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Original Article

Screening of ABCA1 gene polymorphism in Type 2 Diabetes Mellitus patients from Islamabad

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ABSTRACT

Objective: To see the rs 2230808 polymorphisms in ABCA1 gene and find out its associations with the risk of Type 2 Diabetes.

Methodology: A case-control, cross-sectional molecular study was done in the Institute of Biomedical and Genetic Engineering (IB&GE), of KRL Hospital, Islamabad. Genotyping of ABCA1 gene was conducted through Tetra-Primer- Amplification Refractory Mutation System- Polymerase Chain Reaction (ARMS- PCR) and Gel electrophoresis against 45 Type 2 Diabetic patients and 49 controls. The genotypes for polymorphisms followed Hardy-Weinberg Equilibrium.

Results: Homozygous CC genotype was seen in 53.3 % patients (n=24) and in 46.9 % of controls (n=23). Percentage of heterozygous CT genotype was relatively higher in controls 38.7% (n=19) and in patients it was 31.1% (n=14). The homozygous genotype TT was considerably similar in both patients (15.5 %) and controls (14.2%).

Conclusion: Study revealed the non-significant relationship of the respective SNP and the possibility of developing Type 2 Diabetes mellitus.

Keywords: ABCA1 gene, Type 2 Diabetes mellitus, Hardy-Weinberg equilibrium, Polymorhisms.

Introduction

Diabetes is a heterogeneous collection of disorders marked by persistently raised glucose levels. There are two common forms of Diabetes; Type 1 Diabetes, referred to as Insulin- dependent diabetes, while Type 2 Diabetes, also referred to as non-insulin-dependent diabetes.[1] Both genetics and environment play a contributing role in development of diabetes.[2] Diabetes has other forms, which inherit directly, including maturity onset diabetes of the young (MODY). Type 2 diabetes accounts for roughly 85- 90% of cases, whereas type 1 diabetes, an autoimmune disorder, constitutes most of the remainder.[3] Diabetes is generally diagnosed on the basis of either fasting blood glucose or HbA1c.

Data compiled from various laboratories and research stations strongly suggests genetic factors playing role in the onset of Diabetes by altering insulin secretion and sensitivity, which get deteriorated in parallel in most human T2DM incidences.[4] Multiple studies have recognized 11 gene variants to be involved in contribution of diabetes and eight of them necessarily play a role in beta cells dysfunction. Some of the genes are; PPARG, FTO, TCF7L2, ABC8, NOTCH2, KCNJ11, WFS1, SLC30A8, JAZF1, and HHEX).[5]

Prevalence of T2DM is more in indigenous Americans, mainly the Pima Indians who live in Arizona in the US, and in citizens of the Pacific islands, e.g Nauru.[6] Diabetes is linked with a wide range of serious problems which result in poor quality of life and early mortality. Timely diagnosis and proper treatment is one strategy to overcome the burden.[7] According to latest reports and case group studies, the occurrence of type 2 DM in Pakistan is approximately 11.77%. In males, the prevalence is 11.20% while it is 9.19% in females.[8]

Generally the frequency of diabetes tends to increase across age groups; nevertheless, it is observed that the proportion of patients having T2DM significantly increases in patients who are aged between 45–64 years.[9] DM is progressively on the rise every year and a good number of populations are associated with this disease each year.[10] It is widely accepted that several genes control the levels of glucose tolerance and hence contribute to the overall chances of T2DM.[4] Linkage analysis and association studies have gone a long way in identifying certain genes playing role in T2DM. Numerous studies suggest compelling evidence to polymorphisms in ATP-binding cassette transporter A1 (ABCA1) genes as risk factors for

T2D. ABCA1 is an element of a large family, also known as the ABC family, which constitutes around 49 mammalian trans-membrane transporters.[1] There are 2261 amino acids in ABCA1 that are between the cell membrane as an integral protein and the human ABCA1 gene on chromosome 9q31.[11] Patients having T2DM also suffer metabolic complications that include issues with lipid metabolism. ABCA1 functions as a carrier in the change of lipids, especially cholesterol, and it is further associated with the irregularities of serum lipid levels of high-density lipoprotein (HDL) seen in T2D cases.[12] This situation is considered to be implicated in the mechanism of insulin resistance as a reason of T2DM. The objective of the current research was to investigate the relationship between ABCA1 polymorphisms and the possibilities of developing T2D in patients from Islamabad.

Methodology

This case-control, cross-sectional molecular study was done in the Institute of Biomedical and Genetic Engineering (IB&GE), of KRL Hospital, Islamabad. A total of 45 cases from Islamabad and surrounding areas, with T2DM, aged 30-74 years and 49 healthy controls aged 35-80 years of either gender were included. Informed consent was obtained from all willing participants, and the study was approved by KRL Genetics Laboratory. The diagnosis of T2DM was confirmed in patients with fasting blood sugar (FBS) >126 mg/dL and/or HbA1c ≥6.5%, as verified through diagnostic tests and assessment by a relevant endocrinologist or physician.

About 5 ml venous blood was drawn from the study participants by venipuncture, under safe, quality control and safety procedures. It was stored in 5ml Acid Citrate Dextrose (ACD) vacutainers which also serve the purpose of anticoagulant and as preservative (BD Franklin Lakes NJ USA. Samples were stored at 2-8º C before DNA extraction. Anthropometric characters included fasting blood sugar (FBS), total cholesterol (TC), triglycerides (TGs), high-density lipoprotein (HDL), and low-density -lipoprotein (LDL) (in serum from both groups).

DNA was extracted using salting out method. The process included addition of cell lysis buffer three times to the volume of blood and placed it for 30 min in ice. We spun at for 10 min. at 1200 rpm. Discarded the supernatant, re suspended the pellet in 10 ml of cell lysis buffer. It was spun again at 1200 rpm for 10 min and discarded the supernatant. We then re suspended the pellet in 1.8ml of white cell lysis buffer. The suspension was jelly like. We took 800 µl of white cell lysate in a 2ml eppendorf tube and added 300 µl of 6M Nacl. Mixed them well by inverting and vortexing. Placed it on ice for 10 min. Took out the supernatant in a separate 2 ml eppendorf tube. Added 1 ml of chilled Absolute ethanol and mixed well by vortexing. DNA was precipitated and spun at 12000 rpm for 5 min. Discarded the supernatant and added 1 ml chilled 70% ethanol. Spun it for 10 min. at 12000. The supernatant was discarded and the pellet was dried. Resuspended it in 10mM tris pH 8.

We conducted the allele specific amplification of SNP rs2230808 in ABCA1 gene by the use of Tetra-Primer Allele specific (ARMS-PCR). (Table I)

Table I: Sequence of our primers for genotyping of rs2230808.

Primer	Sequence 5'-3'
Forward-inner primer (FI) (T Allele)	AGACAGCGGTTTACCTTGACATTATGTT
Reverse-Inner Primer (RI) (C Allele)	GAAGATTTATGACAGGACTGGACACAAG
Forward-Outer Primer (FO)	CAGATGAGGGAACTGAGGTTTAGATAGG
Reverse-Outer Primer (RO)	AATTTCAGTGAACAAGGTAGTGGCAT

We obtained the upstream and downstream sequence of our SNP rs2230808 through Human Genome present at *Ensemble Genome Browser (link was:* ***http://asia.ensembl.org/Homo_sapiens/Variation?r=9:104800023-104801023;v=rs2230808; vf=1609969)***.

Tetra primers were designed for allele specific (ARMS-PCR) for rs2230808 polymorphisms in the target gene- ABCA1 at the given source:

http://cedar.genetics.soton.ac.uk/public_html/primer1.html

After the optimization for results, we amplified the samples against the target DNA covering our SNP rs2230808. PCR reaction was ran

in the final volume of 25µl which had 2.5 µl of the 10X- PCR- buffer with no $Mgcl_2$ (Fermantas, Lithuania), 1.5µl of $Mgcl_2$ (25mM) (Fermantas, Lithuania), 1µl of dNTPs (2mM) (Fermantes, Lithuania), 0.2 µl of Taq DNA Polymerase (5U/ µl) (Fermantas, Lithuania), 1 µl of 20 20 µM of each of the forward/ reverse inner primers- And 7 µl of 40 ng/ µl of the human genomic DNA and volume was made by us up to 25 µl.

Table II Reagents for PCR- Components and their Concentrations.		
Reagents with Initial Concentration	Reagents with final Concentration	Qty
Distilled Water dH_2O	------	6.6 µl
10X Kcl buffer	1 X buffer	2.5 µl
25 mM- Mgcl2	1.5 mM Mgcl2	1.2 µl
2.5 mM- dNTPs	200 µ MdNTPs	1 µl
5U/ µl Taq DNA Polymerase	0.5 U Taq DNA Polymerase	0.3 µl
20 µM Forward -outer Primer	1.0 µM	1 µl
20 µM Forward- inner Primer	1.0 µM0	1 µl
20 µM Forward -inner Primer	1.0 µM	1.7 µl
20 µM Reverse inner Primer	1.0 µM	1.7 µl
40 ng Sample genomic- DNA	Ng/ µl	8 µl
Total Volume of Reaction	--------	25 µl

We carried out the reaction in a 96 well - thermal cycler (Thermo Electron Corporation- Mill Ford, USA) at the given optimized stages, steps, cycles given in Table III.

Table III Stages and Cycles of PCR				
Stages	Steps	Temperature	Time	Cycles
Stage- 1	Step1	94ºC	4 Min	01 Cycle
Stage- 2	Step 1	94ºC	45 Sec	35 Cycles
	Step 2	58ºC	45 Sec	
	Step 3	72ºC	45 Sec	
Stage- 3	Step 1	72ºC	10 Min	01 Cycle

Gel- Electrophoresis & Gel- Documentation

We added 10 µl 6X gel loading buffer (Fermantas, Lithuania) in each sample and then loaded the sample in gel. Afterwards, we loaded the samples in the gel. 100bp DNA marker was added to the 1st well for reference against the DNA amplification. The gel was ran for 40 min with Mexicell EC-360M electrophoresis system (EC App Corp,St. Petesburg, Florida, USA) under 200 volts with the use of Bio-Rad Power Pack 3000 (BioRad, USA) in a 1X Tris Borate EDTA buffer. The DNA bands were observed under the Ultraviolet illumination, and then they were photographed by Syngene gel-documentation system (Gene Genius, UK).

The statistical analysis was done by conducting and analyzing our results in SPSS (version- 14.0, USA). Following tests were analyzed for our data. The frequency was calculated for each allele of rs2230808 by using Hardy-Weinberg Equilibrium equation:

$$p^2 + 2pq + q^2 = 1$$

Furthermore, the allele frequency of our subjects was calculated by the formula

$$\textbf{Allele Frequency} = \frac{No\ of\ allele\ in\ study\ group}{Total\ number\ of\ the\ allele}$$

H-W frequencies of the two C and T alleles; and their genotypes TT, CT and CC were then considered to see for any significant divergence of our experimental allele and frequencies (genotypes) of the SNP rs2230808 in type 2 diabetic patients and the control groups by using the internet link (*http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html*).

To see the association of the SNP rs2230808 genotype and allele for type 2 diabetes, we used *Chi* square and *p-value* test using SPSS (Version 14.0) program and also explained by Preacher (Preacher, 2001). We considered a *p*-value of <0.05 as statistically significant while *p*-value of > 0.05 as non-significant.

Results

Our study constituted 94 subjects, with 45 Type 2 Diabetic patients and 49 controls. All the patients belonged to Islamabad and associated peripheries. The clinical features of the patients and controls have been mentioned in Table I. Females were in majority on both the T2DM group (67%) and Control group (59%) in comparison to their counterpart males (33% and 41% respectively). The mean age for the Type 2 Diabetic Patients was 55 (30-74) and for controls was 54 (32-76). There was a considerable difference in the Total Cholesterol, TGs, LDL-C,

HDL-C, HbA1C and FBG levels amongst the Diabetic patients and controls. (Table IV)

Table IV: Demographic and Clinical Features of the Subjects		
Variables	T2DM	Controls
Male	15 (33%)	20(41%)
Female	30(67%)	29(59%)
Mean Age Y (Range)	55 (30-74)	54(32-76)
Body Mass Index	30.5 Kg/m^2	≤29 Kg/m^2
Qualifying Disease Factors		
HbA1C level (range)	7- 11 %	≤7%
Fasting blood glucose (range)	130-350 mg/dl	≤126mg/dl
Duration of Diabetes	> 3 yrs	N/A
Family History	62%	30%
Hypertension	45%	18%

Fasting blood glucose, HbA1c and history of Diabetes was noted down for the patients. The BMI for Patients was 30.2±3 kg/m^2. HbA1c for the patients ranged between 7- 11% and for the controls was between 4.5 to 7%. Fasting blood glucose for the Type 2 Diabetics was 130-350 mg/dl.

After doing the Agarose gel electrophoresis of the Tetra-primer Amplification refractory mutation system (ARMS) the PCR products and gel documentation, we located the DNA fragments size on the gel and visualized them under the UV light. We identified the homozygous individuals for C allele (CC genotype) with the presence of 284 bp. While the presence of homozygous individuals for T allele. (TT genotype) were identified by the presence of 220 bp. We visualized the digestion products Digestion products under the Ultraviolet light following agarose gel electrophoresis. (Figure 1)

The distribution of ABCA1 C/T (rs2230808) genotypes among patients and controls showed no significant difference. The CC genotype was most common (53.33%), followed by CT (31.1%) and TT (15.5%). A similar pattern was observed in controls. However, the difference between groups is not statistically significant (p-value > 0.05). (Table V)

The allelic distribution of the ABCA1 gene showed a slightly higher frequency of the C allele in both patients (0.689) and controls (0.663), compared to the T allele (0.31 in patients, 0.337 in controls). While no significant difference was observed in allele frequencies between the two groups (p-value > 0.05). (Table VI)

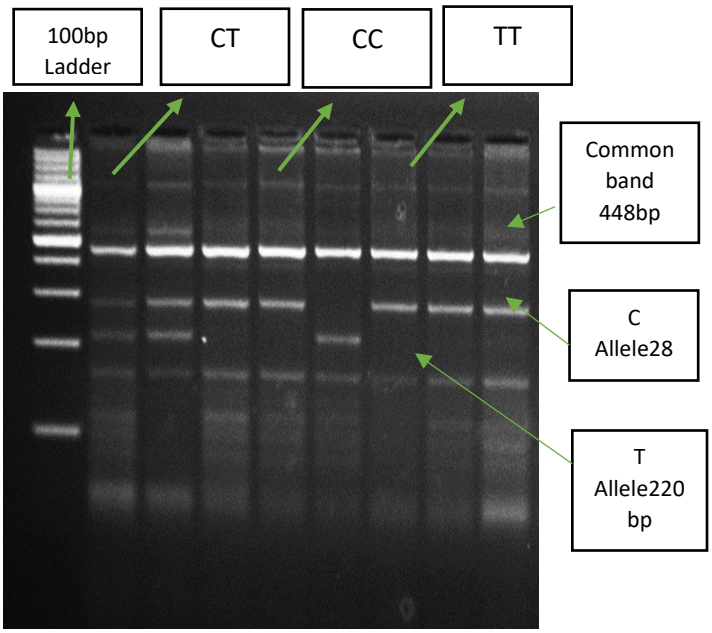

Figure.1 ARMS PCR gel image of ABCA1 R1587K (rs2230808) genotype

Table V: Genotypic Frequencies.			
	ABCA1 C/T Genotypes rs 2230808		
Group	CC	CT	TT
Patients	24(53.33%)	14(31.1%)	7(15.5%)
Control	23(46.9%)	19(38.7%)	7(14.2%)
P- Value	>0.05		

Table VI: Allelic Frequencies		
	Allele Frequencies	
Groups	C	T
Patients	0.689	0.31
Controls	0.663	0.337
P-Value	>0.05	

The CC genotype had odds ratio of 0.744, CT was 1.402, and TT was 0.9048. However, no statistically significant association was noted between genotypes and disease (P>0.05). (Table VII)

Table VII: Odds Ratio Calculations for the study			
Study Group	SNP genotypes		
	CC	CT	TT
Control (N=49)	23	19	07
Patients (N=45)	24	14	07
Odds ratio	0.744	1.402	0.9048
Lower limit	0.344	0.5973	0.2906
Upper limit	1.7418	3.2928	2.8173
p-value	0.537	0.438	0.862
Analysis	Non-significant	Non-significant	Non-significant

Table VIII: Hardy Weinberg Equilibrium Calculations for the Genotypes			
Study group	SNP genotypes		
	CC	CT	TT
Control (Observed Frequencies)	23	19	07
Expected H-W frequency	21.55	21.88	5.55
Stats Result	*p*-value = 0.663 (>0.05)		
Analysis	The genotype frequencies are in H-W equilibrium		
Patients (Observed Frequencies)	24	14	07
Expected H-W frequency	21.355	19.289	4.355
Stats Result	*p*-value = 0.6889 (>0.05)		
Analysis	The genotype frequencies are in H-W equilibrium		

Both patient and control groups were found to be in Hardy-Weinberg equilibrium for the ABCA1 genotypes. The observed and expected genotype frequencies showed stable genetic distribution, with significantly non-significant difference between patients and controls (p>0.05). (Table VIII)

Chi-square analysis for the distribution of both genotype and allele between patients and controls showed non-significant results, with p-values of 0.737 and 0.964 respectively. (p>0.05)

Table IX: P-Value Calculations for Genotype and Allele Frequency					
Study group	SNP genotypes			SNP allele frequency	
	CC	CT	TT	C	T
Control	23	19	07	0.663	0.337
Patients	24	14	07	0.689	0.31
Stats Results	chi-square = 0.61 *p*-value = 0.737 (> 0.05)			chi-square= 0.002 *p*-value = 0.964 (>0.05)	
Analysis	The result is insignificant			The result is insignificant	

Discussion

According to latest report of World Health Organization (WHO), Diabetes remains as the third major risk factor for global mortality followed by high blood pressure and use of tobacco. [13] Nearly 5 million people between 20-79 years of age died because of diabetes during 2015. Nevertheless, current advancements made in the field of Genetics have paved the way for identification of specific genetic polymorphism in human genetic research have facilitated the identification of genetic alterations which confer susceptibility to various genetic diseases, like T2DM, Heart diseases and various neurological disorders. The applications of large number of subjects and genome wide association studies (GWAS) have helped identify numerous risk loci responsible for T2DM. Some of the risk loci include ABCC8, ABCA1, KCNJ11, TCF7L2, SLC30A8 , PPARG and CDKAL1.[14]

Case control studies and other meta-analysis have confirmed that the incidence of Diabetes is on the rise. In Pakistan, almost 7.1 million people are suffering from Diabetes and by the year 2040 Pakistan would be 8th on the list of countries having most Diabetics.

In Pakistan, the genetics-based studies on genes responsible for causing Type 2 diabetes have been few and far between.

Not many studies have been done on ABCA1 gene polymorphisms for susceptibility of Type2DM.

ABCA1 gene is thought to play an important role in the transport of cholesterol to the liver from peripheral tissues.[11] Numerous mutations in the ABCA1 gene have been documented to cause abnormal functions in the transport of HDL-C.[15] Type 2 Diabetes remains as a key risk factor in the development of heart diseases and other micro and macro-vascular complications. Numerous studies have reported that patients having Type 2DM are 2-4 times more prone to developing cardiovascular diseases as compared to people without Diabetes.[16] A high level of cholesterol could be strong risk factor development of T2DM.[17] Numerous studies done on ABCA1 gene have supported the fact that it may cause a glucose derived secretion of insulin.

In this present study, we examined the risk association of ABCA1 polymorphism and Type 2 DM in local population from Islamabad. We believe that not much studies have been done in local population to study this relationship of ABCA1 and Type 2 DM. Our studies found a non-significant relationship between the frequencies of risk alleles. Our results were consistent with a Turkish and Finnish based population studies where no significant relationship was found between ABCA1 gene polymorphisms and T2DM.

Our study enrolled 94 subjects in total. 45 were registered T2DM patients and 49 were controls (normal). We wanted to see if the SNPs in ABCA1

gene are responsible for the onset of Diabetes or not.

Conclusion

In conclusion, rs2230808 genotypes in the type 2 DM patients was CC (53.33%), CT (31.11%) and TT (15.55%), while in the control group was found to be CC (46.93%), CT (38.77%) and TT (14.28%).

Our allele frequencies of C and T allele in type 2 DM patients were 0.689 and 0.31 respectively, while the allele frequency of C and T allele in control group was found to be 0.663 and 0.337 respectively. The p-value was greater than 0.05 which is non-significant.

There have been numerous studies which have shown an association of ABCA1 gene polymorphisms and T2DM.[5] A case control study done with Saudi population also showed a significant link between variations in ABCA1 genes and the risk of having Type 2DM. In order to either confirm our observed findings or to prove otherwise, we need to conduct the study in a relatively larger cohort where we can establish the role of ABCA1 gene polymorphisms in the onset of Type 2 DM.

The role of various genes has been proven in numerous global landmark research trials. More than 20 SNPs have found to play a role in either insulin or beta cell dysfunction. These genes interact with environment and result in the onset of diabetes. In some populations the role of certain genes is more established while in others it is not as much.

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