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Original Article

Biochemistry Section

Association of APOA5 Gene Promoter Region -1131T>C Polymorphism (rs662799) to Plasma Triglyceride Level in Patients with Type 2 Diabetic Nephropathy

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ABSTRACT

Introduction: Diabetic Nephropathy (DN), a serious complication of Type 2 Diabetic Mellitus (T2DM), is progressive and susceptibility to DN varies among T2DM patients. ApoA5-1131T>C polymorphism revealed that is strongly associated with triglyceride levels and proposed as a predisposing factor for DN.

Aim: The purpose of this study was to investigate the association -1131T>C ApoA5 gene polymorphism with serum lipids levels in Type 2 diabetic (DM) patients with or without DN in north of Iran (Mazandaran province).

Materials and Methods: This study comprised patients with established T2DM (n=161) and controls (n=58). Genotyping of APOA5 -1131T>C polymorphisms was performed by PCR–RFLP. Diabetic patients were divided into two groups: with nephropathy (DN+, n = 90) and without nephropathy (DN-, n = 71). Lipids and lipoproteins were assessed by enzymatic methods.

Results: The genotype frequencies were 63.8 % TT, 31 % TC, 5.2 % CC in controls, 33.8% TT, 52.1 % TC, 14.1 % CC in DN- and 44.4 % TT, 36.7 % TC, 18.9 % CC in DN+ patients. The TC genotype and the CC genotype were overexpressed among DN+ and DN-population in comparison to the control group. The highest and the lowest TG levels in both diabetic patients and controls belonged to CC+TC and TT genotypes, respectively. Furthermore in both patients TG increased with this order: TT< TC<CC

Conclusion: These results suggest that APOA5 -1131T>C polymorphisms influence lipid levels in type 2 diabetic patients.

Keywords: Genotyping, Kidney, Lipids, PCR-RFLP

INTRODUCTION

Type 2 Diabetes is a chronic disorder characterized by impaired metabolism of glucose and lipids due to defect in insulin action/secretion from beta cell of pancreas or insulin resistance [1]. The chronic hyperglycaemia in diabetes lead to dysfunction and microvascular events such as nephropathy, retinopathy and neuropathy or macrovascular events like atherosclerosis can produce coronary artery disease and stroke [1].

A principal cause of morbidity and mortality in individuals with type 2 diabetes is Diabetic Nephropathy (DN) that has become a health problem of significant magnitude [2]. DN is the leading cause of end-stage renal disease, a complication characterized by a decreased Glomerular Filtration Rate (GFR), increased proteinuria and high levels of creatinine. New strategies are immediately required to improve the diagnosis of this destructive complication of diabetes [2].

Lipid profile of plasma includes cholesterol (Chl), Low-Density Lipoprotein cholesterol (LDL), High-Density Lipoprotein cholesterol (HDL) and Triglyceride (TG). Patients with DN are characterized by increased plasma fasting TG levels, and it seems that elevated TG precedes DN. Blood lipids modify the decline renal function [3] or TG rich lipoprotein particles such as VLDL might promote progression of DN [4].

Apolipoprotein A5 (APOA5) is located in the apolipoprotein APOA1/C3/A4 gene cluster on chromosome 11q23 [5,6]. APOA5 is primarily expressed in liver cells and secreted into the plasma [7,8]. The APOA5 plays an important function in the metabolism of TG [9].

Increased level of APOA5 is correlated with decreased TG concentration in the plasma [9].

TG interacts with lipoprotein lipase, an enzyme important for the central regulation of circulating TG levels [10]. In plasma, apoA5 is associated mainly with HDL; however, it could shift to Chylomicron (Chy) or very low-density lipoprotein cholesterol (VLDL) in response to a high fat in diet [11]. Polymorphisms in the APOA5 gene are strongly affected by TG levels [12]. Among the genetic variants associated with the expansion of hyperlipidemia there is a natural variant (-1131T>C) in the promoter region of APOA5 gene [12].

Therefore, it is important to understand the role of genetic risk factor for better preventing the development of DN.

Since many studies indicate that there is an ethnic difference in the APOA5 gene variants and due to little information about the APOA5 variants in Iranian population, in the present study, we investigated the association of rs662799 variant of the gene with lipid profile levels in a case-control study involving Type 2 diabetic (DM) patients with or without DN in north of Iran (Mazandaran province).

MATERIALS AND METHODS

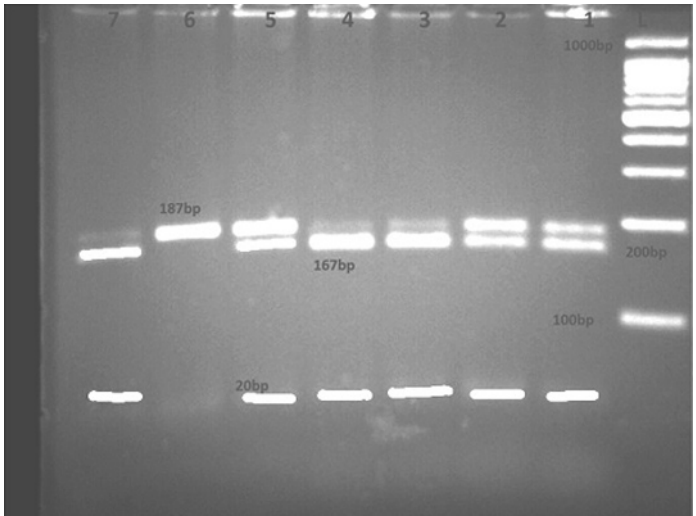
Patients Population

This case-control study comprised of 161 patients with established diagnosis of type 2 diabetes mellitus: 90 with nephropathy (males/females: 35/55), 71 without nephropathy (males/females: 29/42) and a group of 58 healthy (males/females: 24/34) gender- age matched subjects. Patients were recruited from Tooba clinic,

Mazandaran University of Medical Sciences and Andisheh laboratory, Sari, Iran from February 2015 to September 2015. Individuals in the control group were selected from a volunteer population without the history of diabetes, kidney or heart disease History of the disease and other relevant data were collected from all participants with prior permission of attending physician. DN as evidenced by proteinuria >300 mg/l or ACR > 25mg/mmol (albumin to creatinine ratio in 24 urine collection and impaired GFR (60 ml/min/ 1.73 m^2). The study protocol was approved by the local ethics committee and all participants signed an informed consent form prior to the study. Individuals taking lipid lowering drugs or with an evident acute or chronic inflammatory condition, malignant disease, type 1 diabetes, obstructive uropathy, haematuria, urinary tract infection were excluded from the study. Blood specimens were collected from all participants after 12 hour fasting. Samples were collected in two different tubes. EDTA tubes were used for extraction of DNA. Serum from plain tubes was separated by centrifugation and directly stored in freezer (-70° C) until evaluation of lipid profiles. Total cholesterol, High Density Lipoprotein (HDL) cholesterol and triglycerides were assayed using commercial kits by chemistry autoanalyser (Cobas Integra 400, Germany), and then Low Density Lipoprotein (LDL) cholesterol was calculated using the Friedewald equation excluding those samples with triglyceride levels above 400 mg/dl [13].

Genomic DNA was extracted from peripheral blood leukocytes using a salting out method. The -1131T>C polymorphism was determined by PCR–RFLP analysis. For 1131T>C polymorphism, a 187 bp fragment was amplified using the two primers [4,12]: Forward: 5′- GAT TGA TTC AAG ATG CAT TTA GGA C-3′ and Reverse: 5′ –CCCCAGGAACTGGAGCGAAATT-3′.

Gene of interest was amplified by 200 mM dNTP, 1U SmarTaq (Cinagen, Iran), 1× smarTaq buffer, 280 nM each primer 1.5 mM $MgCl_2$ and 1 ul (400ng) of DNA were mixed and the volume was adjusted to 25 ul with water. The PCR conditions included an initial denaturing at 96°C for 4 min, followed by 35 cycles of amplification cycles consisting of denaturation at 93°C for 40 s, annealing at 58°C for 40 s, extension at 72°C for 40 s, and a final extension at 72°C for 5 min. A 6 µl of the PCR products were digested for 12h at 37°C with 5 U of restriction enzyme Tru1I (Promega) to check if the digestion has occurred. Products were resolved on 2% agarose gels post-stained with ethidium bromide and imaged with uvitec (UK) Gel Duct system. According to the digestion patterns, three genotypes were determined: TT genotype resulted in 20 and 167 bp fragments, TC genotype created 20, 167 and 187 bp products and CC genotype produced 187bp fragment [Table/Fig-1].



[Table/Fig-1]: Gel picture of Apo A5 1131T-C PCR-RFLP products resolved on a 2% agarose gel with ethidium bromide staining, heterozygote (TC): lanes 1,2 and 5; Normal homozygote(TT): lane 3,4 and 7 and minor homozygote (CC): lane 6 . DNA molecular weight size ladder is at right end of gel.

STATISTICAL ANALYSIS

SPSS 16.0 software was used for statistical analysis. Normal distribution of the samples was analysed with the Kolmogorov–Smirnov test. Comparisons between groups were performed using t-test or ANOVA for parametric variables and Mann–Whitney U test or Kruskal–Wallis test for nonparametric variables. Chi-square test was used for comparison of the categorical variables. Hardy-Weinberg equilibrium was tested by Chi-square test. Odds Ratios (OR) with 95% of Confidence Intervals (CI) were calculated to assess strength of association.

Lipid levels were expressed in mg/dl and all values were reported as means±SD. Statistical significance was accepted at p value less than 0.05.

RESULTS

The general characteristics and biochemical parameters of the study population, divided into DN-, DN+ and control are summarized in [Table/Fig-2]. Demographic data of cases showed a mean age of 59.2 years in DN+ and 59.01 in DN– and a gender frequency of 38.2% males compared to 66.1% females in DN+ group while 40.8% males and 59.2% females in the DN– group. As expected, diabetic patients had more conventional risk factors than those of controls; both TG and LDL levels were confirmed to be higher but HDL was lower significantly in diabetic patients.

The genotype distribution and allele frequencies of APOA5-1131 T>C genotypes are shown in [Table/Fig-3]. APOA5 -1131 T>C genotypes were in Hardy-Weinberg equilibrium in both diabetic population and controls. The genotypes frequencies were 63.8 % TT, 31 % TC, 5.2% CC in controls, 33.8 % TT, 52.1 % TC, 14.1 % CC in DN- and 44.4 % TT, 36.7 % TC, 18.9 % CC in DN+ patients.

Parameters	Diabetic without nephropathy	Diabetic nephropathy	Control	p-value
Number	71	90	58	
Gender	29(40.8%) male	35(38.9%) male	24(41.3) male	0.09a
	42(59.2%) female	55(61.1%) female	34(58.6) female	0.1 b
Age (years)	59.01±11.94	59.22±9.32	52.90±10.48	0.1a
TG (mg/dl)	169.173±48.508	221.924±77.311	115.71±31.551	0.0001a 0.0001b
Cholesterol (mg/dl)	180.276±34.717	190.391±43.790	179.98±32.762	0.99a 0.22 b
HDL (mg/dl)	44.887±14.475	41.455±13.967	73.38±18.396	0.0001a 0.34 b
LDL (mg/dl)	101.554±35.779	104.550±38.747	84.462±28.155	0.02a 0.85 b

[Table/Fig-2]: Demographic and biochemical features of patients with diabetic and control group.
Values are presented as mean ± SD. a: control vs DN- , b: DN+ vs DN-

	Allele			Genotype		
	C	T	n	CC	TC	TT
No DM (Ctrl)	41%	59%	58	3 (5.2%)	18 (31%)	37 (63.8%)
DN-	40.10%	59.90%	71	10 (14.1%)	37 (52.1%)	24 (33.8%)
p-value : Vs. Ctrl				CC: 0.09	C+ : 0.001	
OR (95% CI)				3 (0.78- 11.4)	3.45(1.6-7.1)	
DN+	37.20%	62.80%	90	17 (18.9%)	33 (36.7%)	40 (44.4%)
p-value : Vs. Ctrl				CC: 0.017	C+ : 0.021	
OR (95% CI)				4.2 (1.1-15.2)	2.27 (1.2-4.3)	
p-value : Vs. DN-				CC: 0.42	C+ : 0.171	
OR (95% CI)				1.42 (0.66-3.3)	0.63 (0.34-1.21)	

[Table/Fig-3]: Frequency of allele apoA5 and genotypes in patients and control subjects.
C+: CT or CC versus TT genotypes. CC: CC versus CT or TT genotypes.

In DN- group, the prevalence of the CC and TC genotypes were found to be higher than control subjects. The TC genotype (OR = 3.45) and the CC genotype (OR = 3) were overexpressed among DN- population in comparison to control group.

Similar results were observed in DN+ group so that the prevalence of the CC and TC genotypes was higher than control subjects. The TC genotype (OR = 2.27) and the CC genotype (OR = 4.2) were overrexpressed among DN+ population in comparison to control group. On comparing genotype distribution between DN– and DN+ patients, results revealed CC carriers were statistically significantly more frequent in the DN+ group with OR=1.42, 95% confidence interval of 1.1–15.2, and p<0.017.

Lipids profile in APOA5 -1131T>C genotype groups in diabetic patients and controls are shown in [Table/Fig-4]. We considered CC and TC genotypes in one group (allele C carriers) and used independent t-test to compare the biochemical parameters of subjects in this group with those of the volunteers with the TT genotype. The highest and the lowest TG levels in both diabetic patients and controls belonged to CC+TC and TT genotypes, respectively. [Table/Fig-5] showed that with comparison between diabetic subjects, TG was higher in DN+ than DN- individuals. So that, in both patients TG increased with this order: TT< TC<CC.

When patients and control groups were divided based on 150 mg/dl as a cut-off value for TG; High TG values were observed in 90% of individuals had CC Genotype compared to 55% of that of the TT or CT carriers with odd ratio= 7.36, (CI=2.16-25.10), p=0.001 [Table/Fig-6].

Due to the existence of the rare allele C (TC+CC subgroup), high TG values were encountered in 63.7%, 76.5%, and 28.6% % of the DN+, DN–, and controls, with an odds ratio of 21,4.87,0.67 when compared with subjects with TT genotype respectively [Table/Fig-7].

Also, we found a higher number of TC heterozygote's in the high TG group as compared with the low TG group in diabetic patients.

Parameters	Control			DN-			DN+		
	TT	TC+CC	p	TT	TC+CC	p	TT	TC+CC	p
Number	37	21		24	47		40	50	-
TG(mg/dl)	113±32	119±29	0.52	152±50	176±47	0.06	175±51	258±75	0.0001
CHL(mg/dl)	178±29	183±39	0.57	179±36	176±31	0.71	189±44	191±43	0.81
LDL(mg/dl)	84±26	85±31	0.91	106±42	95±29	0.41	114±41	97±34	0.03
HDL(mg/dl)	71±19	74±16	0.54	42±16	46±14	0.21	39.6±14	42.9±13	0.26

[Table/Fig-4]: Lipid levels in CC vs TT and TC of APOA5 polymorphism in diabetic patients and controls.

Parameters	DN-				DN+			
	TT	TC	CC	p	TT	TC	CC	p
Number	24	37	10		40	33	17	
TG(mg/dl)	152±50	176±48	182±39	p1:0.14	176±51	222±50	330±63	p1:0.01
				p2:0.25				p2:0.001
				p3:0.95				p3:0.001
CHL(mg/dl)	180±36	177±32	195±41	p1:0.93	189±44	184±39	205±49	p1:0. 1
				p2:0.49				p2:0.01
				p3:0.31				p3:0.01
LDL(mg/dl)	106±42	95±29	114±39	p1:0.44	114±47	97±34	94±37	p1:0.89
				p2:0.83				p2:0.42
				p3:0.29				p3:0.25
HDL(mg/dl)	43±17	46±14	44±9	p1:065	40±14	42±13	44±15	p1:0.71
				p2:0.96				p2:0.46
				p3:0.92				p3:0.85

[Table/Fig-5]: Lipid values according to APOA5 genotype in diabetic patients.
P1: TT vs TC, P2: TT vs CC, P3: TC vs CC

Genotype	Number	TG<150 (mg/dl)	TG>150 (mg/dl)
CC	30	3(10%)	27(90%)
CT+TT	189	85(45%)	104(55%)
			OR=7.36, CI (2.16-25.10), p-value=0.001

[Table/Fig-6]: TG levels in CC vs. TT and CT among three groups.

Group	Genotype	TG<150 (mg/dl)	TG>150 (mg/dl)
Control	TT	5(71.4%)	32(62.7%)
	TC+CC	2(28.6%)	19(37.3%)
		1(CC)+1(TC)	1(CC)+18(TC)
			OR=0.67, CI(0.12-3.82) , p=0.44
DN-	TT	12(23.5%)	19(70.4%)
	TC+CC	39(76.5%)	8(29.6%)
		9(CC)+30(TC)	1(CC)+7(TC)
			OR=4.87, CI(1.61-14.70), p=0.004
DN+	TT	28(36.3%)	12(91.7%)
	TC+CC	49(63.7%)	1(8.3%)
		17(CC)+32(TC)	1(TC)
			OR=21, CI(2.59-17.10), p=0.004

[Table/Fig-7]: High TG values for CC/TC versus TT genotype in control, DN- and DN+

DISCUSSION

More studies have demonstrated that for evaluating the susceptibility to a disorder, especially a multifactor and complex disorders such as diabetes, it is not enough to just determine the routine biochemical parameters, but the genotypes of some expressing functional protein should be measured for early prevention and novel therapeutic intervention.

DN is a severe complication of T2DM, and the occurrence of nephropathy has been increasing worldwide [14]. Therefore, there

is a need to recognize the target to treat and prevent DN [15]. We showed that the frequencies of variants APOA5-1131T > C between patients and control subjects. The frequency of C allele was found to be 41%, 40.1% and 37.2% in controls, DN- and DN+ group, respectively [Table/Fig-3].

These frequencies are similar to the results reported for East Asians (27-37%) [16,17] but are different from other populations such as the Brazilians 16% [18] Turks (13%) and Hispanics (13-16%) [19].

A high fasting triglyceride (TG) level has been implicated as a predictive factor for the development of DN [20,21]. Our results showed that plasma samples of DN- and DN+ group have a higher mean TG values in compared to control groups. The higher mean value of TG was also seen in CC carriers of the DN+ group as well as the DN– and even the CC carriers of the control group, which confirmed the hypothesis of high TG as a marker of nephropathy implicated the 1131T-C polymorphism and its association to high levels of triglyceride. The allele frequency of distribution of APOA51131T-C polymorphism has been widely studied in many studies for evaluating of lipid profile especially triglyceride level in plasma such as young and healthy African Americans [22], in Chinese obese children and adolescents [23], cardiovascular disease [24], metabolic syndrome [25] and diabetic nephropathy [26].

Subgroup analysis revealed that patients bearing the rare allele of C in APOA5 and the Homozygotes for the common allele had a higher risk of hypertriglyceridemia compared to carriers of the other genotype (TT). Also a trend towards higher levels of triglyceride was seen in results that reported for Egyptian diabetic nephropathy [26] and Taiwanese population [27].

Szalai and coworkers believed that the -1131C allele does not yield categorical hypertriglyceridemia in most individuals, but rather play as a modifier allele for plasma TG concentrations [28]. The effect of the -1131C allele on TG concentrations seems to be more marked in patients with severe dyslipidemia, as revealed in familial combined hyperlipidemia [29], severe hypertriglyceridemia [30,31] which proved to be even more definite in CC homozygous subjects [32]. In the present study, subjects did not show extremely high TG levels, which may explain the lack of association between -1131C allele and TG concentrations because in addition to APOA5, well-studied modulators of plasma TG homeostasis include LPL, APOC2, APOC3, glycosylphosphatidylinositol-anchored-high density lipoprotein binding protein 1(GPIHBP1)and others [33].

The effect of the -1131C allele on LDL concentrations seems to be more associated with lower LDLc levels in CC or TC subjects as compared to the TT homozygous, considering the DN-, DN+ and controls. One hypothesis is the -1131T-C polymorphism does not affect LDLc levels [32]. Whereas, another idea explained by Nilsson et al., suggested that the protein APOA5 can interact with the LDL receptor (LRP1 and SrLa), and possible increase in the capture chylomicrons and VLDL by the liver [34]. Subsequently, due to the presence of the - 1131C allele and decrease of APOA5 gene expression significantly decrease the uptake of chylomicrons, VLDL and increase the uptake of LDL molecules, in turn inducing lower plasma LDLc levels [35]. Our data suggest that the functional variant APOA5-1131T>Polymorphism contribute to changed fasting TG levels in subjects that suffer from diabetes with or without nephropathy. In addition, the presence of other gene polymorphism such as APOE, LDL receptor and LPL enzyme and relation to variation of lipid profile in plasma due to considerable heterogeneity and large differences in the allele must be considered in Iranian population.

CONCLUSION

In conclusion, the association between DN and APOA5 1131T-C polymorphism that affects TG levels suggests that TG levels in diabetic patients may contribute to development of DN. This association requires confirmation in other ethnic populations. Furthermore, it could provide an opportunity for the development of a novel therapeutic strategy.

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