Molecular Characterization of *ABCA1* and *CACNA1C* Associated with Type 2 Diabetes

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ABSTRACT

Background and Objectives: *Diabetes mellitus is a chronic metabolic disorder that is mainly characterized by a rise in the plasma glucose levels above the normal range, glucose intolerance and insulin resistance. There are various subtypes of diabetes mellitus of which type 2 diabetes mellitus (type 2 DM) is the most prevalent form. Mutations in the Adenosine Binding Cassette Transporter Proteins Subfamily A Member 1 (ABCA1) have been associated with abnormal lipid levels and certain variants have been linked with type 2 DM. The CACNA1C facilitates calcium channels which are responsible for transporting calcium ions into the cells especially in heart and brain. The objective of the study was to find the association of mutations in ABCA1 and CACNA1C with the risk of developing type 2 DM and to find the genotype and allelic frequency of ABCA1 2230808 and CACNA1C 2239127.*

Methods: *The present study analyzed the association of ABCA1 rs2230808 polymorphism and CACNA1C 2239127 with type 2 DM patients in a local population. The study for ABCA1 2230808 was carried out on 94 subjects who were divided into 49 normal (control) and 45 type 2 DM patients, whereas the sample size for CACNA1C 2239127 was 150 divided into 94 type 2 DM patients and 56 normal samples. Genotyping of ABCA1 rs2230808 polymorphism was carried out by tetra-primers Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) technique, while RFLP technique was used for genotyping of CACNA1C rs2239127.*

Results: *The ABCA1 rs2230808 genotypes in the type 2 DM patients was found to be 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%). While the CACNA1C rs2239127 genotypes in type 2 DM patients was observed as TT (54.25%), CT (34.04%) and CC (11.70%), while in control group it was found to be TT (53.57%), CT (37.5%) and CC (8.92%).*

Conclusion: *The P-value for both genotype and allelic frequency was found to be greater than 0.05% which shows no significant association of ABCA1 rs2230808 and CACNA1C 2239127 polymorphism with type 2 DM in our study group.*

KEYWORDS: *Type 2 DM, ABCA1, CACNA1C, Genetics, SNPs, ARMS-PCR.*

INTRODUCTION

Diabetes Mellitus, also known as hyperglycemia, is a chronic metabolic disorder that is characterized by a rise in the plasma glucose levels above the normal range, glucose intolerance and insulin resistance.[1] More alarming is the fact that by 2030, type 2 DM will be the 7th leading cause of death worldwide. One of the great concerns is the increasing prevalence of type 2 DM in children.[2]

It is expected that in future, the disease will be more pronounced in middle income and lesser developed countries.[3] Both genetic and environmental factors result in type 2 DM.[4] The prominent risk factors responsible for the disease include; obesity, excess body fat, high blood pressure, sedentary life style, age, poor dietary habits with high intake of sugar, smoking, high consumption of alcohol, socioeconomic factors, ethnicity and stress.[5] Concordance value of 100% has been seen in case of monozygotic twins and about 25% of people who develop type 2 DM have a family member affected with it.[6]

According to the International Diabetes Federation, there were more than 400 million people living with diabetes in 2015, and it is expected that by 2040, more than 640 million people will be having diabetes.[7] Patients suffering from diabetes at mid 50's live about 5 years less on average as compared to non-diabetic people, which means that millions of lives globally have been lost due to the disease.[8] The prevalence of

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type 2 DM is on the rise in Pakistan and it is ranked 7th in the world in terms of prevalence of type 2 DM and among top ten nations of the world in terms of diabetic people aged 20-79 years.[9] A number of population based studies and nationwide surveys show that both urban and rural areas of Pakistan show a high prevalence of diabetes, with an overall ratio of 22.04% and 17.15% respectively.[10] HbA1c is one of the most reliable methods for glycemic management in type 2 DM as it takes into account the blood glucose levels during a time period of 3-4 months.[11]

The ATP binding cassette subfamily A member 1 (*ABCA1*) encodes ABCA1 protein, which has an important role in regulating the metabolism of lipids both cholesterol and HDL. It is mainly produced in liver and cells of immune system macrophages. The cytogenetic location of *ABCA1* is at long arm known as q arm of the chromosome 9 at position 31.1. The role of this gene is very important as several inherited have been linked to mutations in this gene.[13]

The regions of the plasma membrane that are more prone to accumulate cholesterol are targeted by ABCA1. The inward cholesterol movement from the plasma membrane toward intracellular storage is brought through the Acyl-Coenzyme A: cholesterol acyltransferase (ACAT), which esterifies cholesterol in the endoplasmic reticulum.[14] ABCA1 protein has 2261 amino acids, 36 exons and comprises of two heterodimers. The SNP rs2230808 (Arg1587Lys) or R1587K is present in the exon 35 of *ABCA1*. It has been reported that *ABCA1* plays an important role in glucose homeostasis, and several studies conducted previously have shown that carriers of heterozygous *ABCA1* mutation have their islet function impaired.[15,16]

The CACNA1C has an important role in providing guidance for making calcium channels which are responsible for transporting calcium atoms/calcium ions that are positively charged into cells. It is also the site where calcium channel blockers including dihydropyridines, phenylalkylamines, and benzothiazepines bind.[17] The gene that encodes the 1C subunit of the L-type calcium channel, *CACNA1C*, is a large gene, nearly 300 kb in size, located on chromosome 12p-13.3.[18] The chromosomal location of *CACNA1C* is 12p-13.33, which is the short (p) arm of chromosome 12 at position 13.33. CaV1.2 is a term used for the calcium channel produced from the *CACNA1C*. Although, a number of cells contain these channels, however, their importance is more pronounced in the normal functioning of heart and brain cells.

METHODS

Blood samples of diabetic and normal subjects were collected, and sampling was carried out at Institute of Biomedical and Genetic Engineering, KRL Hospital. Approvals from ethical committee and institutional review board were taken before starting the research.

Patients were informed about the research objectives and a designed questionnaire was filled from each patient. The questionnaire carried information related to age, family history of diabetes, cholesterol and BP levels and BMI etc. Informed patient consent was an integral part of the research. Anthropometric measurements were recorded in-cluding the gender, diagnosis, duration of disease, age at onset of the disease, ethnicity, obesity, smoking, physical activity, presence of various diabetes related microvascular complications and prior drug treatment. BMI of patients were also recorded. The clinical measurements of the patients were noted which include the blood glucose levels, lipid profiles and any other associated disease.

For DNA extraction, the salting out method[19] was used. The DNA was extracted from blood and was quantified by using Nanodrop 2000c spectrophotometer (Thermo USA). Further, the optical density (OD) of each sample was measured. Reconstitution of primers was carried out by mixing the lyophilized primers with deionized water for preparing stock solution of 100 μM of each primer.

Amplification Refractory Mutation System PCR (ARMS-PCR) was characterized using two primer pairs for amplifying two different alleles of a SNP in a single PCR.[20] Tetra-primer ARMS-PCR approach for the targeted and allele specific amplification of rs2230808 SNP in *ABCA1* was used. For determining the upstream and downstream sequence of rs2230808, the Human Genome at *Ensemble Genome Browser* was used. The ARMS-PCR primers for rs2230808 were designed at the following primer design web service:
http://cedar.genetics.soton.ac.uk/public_html/primer1.html

The sequence of forward inner primer was 5′-AGACAGCGGTTTACCTTGACATTATGTT-3′, sequence of reverse inner primer was 5′-GAAGATTTATGACAGGACTGGACACAAG-3′, the sequence of forward outer primer was 5′-CAGATGAGGGAACTGAGGTTTAGATAGG-3′ and the sequence of reverse outer primer was 5′-AATTTCAGTGAACAAGGTAGTGGCAT-3′. PCR conditions were optimized, amplification of the DNA samples was carried out for target DNA which span rs2230808 SNP.The PCR products were analyzed through using 2% agarose gel through electrophoresis. The different bands of DNA were visualized under the ultraviolet illumination and were photographed using Syngene gel documentation system (Gene Genius, Syngene, UK). The image of agarose gel electrophoresis for rs2230808 using ARMS-PCR approach is shown at (fig. 1).

The genotyping for *CACNA1C* rs2239127 was carried out by PCR-RFLP technique. For amplification, a

forward primer 5′-AACACAGACCC-CACACTCAT-3′ and a reverse primer 5′- TTCTCTCCCAGTGGCTTG-TT-3′ was used. After amplification the product was digested with the restriction enzyme HinfI. Fragments were separated through 2% agarose gel electrophoresis. The three genotypes TT, CC and CT were distinguished.

STATISTICAL ANALYSIS

For analyzing the data, different statistical tools were used including Statistical Package for the Social Sciences (SPSS). Different tests were applied including the *P-value* for significance, HW-Equilibrium, chi-square test and the odds ratio test to see the association of a particular exposure with an outcome.

RESULTS

Thegenetic polymorphism of *ABCA1* rs2230808 was investigated in type 2 DM patients. Sample size of 94 subjects was used during the current study. The subjects were divided into 45 patients with type 2 DM and 49 control subjects. 5 ml of whole blood was collected in ACD vacutainer tubes. The mean values of age, fasting and random blood glucose and cholesterol levels were calculated. About 67% of the type 2 DM patients in our study group comprised of females. The mean age in years was calculated as 50 ± 8.76, the mean fasting blood sugar in mg/dl was calculated as 212.89 ± 66.8, the mean of random blood sugar in mg/ dl was 260.5 ± 131.64, while the mean cholesterol value was 229.1 ± 29.95 mg/dl among the type 2 DM patients. Age and sex matched healthy individuals were recruited as controls for the current study program. The results from our study group are in support of the null hypothesis. No marked difference has been observed between the control group and type 2 DM patients. The genotypes & allelic frequencies and odds ratio for *ABCA1* rs2230808 are provided at (Table- 1 & 2) respectively. The genotypes & allelic frequencies and odds ratio for *CACNA1C* 2239127 are provided in (Table- 3&4) respectively. The results suggest that P-value for SNP genotypes and allelic frequencies is greater than 0.05 thus yielding insignificant result.

Table- 1: *Genotypes and Allele Frequency for ABCA1 rs2230808.*

P-Value Calculation for Genotypes 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
Stat 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	

Table- 2: *Odds Ratio Calculations for ABCA1 rs2230808.*

Odds Ratio Calculation for the Study			
Study Group	*SNP Genotypes*		
	CC	*CT*	*TT*
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- 3: *Genotypes and Allelic Frequencies for CACNA1C rs2239127.*

P-Value Calculation for Genotypes and Allele Frequency					
Study Group	*SNP Genotypes*			*SNP Allele Frequency*	
	TT	*CT*	*CC*	*C*	*T*
	51 (54.25%)	32 (34.04%)	11 (11.70%)	0.71	0.29
Patients (N = 94)	30 (53.57%)	21 (37.5%)	5 (8.92%)	0.72	0.28
Stat Results	chi-square =0.546 *P*-value = 0.7610(p>0.05)			chi-square=0.025 *P*-value =1(p>0.05)	
Analysis	The result is insignificant			The result is insignificant	

The *CACNA1C* rs2239127 genotypes in type 2 DM patients was observed as TT (54.25%), CT (34.04%) and CC (11.70%), while in control group it was found to be TT (53.57%), CT (37.5%) and CC (8.92%). The allelic frequencies for T and C in type 2 DM group was found to be 0.71 and 0.29 respectively while in control group it was observed to be 0.72 and 0.28 respectively. The p-value for both genotype and allelic frequency was found to be greater than 0.05% which shows no significant association of rs2239127 polymorphism with type 2 DM.

DISCUSSION

Frikke-Schmidt reported that R1587K (rs-2230808) polymorphism was over expressed in subjects with low HDL cholesterol levels. Similar results were observed by different reports.[21-23] The findings of another study carried out at Greek nurses suggested that R1587K polymorphism of ABCA1 was linked to lipid variables, age, and gender.[24]

In a study carried out at Malaysian population, no significant genetic association for R1587K polymorphism of *ABCA1* with type 2 DM was found, which is in support of our results.[25]

Table-4: *Odds Ratio Calculations for CACNA1C 2239127.*

Odds Ratio Calculation for the Study			
Study Group	*SNP Genotypes*		
	TT	*CT*	*CC*
Odds Ratio	1.027	0.860	1.35
Lower Limit	0.589	0.482	0.539
Upper Limit	1.7418	3.2928	2.8173
p-value	0.537	0.438	0.862
Analysis	Non-significant	Non-significant	Non-significant

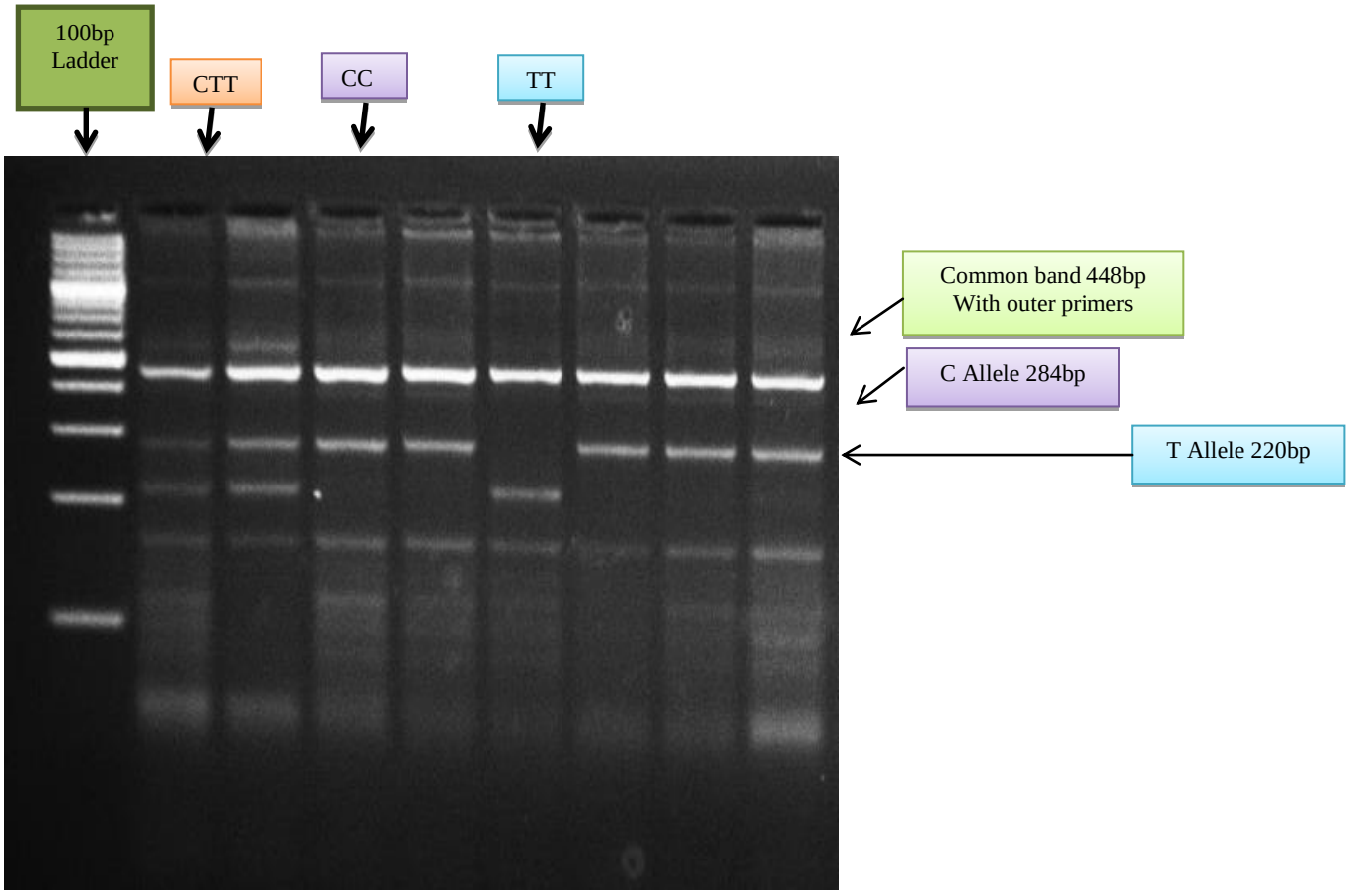

Fig. 1: *Image of Agarose Gel Electrophoresis of ABCA1 rs2230808 through ARMS-PCR.*

However, in a study carried out at Saudi population, the association between ABCA1 C69T polymerphism and type 2 DM was investigated and it was observed that the T allele frequency of the *ABCA1* C69T was greater in control subjects as compared to the type 2 DM patients. Hence, it was inferred that the T allele may have a protective role against type 2 DM in the Saudi population under study.[26]

Mexican researchers in a study carried out at the Mexican population reported that the *ABCA1* R230C variant was significantly associated with type 2 DM. Similarly, in a study carried out at the Han Chinese population of Taiwan, it was observed that the polymorphisms in *ABCA1* rs10121901 were associated with development of early onset of diabetes.[27]

It was reported that in type 2 DM patients, the *ABCA1* expression was reduced in leukocytes[27], hence this study suggested a link between *ABCA1* polymorphism and type 2 DM. In another study carried out in Turkish patients, a significantly higher frequency of

both T genotype and allele was observed in control group in comparison to diabetic patients, which may lead to possibility of T allele being a protective factor against the disease. [28]

Ergen et al.[29] reported that the GG allele and genotype differed significantly between the control and type 2 DM patients, hence G allele of the *ABCA1* rs4149313 polymorphism was associated with a higher risk of Type 2 DM; however, no link could be established between the *ABCA1* rs2230806 polymorphism and Type 2 DM in the study group.

Furthermore, it was also reported that high levels of cholesterol inhibit the secretion of insulin.[30] This study may lead to discovering new mechanisms contributing to dysfunction of the β-cell and developing diabetes in patients which are obese. β-cell function studies (in-vivo) are needed to confirm this effect.

Timothy syndrome and Brugada syndrome are caused by mutations in CACNA1C.[31] Polymorphism in this gene has also been linked to diabetic cataract.[32] Apart from this schizophrenia, autism, bipolar disorder and depression have also been linked to CACNA1C polymorphism. The Genome Wide Associations Study (GWAS) have identified that SNP in rs1006737 of *CACNA1C* has association with schizophrenia and bipolar disorder.[33]

CONCLUSION

Variants of *CACNA1A* and *CA-CNA1C* were associated with blood pressure changes and incidence of hypertension.[34] The gene base study reported that *CACNA1C* had a significant association with systolic blood pressure change over a period of time.

LIMITATIONS OF STUDY

This study had small sample size for the genetic studies and molecular mechanisms. A larger study of complex diseases such as type 2 DM will undoubtedly improve the outlook for a more targeted treatment and ultimately aiming at prevention of disease development.

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AUTHOR'S CONTRIBUTION

MEB: Conception and design of the study.
AA, KA, NB, AA: Acquisition of data.
MI, TH, ARK, QM: Analysis of data & Drafting of the article.
MEB: Critical revision of the data

CONFLICT OF INTEREST

None to declare.

GRANT SUPPORT AND FINANCIAL DISCLOSURE

None to disclose.

REFERENCES

1. Sicree R, Shaw J, Zimmet P. Prevalence and projections. Diabetes Atlas. 2006; 94(3): 16-104.
2. Bloomgarden ZT. Type 2 diabetes in the young: the evolving epidemic. Diabetes Care. 2004; 27(4): 998-1010.
3. Wild S, Roglic G, Green A, Sicree R, et al. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004; 27(5): 1047-53.
4. Ripsin CM, Kang H, Urban RJ. Management of blood glucose in type 2 diabetes mellitus. Am Fam Physician. 2009; 79(1): 29-36.
5. Te Morenga L, Mallard S, Mann J. Dietary sugars and body weight: systematic review and meta-analyses of randomised controlled trials and cohort studies. BMJ. 2012; 345(3): 7492-99.
6. Rother KI. Diabetes treatment—bridging the divide. N. Engl. J. Med. 2007; 356(15): 1499-1506.
7. Atlas D. International diabetes federation. IDF Diabetes Atlas. 7th Ed. Int. Diabetes Federation; 2015.
8. Loukine L, Waters C, Choi BC, Ellison J. Impact of diabetes mellitus on life expectancy and health-adjusted life expectancy in Canada. Popul Health Metr. 2012; 10(1): 7-13.
9. Guariguata L, Nolan T, Beagley J, Linnenkamp U, et al. The IDF Diabetes Atlas methodology for estimating global prevalence of hyperglycaemia in pregnancy. IDF Diabetes Atlas. 2014; 103(2):186-96.
10. Zahid N, Claussen B, Hussain A. Diabetes and impaired glucose tolerance in a rural area in Pakistan and associated risk factors. Diabetes Res Clin Pract. 2008; 79 (1): 124-7.
11. Stratton IM, Adler AI, Neil HAW, Matthews DR, et al. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. BMJ. 2000; 321(7258): 405-12.
12. McCarroll SA, Altshuler DM. Copy-number variation and association studies of human disease. Nat Genet. 2007; 39(7): 37-42.
13. Pohl A, Devaux PF, Herrmann A. Function of prokaryotic and eukaryotic ABC proteins in lipid transport. Biochim Biophys Acta. 2005; 1733(1): 29-52.
14. Maxfield FR, Tabas I. Role of cholesterol and lipid organization in disease. Nature. 2005; 438(7068): 612-21.
15. Brunham LR, Kruit JK, Pape TD, Timmins JM, et al. β-cell ABCA1 influences insulin secretion, glucose homeostasis and response to thiazolidinedione treatment. Nat Med. 2007; 13(3): 340-7.
16. Kruit JK, Brunham LR, Verchere CB, Hayden MR. HDL and LDL cholesterol significantly influence β-cell function in type 2 diabetes mellitus. Curr Opin Lipidol. 2010; 21(3): 178-85.
17. Abernethy DR, Schwartz JB. Calcium-antagonist drugs. N Engl J Med. 1999; 341(19): 1447-57.
18. Soldatov NM. Genomic structure of human L-type Ca2+ channel. Genomics. 1994; 22(1): 77-87.

19. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A laboratory Manual. 2nd Ed. Cold Spring Harbor (NY): Laboratory Press. 1989: 9-16.
20. Ye S, Dhillon S, Ke X, Collins AR, et al. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res. 2001; 29(17): 88-93.
21. Frikke-Schmidt R, Nordestgaard BG, Jensen GB, Tybjærg-Hansen A. Genetic variation in ABC transporter A1 contributes to HDL cholesterol in the general population. J Clin Invest. 2004; 114(9): 1343-53.
22. Clee SM, Zwinderman AH, Engert JC, Zwarts KY, et al. Common genetic variation in ABCA1 is associated with altered lipoprotein levels and a modified risk for coronary artery disease. J Circ. 2001; 103(9): 1198-205.
23. Slatter T, Jones G, Williams M, Van Rij A, et al. Novel rare mutations and promoter haplotypes in ABCA1 contribute to low-HDL-C levels. Clin Genet. 2008; 73(2): 179-84.
24. Kolovou V, Marvaki A, Boutsikou M, Vasilopoulos G, et al. Effect of ATP-binding cassette transporter A1 (ABCA1) gene polymorphisms on plasma lipid variables and common demographic parameters in Greek nurses. Open Cardiovasc Med J. 2016; 10(3): 233–9.
25. Haghvirdizadeh P, Etemad A, Ramachandran V, Ghodsian N, et al. Analysis of R1587K polymorphism of ATP-binding cassette transporter A1 gene among type 2 diabetes mellitus Malaysian subjects. Biomed Res. 2017; 28(7): 3125-8.
26. Alharbi KK, Khan IA, Al-Daghri NM, Munshi A, et al. ABCA1 C69T gene polymorphism and risk of type 2 diabetes mellitus in a Saudi population. J Biosci. 2013; 38(5): 893-7.
27. Lu CH, Liao WL, Wang TY, Chen CC, et al. Associ-ation of adenosine triphosphate-binding cassette trans-porter A1 gene polymorphism with lipid profiles and early-onset type 2 diabetes. Sci Asia. 2014; 40(3): 212-8.
28. Ergen HA, Zeybek Ü, Gök Ö, Karaali ZE. Investigation of ABCA1 C69T polymorphism in patients with type 2 diabetes mellitus. Biochem Med. 2012; 22(1): 114-20.
29. Saravani R, Galavi HR, Ranjbar N, Alamdari AR. ATP-binding cassette transporter A1 polymorphisms and haplotypes in risk of type 2 diabetes. Gene Cell Tissue. 2017; 4(1): 13326-33.
30. Hao M, Head WS, Gunawardana SC, Hasty AH, et al. Direct effect of cholesterol on insulin secretion: a novel mechanism for pancreatic β-cell dysfunction. Diabetes. 2007; 56 (9): 2328-38.
31. Boczek NJ, Miller EM, Ye D, Nesterenko VV, et al. Novel timothy syndrome mutation leading to increase in CACNA1C window current. Heart Rhythm. 2015; 12(1): 211-9.
32. Chang C, Zhang K, Veluchamy A, Hébert H L, et al. A genome wide association study provides new evidence that CACNA1C gene is associated with diabetic cataract. Invest Ophthalmol Vis Sci. 2016; 57(4): 2246–50.
33. Eckart N, Song Q, Yang R, Wang R, et al. Functional characterization of schizophrenia associated variation in *CACNA1C*. PLoS ONE. 2016; 11(6): 157086.
34. Hu Z, Liu F, Li M, He J, et al. Associations of variants in the CACNA1A and CACNA1C genes with longitudinal blood pressure changes and hypertension incidence: The gensalt study. Am J Hypertens. 2016; 29(11): 1301-6.

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