The elevated level of total cholesterol and low density lipoprotein (LDL) cholesterol is known as hypercholesterolemia. Familial Hypercholesterolemia (FH) occurs in an autosomal dominant manner and is characterized by substantially elevated levels of low density lipoprotein cholesterol (LDL-C). The risk of coronary heart disease increases five to eight folds in FH cases (Marks *et al.*, 2003). Prolonged elevated cholesterol begins to deposit in peripheral tissues and accelerate the atherosclerosis leading to premature cardiovascular events (Alonso *et al.*, 2009). The Simon Broome Register criterion is used for clinical diagnosis of FH which includes elevated LDL-C level (>4·9 mmol/L) and family history of high cholesterol or cardiovascular disorders.

Such patients are designated as having possible FH. Those patients who manifest prolong hypercholesterolemia resulting in tendon xanthomas are categorized as having definite FH (Marks *et al.*, 2003, Wierzbicki *et al.*, 2008). The prevalence of FH is about 1 in 500 people in most of the Caucasian population. FH is known to be caused by genetic variations in LDLR (Low density lipoprotein receptor), *ApoB (Apolipoprotein B) and PCSK9 (Proprotein convertase subtilisin/kexin type 9)* genes (Rader *et al.*, 2003). Mutation detection rate in above described three genes is about 40% and remaining 60% of the patients might have polygenic cause of FH (Taylor *et al.*, 2010). Meta-analysis of genome wide association studies have identified a number of variants linked with LDL-C concentration (Teslovich *et al.*, 2010) and cumulative effect of multiple variant have shown FH as a polygenic trait (Kathiresan *et al.*, 2009).

The *ApoB* gene is located at 2p24 position (Blackhart *et al.*, 1986) and spans 43 kb with 29 exons (Knott *et al.*, 1985). The *ApoB* mRNA is 14.1 kb long which encodes 4563 amino acid polypeptide molecule including a peptide signal of 27 amino acids (Chen *et al.*, 1986). *ApoB* gene expresses in two isoforms as ApoB-48 in chylomicron and ApoB-100 in the liver. ApoB-100 is an important protein moiety of LDL. ApoB-100 protein is

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non-exchangeable largest known monomeric protein molecule comprised of five domains. ApoB protein molecule is amphipathic (hydrophobic and hydrophilic) in nature and can interact with aqueous environment as well as with the lipid part of lipoproteins (Segrest *et al.*, 1994a,b, 2001). Inability of *ApoB* mRNA editing leads towards high level of LDL and atherosclerosis in coronary vessels (Powell-Braxton *et al.*, 1998). ApoB level determination is more informative for CHD risk assessment than lipids and lipoproteins (Demacker *et al.*, 2000). Genetic variations within *ApoB* gene execute impact on lipid metabolism and relate with susceptibility to CHD in effected individuals (Ye *et al.*, 1995). Genetic variations in *ApoB* may associate with its increased or decreased levels and with LDL-C (Humphries, 1988).

The aim of this study was to investigate the genetic variations in coding region of exon 29 of ApoB-100 encoding gene in hypercholesterolemic families and their prevalence among CHD patients of Punjab origin.

MATERIALS AND METHODS

Sample selection

Based on family history and estimates of lipid levels, ten families with hypercholesterolemia were identified from Punjab, Pakistan. Hypercholesterolemia was diagnosed following the Simon Broome Register criterion of LDL cholesterol > 4.9mmol/L and total cholesterol > 7.5mmol/L (Marks *et al*., 2003; Wierzbicki *et al.*, 2008). Tendon xanthomas were not present in any family while strong history of premature coronary heart disease was present in all families (http://www.ncbi.nlm.nih.gov/books/NBK53810/table/appendixes.app6.t1/?report=objectonly).

Pedigrees were drawn to analyze the pattern of inheritance. Family members with hypercholesterolemia and normal cholesterol levels were screened after their willingness and were included in the study after informed consent.

We also selected clinically diagnosed 95 CHD patients (62 males and 33 females) from Mayo Hospital, Lahore. CHD patients were diagnosed on the basis of clinical symptoms, changes in electrocardiogram and biomarkers values (Antman *et al.*, 2000). The control group was selected by eliminating the individuals having family history of CVD or hypercholesterolemia. Control group consisted of 96 individuals (56 males and 40 females) with the same age range and was chosen by random selection after informed consent. This study was approved by the ethical committee of Bioequivalence Study (BeSt) Center, University of Veterinary and Animal Sciences, Lahore. Blood samples were collected from all the subjects after a fast of 12-16 h. Serum samples were stored for lipid assay.

Determination of lipid levels

Serum samples were used for estimation of total cholesterol, triglycerides and HDL-C by using UV-spectrophotometry after following the manufacturer's instructions. LDL-C was calculated by using the formula (Friedewald *et al.*, 1972). Lipid values of FH and control group are given in Table I.

Table I.- Mean age (year ± SD) and lipid values (mmol/L ± SD) for individuals included in this study. SD: Standard deviation, TC, total cholesterol; TGL, triglycerides; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.

Variables	Control (n=96)	FH-probands (n=10)	*P*-value	
Age	39.7±3.5	41±4.9	0.072	Not significant
TC (mmol/L)	4.4±0.6	7.4±1.1	0.001	significant
TGL (mmol/L)	5.3±2.3	5.1±1.9	0.082	Not significant
HDL-C (mmol/L)	1.2±0.2	1.1±0.1	0.075	Not significant
LDL-C (mmol/L)	2.1±0.5	6.1±1.6	0.001	significant

DNA extraction and amplification

Blood samples were collected in vacutainers containing EDTA (Ethylene Diamine Tetra Acetic acid). DNA was extracted by standard organic method and quantified using NanoDrop (ND2000, Thermo Scientific) and agarose gel electrophoresis. Forward primer 5' TCAAAAGAAGCCCAAGAGGT3' and reverse primer 5'GGGAAGTAAAGTTAGAGGCACTG3' were designed using primer3 software (Koressaar and Remm, 2007) to PCR amplify the 475 bp segment of exon 29 of *ApoB* gene. Initial denaturation of template DNA at 95°C for 5 min and cyclic denaturation for ten cycles at 94°C for 30 sec, annealing temperature at 60°C for 30 sec with decreasing 1°C for every cycle, extension at 72°C for 45 sec were carried out in Bio-Rad thermocycler. Additional twenty five cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec were repeated with a final 10 min extension. PCR mixture for each reaction was prepared using *Taq* DNA polymerase.

Sequencing of amplicons

PCR amplicons of exon 29 of *ApoB* gene of all

probands were purified and precipitated with 64% ethanol and then sequenced by Sanger method using genetic analyzer ABI 3130xL.

Restriction analysis of myocardial infarction patients

CHD patients and control individuals were screened for S4338N polymorphism using restriction fragment length polymorphism (RFLP) analysis using DdeI (Fermentas, USA) enzyme. DdeI recognizes 5'CTNAG3' sequence, therefore a single base change was induced (T to C) in forward primer sequence 5'AGATGAGATCAACACAATC**C**TCA3' to create the restriction site; the sequence of reverse primer was 5' GGGAAGTAAAGTTAGAGGCACTG3'. PCR was performed on the same conditions as described above to amplify the 314 bp segment of exon 29 of *ApoB* gene. To analyze the restriction fragment length polymorphism, 18 µL of amplicons, 2U of enzyme, 1 µL (10×) Tango buffer in a final volume 20 µL were incubated overnight at 37°C. Ethidium bromide stained agarose gel (4%) was used to visualize the fragments.

Statistical analysis

Allele frequency was computed and level of significance for Hardy-Weinberg Equilibrium (HWE) was determined by Chi-square test using PopGene software (https://www.ualberta.ca/~fyeh/popgene.html). Fisher's exact *p*-value, Relative Risk and Odds Ratio with 95% confidence interval were calculated to find out whether the polymorphism is associated with the disease (http://www.socscistatistics.com/tests/fisher/default2.aspx).

RESULTS

Sequencing of *ApoB* gene of all FH probands revealed a guanine to adenine change (c.G13013A; rs1042034) in 9 families (Fig. 1), resulting in serine to asparagine change (p.Ser4338Asn, S4338N). Four families (PAFH1, 4, 7 and 8) were heterozygous and five (PAFH 2, 3, 5, 6 and 10) were homozygous for allele A. Only one family (PAFH9) showed homozygousity in the sequence at G allele. Genotyping pattern of ten families did not differ significantly from the Hardy-Weinberg principle.

According to RFLP analysis, 9 (8.5%), 39 (39.9%) and 47 (46.6%) of CHD patients had GG, GA and AA genotypes, respectively, while 23 (23.9%), 44 (45.8%) and 29 (30.2%) of control individuals had GG, GA and AA genotypes, respectively. The observed genotype frequencies did not significantly differ from Hardy-Weinberg Equilibrium as shown in Table II.

The CHD and control groups had different allele frequency for G (30%, 47%) and A (70%, 53%) alleles, respectively. To test the significance of risk and normal allele in both groups, Fisher's exact test was used (http://www.socscistatistics.com/tests/fisher/Default2.aspx). Fisher's exact test with 95% confidence level at 1 degree of freedom showed association of the polymorphism with the disease (Table III). Values of relative risk (95% CI) = 0.685 (0.543, 0.864) and odds ratio (95% CI) = 0.486 (0.319, 0.739) also suggested an association between S4338N polymorphism in the *ApoB* gene and coronary heart disease.

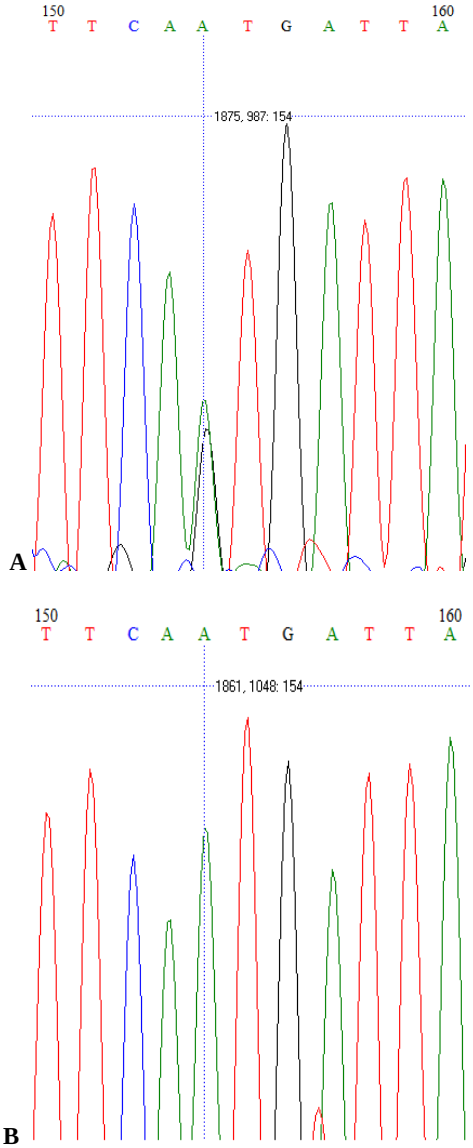

Fig. 1. Sequence of exon 29 of *ApoB* gene heterozygosity (A) and homozygosity (B) for nucleotide change G>A.

Table II.- Chi-square test for testing significance of relation of S4338N polymorphism with CHD (CHD: Coronary heart disease. Sig: Significance, NS: Not significant, df: degree of freedom).

ApoB gene	Genotype and Allele	CHD Group n=95	%	Chi-square value Obs	Exp	*P*- value	Sig. level	Control Group n=96	%	Chi-square value Obs	Exp	*P*- value	Sig. level
4338	GG	9	8.5	9	8.5	0.83	NS at 1 df	23	23.9	23	21.1	0.4346	NS at 1 df
	AG	39	39.9	39	39.9			44	45.8	44	47.8		
	AA	47	46.5	47	46.5			29	30.2	29	27.1		
Allele	G	57	30.0					90	47.0				
	A	133	70.0					102	53.0				

DISCUSSION

Cholesterol in plasma correlates with LDL (low density lipoprotein) concentration. ApoB-100 is a key structural part of LDL. *ApoB* gene codes for 4563 amino acids and first 27 amino acids act as peptide signal and are cleaved off during maturation (Chen *et al.*, 1986). Single nucleotide polymorphisms in *ApoB* gene have a significant effect among the individuals with high and low risk of coronary artery disease (Heng *et al.*, 1999). In the current study, sequencing of exon 29 of *ApoB* gene in members of Hypercholesterolemic families with elevated level of TC and LDLC showed a single nucleotide base change G>A (c.G13013A), resulting in change of amino acid serine to asparagine (p.S4338N). G allele encodes for serine while A encodes for asparagine. This polymorphism is designated as rs1042034 and resides at 4311 position of processed protein molecule sequence. Coronary heart disease (CHD) and normal individuals were also genotyped for S4338N polymorphism. Genotype frequency did not differ significantly in both groups and they were in Hardy-Weinberg equilibrium. To the best of our information, S4338N has not been screened in Pakistani FH and Cardiovascular patients. Allele frequency of G allele was 30% and 47%, and of A allele was 70% and 53% in CHD and control groups, respectively. The allele frequencies differed significantly between two groups ($P < 0.05$), suggesting a relation of S4338N with CHD. Asparagine to serine mutation was found in 24% of 81 unrelated individuals (Navajas *et al.*, 1990). A diverse population based genotyping data with respect to one base change A>G at 4338 amino acid residue position suggest that recombination at the 3' end of *ApoB* gene is less frequent in human (Dunning *et al.*, 1992). Serine allele is more frequent in Caucasian population of Taiyuan (Evans *et al.*, 1993). Japanese population also has 68.9% serine allele frequency for *ApoB* gene. In a control study, the asparagine allele at 4338 position of *ApoB* gene was associated with elevated level of ApoB than to the serine allele. A higher Asn/Asn genotype frequency was found in a group with higher values of total cholesterol (>200 mg/dL) and apoB (>85 mg/dL) than in a group with lower values of total cholesterol (<200 mg/dL) and ApoB (<85 mg/dL) (Wu *et al.*, 2001). Significantly higher serum fractions of apoB proteins among Pakistani MI patients support this molecule as a risk predictor marker (Siddiqui and Cheema, 2009). Association between twelve polymorphisms of *ApoB* gene and plasma apoB levels in 1442 European subjects, suggests polymorphism at 4338 position as an informative polymorphism in relation to ApoB levels (Tahri-Daizadeh *et al.*, 2004). *ApoB* gene polymorphism S4338N was studied in French and Irish

Table III.- Fisher's exact test for testing significance of relation of S4338N polymorphism with CHD. CHD, Coronary heart disease; RR, Relative Risk; CI, Confidence Interval.

ApoB gene	Genotype and Allele	CHD group		Control group		Fisher's exact *P*-value	RR	CI	Odds ratio	CI
		n=95	%	n=96	%					
Allele	G	57	30.0	90	47.0	0.0008	0.685	0.543, 0.864	0.486	0.319, 0.739
	A	133	70.0	102	53.0					

population and associated with lipoprotein profile variation in coronary artery disease patients (Moreel *et al.*, 1992).

In conclusion, our hypercholesterolemic families' data suggest that S4338N polymorphism in *ApoB* gene is related with higher levels of total cholesterol and LDLC. Our finding of significant difference ($P<0.05$) of S4338N alleles frequency between coronary heart disease patients and control individuals demonstrates its contribution as a risk factor for CVD development. Moreover, values of relative risk (0.685) and odds ratio (0.486) demonstrate that individuals carrying allele A are at ½ times more risk for developing CHD compared with those carrying allele G.

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