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Study on Association of APOB Gene Polymorphism with Glycation of Low Density Lipoproteinin Type 2 Diabetes

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Abstract

Objectives: Sustained hyperglycemia results in non-enzymatic glycation of Apo B or LDL particle which may affect its recognition and uptake. Increase in circulating LDL levels is a vital contributor for atherosclerosis. APOB gene (c.12669 G>A, p.GLN4154LYS) polymorphism is believed to be associated with coronary artery disease. We thus designed our study to evaluate association of APOB gene polymorphism with Apo B glycation in type 2 diabetic patients.

Methods: A total of 45 non diabetic controls and 45 type 2 diabetic patients participated in this study. Following an overnight fast, venous blood was collected and analyzed for glycemic status, lipid profile and other biochemical parameters. Apo B was estimated using nephelometry, Glycated LDL was estimated by ELISA. PCR-RFLP was used to determine the DNA polymorphism in the APOB gene using EcoR1.

Results: Polymorphic analysis of APOB gene in diabetic population showed 73.3% wild type (R+/R+), 20.0% heterozygous mutant (R+/R-) and 6.7% homozygous mutant (R-/R-). Significant associations of glycated LDL was observed with R-/R- and R+/ R- when compared with R+/R+. Significant association was not observed between Apo B levels and of genotypes.

Conclusions: Presence of polymorphism may not affect the expression Apo B level but acts as an important contributor to LDL modification and increases its glycation. Since glycation of LDL reduces uptake of LDL by LDL receptors, it may increase the risk of atherosclerosis.

Keywords: Apo B; Glycated LDL; Percent glycated apoB; Diabetes mellitus; Gene polymorphism; Lipid profile

Abbreviations: Apo B: Apolipoprotein B; Ages: Advanced Glycated End Products; T2DM: Type 2 Diabetes Mellitus; CAD: Coronary Artery Disease; ADA: American Diabetic Association; BMI: Body Mass Index; ELISA: Enzyme Linked Immunosorbent Assay; GOD-POD: Glucose Oxidase- Peroxidase; Hb: Hemoglobin; HPLC: High Performance Liquid Chromatography; HDL-C: High Density Lipoprotein Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol; PCR: Polymerase Chain Reaction; SNP: Single Nucleotide Polymorphism; WC: Waist Circumference

Introduction

Chronic hyperglycemia is an important etiologic factor resulting in macro and microvascular complications in diabetes mellitus (DM). The most common and life threatening disorder in type 2 diabetics is cardiovascular disease (CVD). The risk for death by CVD among diabetic subjects is greater by three folds when compared to non-diabetic subjects [1]. The risk of CVD is markedly increased in patients with poor glycemic control [2]. Increased extracellular glucose leads to non enzymatic glycation of various proteins resulting from interaction of glucose with free amino groups of lysine residue of protein. Sustained hyperglycemia, through advanced glycated end products (AGEs) is potential contributor for glycation of low density lipoprotein (LDL) [3]. The modification by AGEs can occur at Apo B as well as phospholipids components of LDL [3]. Increased non-enzymatic glycation of Apo B containing lipoproteins is one of the post-secretory modifications which impair its uptake and metabolism by the high affinity low density lipoprotein (LDL) receptors. This increases the residence time for the modified LDL in circulation and subendothelium, which is taken up by macrophages resulting in formation of foam cells and thus may contribute to increased risk of atherosclerosis [3].

Apolipoprotein B (Apo B) plays a central role in human lipoprotein metabolism and is encoded by APOB gene located on chromosome 2 [4]. Apo B-100 is ligand for LDL-receptor-mediated endocytosis of LDL particles. Raised serum ApoB levels are associated with increased risk of coronary artery disease (CAD) [5,6] and several APOB polymorphisms have been evaluated for their association with lipid levels and CAD in different populations [7-10]. One such polymorphism in the 29th exon (c.12669 G>A) results in Glu4154Lys amino acid substitution. This polymorphism can be detected by using EcoRI restriction enzyme. Gene polymorphism may have its consequences ranging from effect on structure function relationship of a protein or alteration in protein expression level. Role of the APOB gene polymorphism in coronary artery disease in the Indian Punjabi population has been evaluated by Sharma et al. [8]. Thus we designed this study to evaluate the effect of APOB gene polymorphism on levels of Apo B containing lipoproteins and their glycation.

Methods

The present cross sectional case control study was carried out in

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the Department of Biochemistry and Medicine, at University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi-110095, between Nov 2012 and Apr 2013. Ethical clearance was obtained from the institutional ethical committee for human studies (UCMS/IEC-HR/BIO/261012 dated 26 Oct, 2012). All the patients were recruited from the Diabetic clinic and Medicine outpatient department (OPD) of GTB Hospital

Recruitment of patients and control

We recruited 45 diabetic patients in the age group of 40-60 years irrespective of the gender, as per to new guidelines of American Diabetes Association (ADA) 2013 [11].

All patients on lipid lowering therapy, Insulin therapy and pioglitazone therapy were excluded from the study. Patients on corticosteroids, retinoid drugs, immune suppressive drugs, beta-blockers, and steroid hormones were also excluded from the study. Forty five apparently healthy non diabetic non hypertensive controls in age groups of 40-60years were also recruited.

An informed written consent was obtained from each participant before recruitment, and each participant was provided with a patient information sheet. A detailed history was recorded and clinical examination including general physical examination was carried out before collecting blood samples.

Blood collection and biochemical analysis

For all subjects, blood samples were collected after an overnight fasting. Blood collected in plain vials were allowed to clot for 30 minutes and then it was centrifuged at 3000rpm for 10 min for serum separation. The serum thus separated was analyzed for routine biochemical parameters using Olympus AU400, Japan. Serum cholesterol [12], HDL cholesterol [13], and triglycerides [14] were analysed using commercially available kits on the autoanalyzer. Blood in fluoride vial for plasma glucose (fasting and 2 hour post prandial) was analysed using standard enzymatic method (GOD-POD method) [15]. LDL cholesterol was calculated using Freidwald formula [16]. Serum Apo B was estimated by nephelometry, using NEPHSTAR apolipoprotein B kit. Whole blood with EDTA was used for HbA1c estimation by HPLC method (BIO RAD, D-10). EDTA blood was subjected to centrifugation at 3000 rpm for 10 minutes to separate the plasma. The plasma thus separated was stored in aliquots at -80°C for estimation of glycated Apo B using commercially available kit (Glycacor TM by Exocell Philadelphia, USA) based on competitive ELISA. The glycated LDL was measured in apolipoprotein B equivalents, and was expressed as mg/dl. Percent glycated Apo B was calculated as Glycated Apo B/ Apo B

For genomic study 300 µl whole blood in EDTA was kept separately at 4-8°C. This blood was subjected to DNA isolation within 2 weeks of collection.

Polymorphic study for APOB gene

Genomic DNA was extracted from whole blood by commercially available kit (SIGMA DNA isolation kit™) as per the instructions provided by the manufacturer. PCR reactions were carried out in 20 µl reaction mixture using the forward and reverse primers for the 376 bp sequence with restriction site for EcoR1 in Apo B gene. 20 µl reaction mixture was prepared containing 2.5 µl genomic DNA, 200 µM dNTPs, 1.5 mM $MgCl_2$, Taq reaction buffers and 2 U Taq DNA Polymerase. The primer sequences were: forward primer (F) 5'--GCTCACCCTGAGAGAAGTGTCTFCA-3' and reverse primers (R) 5'--CATAGTGCAAAGTTCCTCCCTAGTG-3'. After initial melting temperature of 94°C (5 min), 30 cycles of denaturation, annealing, and extension (2 min at 94°C, 1 min 59°C and extension for 1min at 72°C) were carried out on a programmed thermocycler (Eppendorf Mastercycler Gradient-5331). A final 10 min extension step at 72°C terminated the process.

The PCR-products were then digested in 45 µl reaction mixture using 4U of EcoR1, BSA, (Genei Merck) and 12.5 µl of PCR mix. After activation step at 37°C for 45 minutes was followed by inactivation step at 65°C for 10 minutes. The products were run on 2.5% agarose gel in 1X Tris-acetate ethylenediamintetraacetic (TAE) buffer and ethidium bromide dye at a constant voltage of 50 V and the gel was visualised on the gel documentation system (UVITEC). Three types of bands were visualized. The cutting site for EcoRI was based on a single base-pair mutation atgene (c.12669 G>A). An uncut fragment with a size of 376 bp was defined as the R - allele; when digested, the two resulting sub fragments of 260 and 116 bp were defined as the R+ allele (Figure 1).

Statistical analysis

Data was analysed using SPSS software version 20.0. The data in the two groups were analysed using students't' test. The allele frequency was calculated using gene counting method. Comparison of the categorical data i.e., different APOB genotypes among controls and obese subjects was done by Fischer's exact test and $\chi 2$ test. Odd's ratios were calculated with a 95% confidence interval limit using 2×2 contingency table. Two way ANOVA was applied for all parameters taking group as 1st factor and polymorphism as 2nd factor. If interaction between groups and polymorphism was <0.15, we applied one way ANOVA to compare within group and Tukey's test for multiple comparisons. To compare between groups, t test was applied and p-value was adjusted as Bonn Ferroni correction i.e. 0.05/3≈0.02.

Results

The mean age in control group was 47 ± 7.18 years and in diabetic group 48.8 ± 5.84 years. In diabetic group, there were 26 (57.8%) males and 19 (42.2%) females, where as in control group, 29 (64.4%) males and 16 (35.5%) females were present. Hypertensive patients were identified according to the seventh report of the Joint National Committee on prevention, detection, evaluation and treatment of high blood pressure (JNC VII) [17]. 23 individuals (51%) in diabetic group were found to be hypertensive. Using waist circumference as the criteria for diagnosis of obesity [18], it was observed that 33 (73.4%) in diabetic group and 30 (66.6%) subjects in control group were obese. There were 4 (8.8%) smokers in diabetic group and 3 (6.6%) smokers in control group. Other clinical and anthropometric variables are compared in Table 1. Statistically significant difference was not

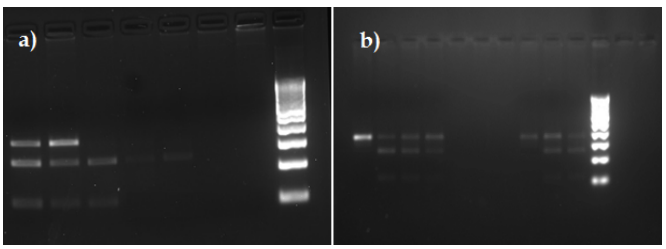

Figure 1: Agarose Gel Electrophoresis of Eco R1digested APOB gene after PCR amplification: (a) shows DNA marker in 11, R-/R- in 1 and 8, and R+/R- in 2,3,4,7,9,10; (b) shows DNA marker in 8, R+/R- in 1 and 2, R+/R+ in 3, 4 and 5.

observed in lipid profile between the two study groups (Table 1). Table 2 shows comparison of Apo B levels and glycated LDL between two groups. The frequency of R+ and R– alleles was calculated (with an allele counting method) in diabetic and non-diabetic group. Genotype variants and allele frequency in the two groups is given in Table 3. No deviation was observed on applying Hardy Weinberg Equilibrium test in the control group (p=0.196) as well as in diabetes group (p=0.067). Odds ratio revealed no significant association between genotypes with diabetes. On applying two way ANOVA, interaction was found to be significant only for Apo B, glycated LDL and percent glycated Apo B (<0.15). A significant difference was observed for glycated LDL and percent glycated apo B amongst genotypes in diabetic groups as shown in Table 4. While carrying out intergroup comparison for each genotype, it was observed that glycated LDL was higher in diabetic group in each genotype although difference was statistically significant only for R+/R+ (p=0.000). Similar pattern was observed for percent glycated apo B in which significantly higher values were observed for R+/R+ (p=0.010) in diabetic group. However significant difference was not observed in Apo B concentration for each genotype while carrying out within group and between groups comparison.

Discussion

Patients with diabetes frequently suffer from dyslipidemia which is characterized in part by defective lipoprotein uptake and metabolism [19-21]. Since earlier studies have indicated that APOB polymorphism is associated with CAD, we designed our study to analyse the effect of APOB polymorphism on lipid profile and extent of glycation of LDL. R^+ allele was found to be more common in our population. Similar results were reported from Punjab and Tamil Nadu also [8,22]. There was no statistically significant difference in the LDL-C or any other lipid parameters among different genotypes of APOB in our study population. This is in line with other studies on different ethnic populations in India [8,22]. Both studies observed no association between total or LDL cholesterol levels with different APOB genotypes in CAD patients as well as normal subjects [8,22]. In addition, Delghandi et al studied DNA polymorphisms of the APOB gene (XbaI, EcoRI, and MspI RFLPs) in Norwegians population and concluded that variations in the EcoR1 APOB gene polymorphism, does not affect the circulating blood lipids [7]. Contrary to all these studies, Timirci et al. observed that polymorphism of this gene affects total cholesterol and LDL-cholesterol levels in obese children in Turkish population [23]. These results indicate that G> A polymorphism has no effect on circulating lipids in most populations. However, association of genotypic variation with circulating lipids may vary in different ethnic populations.

Variables	Control group (n=45)	Diabetic group (n=45)	p value
Height (cms)	162.28 ± 7.75	154.42 ± 8.24	.000
Weight(kg)	70 ± 11.37	63.06 ± 10.12	0.003
Body Mass Index (kg/m2)	26.56 ± 4.29	25.63 ± 3.78	0.278
Waist circumference(cms)	90.12 ± 15.38	88.95 ± 14.83	0.323
Hip circumference (cms)	90.4 ± 17.00	89 ± 14.02	0.104
Waist Hip Ratio	0.98 ± 0.056	1.00 ± 0.057	0.134
Fasting plasma glucose (mg/dl)	89.73 ± 10.49	131.15 ± 40.41	0.000
Post prandial plasma glucose(mg/dl)	128.77 ± 16.30	199.97 ± 56.72	0.000
Hb1Ac (%)	5.38 ± 0.39	7.56 ± 1.99	0.000
Total cholesterol (mg/dl)	187.08 ± 24.014	197.24 ± 39.50	0.145
HDL- cholesterol (mg/dl)	45 ± 8.68	43.37 ± 7.45	0.344
LDL- cholesterol (mg/dl)	118.96 ± 19.76	128.25 ± 34.24	0.119
Triglycerides (mg/dl)	115.62 ± 39.60	128.044 ± 49.23	0.191
Values are expressed as mean ± SD, p<0.05 is significant			

Table 1: Clinical and biochemical profile among study groups.

Variables	Control group (n=45)	Diabetic group (n=45)	p value
Apo B (g/L)	1.08±0.37	1.20±0.42	0.887
Glycated LDL (mg/dl)	0.95±0.05	1.83±1.00	0.000
Percent glycated apo B	0.90±0.00	1.68±1.12	0.000
Values are mean ± SD, p<0.05 is significant			

Table 2: Comparison of Apo B and glycated LDL among study groups.

Polymorphic variants	Control group(n=45)	Diabetic group(n=45)	Odds ratio	95% CI	p value chi sq	
R+/R+	28(62.2%)	33(73.3%)	1.0		0.259	0.982
R+/R-	13 (28.9%)	9 (20.0%)	0.913	0.328-2.55	0.527	
R-/R-	4 (8.9%)	3 (6.7%)	0.913	0.185-4.50	1.00	
R+ Allele	69	75	0.776	0.371 -1.625		0.502
R- Allele	21	15	1			

Table 3: Polymorphic variants and allele frequency in study groups.

Parameters	Genotypes	Control group	Diabetic group
Total cholesterol (mg/dl)	R^+/R^+	180.18±25.39	200.33±38.79
	R^+/R^-	199.79±17.17	179.25±27.44
	R/R^-	188.50±19.09	214.00±79.64
HDL - Cholesterol(mg/dl)	R^+/R^+	43.93±9.10	43.33±7.65
	R^+/R^-	49.57±10.95	43.38±7.84
	R^-/R^-	55.00±7.70	44.67±8.08
LDL- Cholesterol(mg/dl)	R^+/R^+	114.96±20.63	129.46±33.58
	R^+/R^-	122.41±17.43	113.45±22.05
	R^-/R^-	118.35±9.24	143.73±65.36
Triglycerides (mg/dl)	R^+/R^+	106.64±38.55	137.73±50.9
	R^+/R^-	109.71±31.63	97.13±26.54
	R^-/R^-	117.00±44.74	128.0±38.12
Apo B(g/L)	R^+/R^+	1.11±037	1.24±0.44
	R^+/R^-	1.23±0.41	1.09±0.39
	R^-/R^-	1.06±0.17	1.10±0.44
Glycated LDL(g/L)	R^+/R^+	0.96±0.62	1.51±1.04
	R^+/R^-	0.86±0.51	1.84±0.97
	R^-/R^-	0.79±0.47	3.46±0.08*
Percent Glycated apo B	R^+/R^+	0.99±0.64	1.58±0.93
	R^+/R^-	0.83±0.61	1.62±1.16
	R^-/R^-	0.73±0.39	3.94±2.19[a,b]
**** R+/R- vs R-/R- p=0.043, [a]R+/R- vs R-/R- p=002, [b]R+/R+ vs R-/R- p=0.009***			

Table 4: Lipid profile among different genotypes in control and diabetic group.

The results of our study also reveal that glycation of LDL and ApoB is higher in diabetes and similar results have been reported earlier also [24-26]. On comparing the association of glycated LDL and percent glycation of Apo B with different genotypes, significantly higher glycation of LDL and ApoB was observed in individuals with R^- allele, being highest in individuals with homozygous R^- genotypes. This finding indicates that presence of SNP (G>A) i.e. substitution of Glu →Lys at codon 4154 in exon 29 of Apo B increases susceptibility of Apo B to undergo glycation and thus acts as an important contributor to LDL modification. Increased non-enzymatic glycation of Apo B containing lipoproteins impairs uptake and metabolism by the high affinity low density lipoprotein (LDL) receptor, and is one of the post-secretory modifications contributing to accelerated atherosclerosis in diabetes [27]. Glycated LDL is more susceptible to oxidative modification than

non-glycated LDL. Therefore, glycated-LDL accumulates in plasma and may enhance cholesterol ester accumulation in macrophages and thus may increase the risk of atherogenic complications [3,27]. In addition the modified LDL also stimulates vascular smooth muscle cells to produce pro oxidant and pro inflammatory state and thus contributes to atherosclerosis in diabetes [27].

Since LDL and Apo B levels are similar in all genotypes, we suggest that presence of polymorphism may not affect the expression of Apo B level but increases its susceptibility to glycation and thus may increase the risk of cardiovascular complications. The limitation of this study is relatively small sample size. So potential for selection bias may raise some concerns on the statistical precisions of the estimates.

Conclusions

The present study indicates that, R+/R+ is the most common genotype and R-/R- is the least common. Though presence of SNP (G>A) may not alter phenotypes of apoB and LDL, it acts as an important contributor to LDL modification and hence may predispose the individuals to complications like atherosclerosis.

Conflicts of Interest and Competing Interest Statement

Authors declare that there is no financial or non-financial conflict of interest and competing interests.

Authors' Contribution

KD, SG, SBS, and SVM designed the study, KD and SG carried out experiments and acquired data; AA, and SVM helped in recruiting patients and performed CIMT and HbA1c analysis; SG, SBS, KD, AA and SVM contributed to draft the whole project. All authors have read and approved the final manuscript.

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