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Genetic Mutation

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SCREENING OF GENETIC MUTATION IN ABCA1 GENE AND RELATIONSHIP OF LOW-DENSITY LIPOPROTEIN AND TRIGLYCERIDES IN PATIENTS WITH TYPE 2 DIABETES WITH AND WITHOUT DYSLIPIDEMIA

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ABSTRACT

Objective: To find the genetic mutation in exon-9 of the ABCA1 gene and relationship of serum low-density lipoprotein levels and serum triglycerides in patients with type 2 diabetes mellitus with and without dyslipidemia.

Study Design: Cross-sectional comparative.

Place and Duration of Study: Department of Biochemistry and Molecular Biology, Army Medical College Rawalpindi, from Jan to Dec 2017.

Methodology: Ninety subjects were selected and divided into three groups, thirty in each. The division was based on newly diagnosed patients of type 2 diabetes with dyslipidemia, newly diagnosed patients of type 2 diabetes and healthy individuals. Genomic DNA was extracted from the blood samples of all subjects. Exon-9 of the ABCA1 gene was amplified through polymerase chain reaction and sequenced on automated DNA sequencer. The biochemical data was analyzed by SPSS-20 and presented in percentage and mean ± SD.

Results: The pattern of sequences of ABCA1 gene was found normal in patients of the group I and II through DNA sequencing. The levels of triglycerides were found elevated and mean value was 2.45 ± 0.57 for patients with diabetic dyslipidemia as compared to diabetic and control groups. The levels of low-density lipoproteins were observed as normal with mean value of 1.50 ± 0.22.

Conclusion: The ABCA1 gene did not carry any genetic mutation in exon-9 in studied subjects. The raised level of triglyceride lipoproteins was a typical sign of dyslipidemia with a normal profile of low-density lipoproteins.

Keywords: ABCA1 gene, DNA sequencing, Dyslipidemia, Low-density lipoproteins, Triglycerides, Type 2 diabetes mellitus.

INTRODUCTION

Diabetes mellitus is being treated as an epidemic now a days[1]. According to the International Diabetes Federation, an approximated 382 million people are diabetic and the number will increase to 591 million by the year 2035. In type 2 diabetes mellitus, disturbance in different metabolic activities in various tissues leads to complications such as vascular disease, nephropathy, neuropathy and retinopathy[1].

Dyslipidemia is observed as a common seen complication with diabetes mellitus and a risk factor towards atherosclerosis[2]. Pakistan is at a high prevalence of diabetes at seventh position in WHO list[4]. Therefore, risk factors other than hyperglycemia are being targeted in patients with diabetes to counter the vascular complications. The prevalence of dyslipidemia increases as a complication of type 2 diabetes mellitus in Pakistan. Dyslipidemia is a major threat for progression to cardiovascular disease (CVD) and regarded as raised levels of plasma triglyceride with a low level of high-density lipoprotein (HDL-C). HDL is an important particle to mediate free cholesterol transport out of the body[4,5].

ATP-binding cassette transporter subclass A type 1 (ABCA1) is a transmembrane protein and acts as a cell surface receptor with 240kDa molecular weight and 2261 amino acids[6,7]. The human ABCA1 gene is located on the 9th chromosome with position; 9q31, contain 50 exons and spans about 149kb of genomic DNA. Abundantly in macrophages, liver and brain[6], it is also

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expressed in various other tissues. ABCA1 protein functions in promoting the cellular cholesterol transport to lipid poor apolipoprotein A-1 through HDL and HDL biogenesis. The defects in cholesterol efflux mediate different pathological conditions in different cells and organs. The structure of human ABCA1 transporter is symmetrical[8], embodying two transmembrane domains, nucleotide binding domain, highly glycosylated and two regulatory domains connected to amino and carboxylic terminals found in cytosol. Mutations in ABCA1 gene result to the disruption in plasma HDL homeostasis[9]. Due to these mutations, cholesterol cannot be transported to APOA1 and therefore result in an intracellular accumulation of cholesterol. ABCA1 protein loads the free cholesterol to the free APOA1 by promoting the cellular efflux by involving APOA1 binding to the cell surface and translocation of phospholipid.

The rs1800977 (C69T), rs2230806 (R219K) and rs9282541 (R230C) polymorphisms in ABCA1 gene were found as the key regulators of gene transcription in type 2 diabetes. In another study, -565C/T polymorphism in the ABCA1 gene was found associated with decreased HDL-C, increased TG, IL-6 and hypoalphalipoproteinemia. Moreover, R1587K polymorphism identified in young Greek nurses had shown association with lipid variables, age, gender and BMI.

So, keeping in view the importance of the ABCA1 gene, this research was conducted to study the ABCA1 gene in patients with type 2 diabetes mellitus.

METHODOLOGY

In this cross-sectional comparative study, ninety subjects were enrolled after approval of the ethic review committee of the institute. WHO calculator was used using the prevalence of diabetes in Pakistan as a reference. Sample collection was done in collaboration with Pak-Emirates Military Hospital, Rawalpindi. The experimental work was performed at CREAM Lab, the Department of Biochemistry and Molecular Biology, Army Medical College, Rawalpindi from January to December, 2017. The sampling technique was non-probability purposive and written consent from each study participant was obtained. In the group I, thirty newly diagnosed patients with diabetic dyslipidemia were included. The group II comprised of thirty newly diagnosed patients with diabetes without dyslipidemia while group III comprised of thirty healthy individuals.

The criterion for diagnosis of diabetes was finalized according to the clinical assessment of the attending physician. Laboratory tests such as fasting blood glucose levels and/or HbA1c were used to evaluate the diabetic profile of the patients visiting the OPD. HbA1c levels of 6.5% or higher on more than two occasions or fasting plasma glucose (FPG) of 126 mg/dL (7 mmol/L), or higher on two separate tests was considered conclusive (Type 2 Diabetes Diagnostic Criteria by the ADA 2019). Patients having comorbid illnesses such as cardiovascular disease, hypertension, liver disease, non-diabetic dyslipidemia and taking lipid-lowering drugs were excluded. Patient's age, BMI, weight and height were noted. Their family history, physical activity, and other comorbid conditions were also registered. The clinical data was collected and levels of triglycerides and LDL were analyzed in all groups. The demographic data consisting of age, glycemic control and socioeconomic status were also apprehended.

Genomic DNA from the blood sample of each subject was isolated by phenol/chloroform method. Exon-9 of the ABCA1 gene was amplified using the polymerase chain reaction technique. DNA-based primers of exon-9 of the ABCA1 gene were designed using online bioinformatic software, Primer[3].

The sequence of left primer was 5′-CTTCTCATCCCCAACCCTTG-3′ and the right primer was 5′-CCAAGGCCAGAACTAGGGA-3′. The PCR program was optimized by using reagents in different concentrations and programming on a gradient PCR machine (Corbet Inc., USA). Further, the amplified PCR products were

visualized on 1.5% agarose gel on horizontal electrophoresis apparatus (Bio-Rad). The PCR purification kit (ThermoFisher Scientific, USA) was used to purify the PCR products as stated in the protocol provided by the kit manufacturer.

DTCS Quick Start kit (Beckman and Coulter, USA) was used to perform the sequencing PCR. The sequencing PCR reaction mixture was prepared using the following reagents: 3.2µmol forward and reverse primers, 8µl sterile distilled water, 5µl DTCS mixture (Dye terminator cycle sequencing mixture) and 100ng purified PCR product as a template. The forward and reverse primers were used in the separate sequencing reactions. The sequencing protocol was followed as described in instruction manual and annealing was done at primer specific temperature i.e. 57.5°C. The purification of the sequencing PCR product was carried out by ethanol precipitation method. The DNA pellets obtained by purifying sequencing PCR products of the samples were resuspended in the sample loading solution (SLS). After the incubation for 15 minutes, they were loaded onto 96 well plate and run on Automated DNA Genetic Analyzer CEQ 8000 (Beckman and Coulter, USA). BioEdit 7.9 biological software was used for sequence analysis and comparison of genetic variations in the ABCA1 gene. The comparison was done between the DNA sequencing data of patients and controls and ABCA1 gene sequence download from a human genome database (NCBI). The biochemical parameters based data was analyzed by SPSS v.20 and presented in percentage and mean ± SD.

Clinical data of the study participants comprising the fasting blood glucose and lipid profile was collected. SPSS v.20 was used for the analysis of the data. The comparison of TG and LDL levels was made by using ANOVA and Post Hoc Tukey Test. The categorical data was presented in percentages and variable parameters were presented in mean ± standard deviation. The *p* value ≤0.05 was regarded as statistically significant.

RESULTS

The DNA quality and quantity was checked through gel electrophoresis and spectrophotometer. The DNA quantity ranged between 500ng-3µg/µl without any sharing and protein contamination (fig-1a).

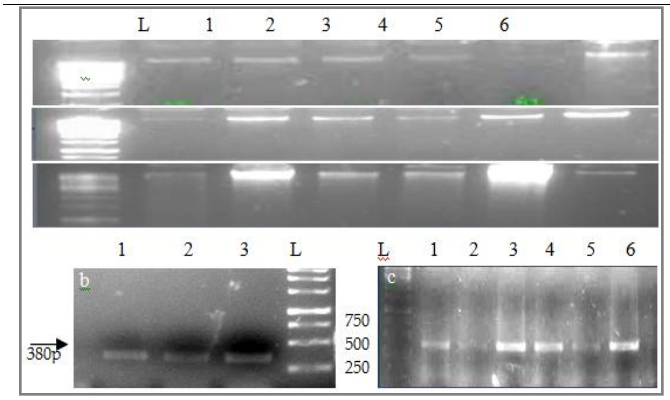

Figure-1: DNA isolation and PCR of ABCA1 gene of Subjects of Groups I, II and III. a): Lane 1-6, DNA samples; Lane L: 1kb DNA ladder, b): Lane 1-6, PCR amplification; Lane L: 1kb DNA ladder, c): Lane 1-3, PCR product purification; Lane L: 1kb DNA ladder.

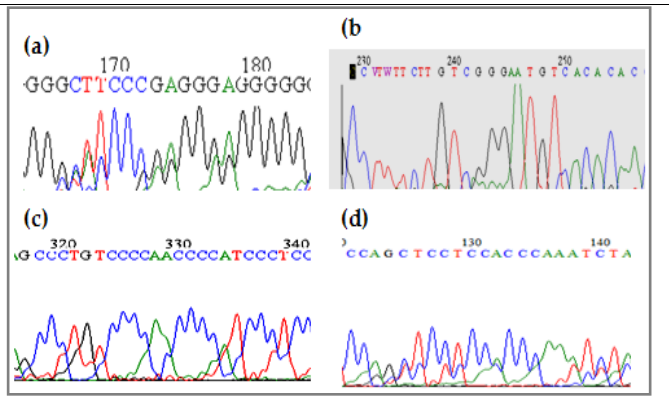

Figure-2: DNA sequencing of Groups I, II and III. a-c: DNA Sequencing of group I and II, d: DNA sequencing of group III.

The 380bp desired fragment (fig-1b) was amplified using reagents in concentration of 1xTaq buffer, 1.5mM MgCl2, 0.2µM dNTPs, 1pmol reverse and forward primers, 1 unit Taq DNA polymerase, 100ng genomic DNA in 25µl total volume of PCR mixture. The PCR program was optimized as 1 cycle for 5 minutes at 95°C, denaturation at 95°C for 30 seconds, annealing at 57.5°C for 30 seconds, chain extension at 72°C for 30 seconds with 35 repeats and final extension at

72°C for 8 minutes. The amplified PCR products were then purified by using kit with protocol provided by the manufacturer of the kit (Thermo-Fisher Scientific, USA).

The sequencing of the desired region (380pb) of exon-9 of the ABCA1 gene was performed. The comparison analysis of the sequencing data has shown no occurrence of a novel and recurrent mutation in exon-9 in the studied samples (fig-2a-c). The DNA sequence of samples of a group I and II was compared with a DNA sequence of healthy individuals of group III (fig-2d). The concentration of sequencing PCR reagents were also optimized for the ABCA1 gene. The primers were designed with flanking region of introns to amplify full length exon sequence. Therefore, a complete sequence of exon-9 of ABCA1 gene was obtained in DNA sequencing.

The derangements were observed in levels of triglyceride in patients with diabetes mellitus. The TG levels were found elevated with mean values of 2.45 ± 0.57mmol/L in patients with diabetic dyslipidemia. The LDL levels were 2.96 ± 0.81mmol/L respectively. The diabetic patients without dyslipidemia were having TG levels in range of 1.50 ± 0.22mmol/L and LDL 2.63 ± 0.73mmol/L.

The TG levels in healthy individuals were observed as 1.3297 ± .326mmol/L and LDL levels were 2.62 ± 0.64mmol/L respectively. The TG levels were remarkably observed high in patients with diabetic dyslipidemia (p<0.000) but without abnormality in LDL levels (p>0.137) between three groups (table). In addition, type 2 diabetes mellitus with dyslipidemia and without dyslipidemia was common among 40 to 60 year age group. The younger and middle-aged patients displayed poor glycemic control in contrary to older age group. The data predicted very poor glycemic control in age group 40 to 60 years.

DISCUSSION

Pakistan is a developing country with a prevalence rate of 6.9% of diabetes according to the International Diabetes Foundation. Dyslipidemia cohere with type 2 diabetes, is a prevailing complication and producing a risk of CVD[10].

It has been demonstrated by various genetic studies that the phenotypes of both diabetes and lipid levels are exceedingly inherited phenotypes. In the recent times, several vulnerable genetic loci have been publicized to reveal an association with lipid levels among general population[5,7,10-14]. Nevertheless, there is a need to replicate these findings in type 2 diabetic patients.

The rs4743763, rs4149339 and rs2472386 single nucleotide polymorphisms in the ABCA1 gene were found to be associated with coronary artery disease along with physical activity and food intake in patients with in Southern China. Genetic mutations have <1% chance in a population comparative to polymorphism. However, we could not find any genetic variations in the studied samples; neither mutation nor single nucleotide polymorphism. DNA sequencing revealed the normal sequence of exon-9 of the ABCA1 gene. There are 50 exons in ABCA1 gene and genetic mutations could possibly be present in other exons. These findings reported an insignificant role of exon-9 of the ABCA1 gene in diabetic dyslipidemia beside of elevated TG levels in these patients. It is pertinent to mention that PPARα is a transcription activator[11] of the ABCA1 gene at transcriptional level. The anomalies in the expression of PPARα can lead to

Table: Mean ± SD of TG and LDL among three groups and comparison using ANOVA and Post Hoc Tukey test.

Variables in (mmol/L)	Group I Diabetic Dyslipidemia	Group II Diabetic only	Group III Controls	Group I vs. Group II *p*-value	Group I vs. Group III *p*-value	Group II vs. Group III *p*-value
TG	2.45 ± 0.57	1.50 ± 0.22	1.3297 ± .032	0.000*	0.000*	0.211
LDL-c	2.96 ± 0.81	2.63 ± 0.73	2.62 ± 0.64	0.200	0.188	0.999

**p-value equal or less than 0.05*

down regulation of the ABCA1 gene, in addition to mutations[15]. Anomalies in reverse cholesterol uptake transport in diabetes might be a result of HDL deficiency having genetic variations in the ABCA1 gene. A pooled analysis carried out on the Caucasian population suggested that there is a notable coalition of ABCA1 (R230C) C allele with a higher risk of DM, also susceptibility to diabetes might be provided by R230C/C230C genotypes through the mechanism of secretion of insulin and adipocyte function[13,16,17].

Among the Mexican school children, a non-synonymous variant R230C in ABCA1 gene was reported to be linked with a decline in the transport of cholesterol in RCT due to decrease HDL-C and increased TG. Another study detected a novel nonsense mutation in the ABCA1 gene with the formation of truncated protein and caused the onset of Tangier disease and HDL deficiency in an Italian family. However, the study also reported a negative association between genetic polymorphism in the ABCA1 gene and coronary artery disease.

In the era of biotechnology, molecular biology has revolutionized the medical sciences and converted it to personalized medicine. Though, we have not found any genetic mutation in our studied subjects; the utmost need is to focus our understanding on the molecular genetics of the disease. The present study suggested that the other exons of ABCA1 gene need to be studied. Moreover, among the genetic risk factors causing abnormalities in lipid metabolism, ABCA1 accounts only as a single host. The different factors like insulin, glucose, leptin, cytokines and ghrelin may also play their role in causing abnormal lipid metabolism in diabetic population. Therefore, there is a need to conduct high-quality studies to signify the true effects of ABCA1 gene mutations.

However, this study demonstrated that the patients of group I were having increased levels of TG as compared to other two groups. It was noticeable that the group I individuals with diabetic dyslipidemia have shown more deranged lipid profile and such patients should take heed of dyslipidemia to hold back from coronary artery disease in the future. The pathophysiology of diabetic dyslipidemia is still under investigation. A study has reported the reduction in sphingosine-1-phosphate content of HDL due to glycation of HDL in type 2 diabetes[12,13] thus, HDL reduces the ability to encounter oxidative stress through intracellular survival pathways. It has been proved that lipid toxicity is significantly involved in the pathogenesis of diabetes[18]. During the study course, different confounding factors such as gender, smoking and drinking which may depict different effects on results validity were not considered so in future more studies are required to disregard these factors. To confirm the results, more studies still need to be undertaken in larger populations of different ethnicities as the sample size used in the study was relatively small.

These findings suggested that routine medical checkup should be a part of daily life activities of middle age to circumvent the likelihood of developing other complications of type 2 diabetes. Another secondary observation of this study revealed a lack of physical activity. Most of our enrolled subjects, even the healthy individuals, did not provide any recognizable history of daily walk or exercise/physical activity. Therefore, sedentary life style can also be a basis of derangement in the metabolic pathways, apart from genetics[16,17].

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CONCLUSION

It was concluded from the study that exon-9 of the ABCA1 gene did not carry any genetic variations related to dyslipidemia in type 2 diabetes. The TG levels were exceptionally higher in patients with diabetic dyslipidemia as compared to the patients with type 2 diabetes mellitus and an unpreventable risk factor to develop cardiovascular disease[12,14]. Therefore, it is proposed that other exons of the ABCA1 gene should be

studied to find out genetic mechanism of dyslipidemia in type 2 diabetes. The lack of funds was a limitation of study and we could not screen the remaining exons.

Discolure

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CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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