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ORIGINAL ARTICLE

Lack of significant association of ARHGEF11 gene polymorphism (rs945508) with risk of type 2 diabetes mellitus in north Indian population.

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ABSTRACT
Rho guanine nucleotide exchange factor 11, encoded by ARHGEF11 gene, is an activator of Rho GTPases that plays vital role in the regulation of G protein and insulin signaling, insulin secretion & thus associated with risk of T2DM. The aim of present study is to evaluate the association of single nucleotide polymorphism (SNP) rs954408 (R1467H) G/A in ARHGEF11 gene with type 2 diabetes mellitus (T2DM) in north Indian population. G/A polymorphism was genotyped in 168 T2DM patients and 102 healthy control subjects, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Significant association could not be observed between genotypic distribution of SNP rs954408 in T2DM cases and control subjects ($\chi 2=1.13$, p= 0.567). Minor allele (A) frequency was found to higher in cases as compared to control subjects ($\chi 2=0.96$, OR (95% CI) =1.21 (0.82-1.80), p= 0.327). Clinical characteristic like fasting plasma glucose (FPG), glycated hemoglobin (HbA1c%), low-density lipoprotein cholesterol (LDL-C), total cholesterol (CHL), insulin, homeostasis model assessment for insulin resistance (HOMA-IR) were significantly higher in T2DM cases compared to control subjects (p <0.05) and clinical characteristic like high-density lipoprotein cholesterol (HDL-C), homeostasis model assessment for β cell function (HOMA-β) were significant higher in control subjects as compared to T2DM cases (p<0.05). Regression analysis showed HbA1c%, HDL-C, Total CHL, Insulin, HOMA-IR to be independently associated with increased risk of T2DM.
***Keywords**: Type 2 diabetes mellitus, Polymerase chain reaction, Restriction fragment length polymorphism, ARHGEF11, Single nucleotide polymorphism*

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INTRODUCTION

Diabetes affects an approximate 422 million people worldwide and out of that about 95% people have type 2 diabetes mellitus (T2DM) while remaining 5% have type 1 diabetes mellitus (T1DM). It is a major cause of morbidity and mortality Worldwide [1]. According to International diabetes federation (IDF), atlas (9th edition), 463 million individuals have T2DM in 2019 and this number will be raised to 578 million in 2030 and 700 million in 2045 [2]. T2DM is metabolic condition which is characterized by increased level of blood glucose due to impairment in insulin metabolism [3]. The characteristic symptoms of diabetes are polyuria, polydipsia, polyphagia and loss of weight [4].

Rho guanine nucleotide exchange factor 11 (ARHGEF11) gene encodes 1522 amino acid containing protein, having molecular mass of 168.6 KD. ARHGEF11 gene has 41 exons in humans and it is located on chromosome no.1q21-q24[5]. ARHGEF11 protein activity is regulated by its C- terminal region and it acts as an activator of Rho GTPases thus regulates G protein signaling. It is also involved in insulin signaling through Jun NH2-terminal kinase and mitogen activated protein kinase pathways [6-11] and lipid metabolism [12]. Activation of phosphatidylinositol 3-kinase and Rho family GTP-binding protein TC10

regulates the dynamic actin rearrangement which is required for insulin-stimulated translocation of GLUT4 [13, 14]. It is therefore suggested that ARHGEF11 protein may play an important role in glucose homeostasis.

ARHGEF11 is highly expressed in liver, pancreas and adipose tissues [15]. Polymorphism in ARHGEF11 gene at SNP rs945508 was previously associated with increased risk of T2DM or insulin resistance in Chinese population [16] but the studies are lacking in Indian population. So the present study was planned to evaluate the association of ARHGEF11 gene polymorphism at SNP rs945508 with risk of development of T2DM in Indian (North) population.

MATERIAL AND METHODS

Study population

In the present study 168 T2DM subjects (100 men and 68 women; age range ≥ 45 years) and 102 healthy subjects as control (57 men and 45 women; age range ≥ 45 years) were enrolled as per the guidelines of Indian council of medical research (ICMR). The patients with hormonal therapy, diabetes (type 1, gestational, maturity-onset diabetes of the young **(**MODY), latent autoimmune diabetes in adults (LADA), cardiovascular, liver and any other major diseases were excluded from this study. The present study was given ethical approval by committee of Kurukshetra University, Kurukshetra and all the subjects under the study gave written informed consent. Ethnicity of all the subjects under study was state of Haryana (North India).

Demographic and Biochemical measurements

Demographic and anthropometric data were collected from all subjects by a standardized questionnaire. Five ml peripheral venous blood was drawn from all subjects in two tubes (one EDTA and other without EDTA) after an overnight fasting. Ethylene diammine tetra acetate (EDTA) samples were used for fasting blood glucose (FPG) and non-EDTA samples were used for serum collection. Insulin concentration was determined by enzyme linked immunosorbent assay (ELISA). HbA1c, serum total-cholesterol (CHL), HDL-cholesterol, triglycerides (TG), low density lipoprotein (LDL) were determined by enzymatic methods. The formula - fasting insulin (IU/ml) X fasting blood glucose (mg/dl)/405 was used to calculate HOMA-IR i.e. Homeostasis model assessment for insulin resistance.

DNA extraction and genotyping of ARHGEF11 gene at SNP rs945508 in T2DM cases and control subjects

Genomic DNA was extracted from EDTA coated tubes using Medi G Blood Genomic DNA Miniprep Kit (mdi. Ambala cantt, India) as per the instructions and extracted DNA was stored at minus 20°C for further use. Genotypes were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. PCR mastermix (25ul) contains 12.5µl master mix 2X (GoTaq® Green Master Mix, Promega, India), 0.5 µl of each forward primer (5'-ACAGGAACGGGAGGATAGC-3') and reverse primer (5'-GGAATGGTTTTGGGGTGATG-3') (conc.15pmol/µl), 1.5 µl of 40-50 ng of genomic DNA, 10µl nuclease free water. PCR Amplification was carried out for 35 cycles and each cycle consists of denaturation at 95°C for 30 seconds, annealing at 54.5 °C for 30 seconds and extension at 72°C for 45 seconds. There was initial denaturation step at 95°C for 5 minutes with a final extension step at 72°C for 6-10 minutes in a thermal cycler. The amplified products were checked on 1.5% agarose gel and bands of 377 bp were observed under UV transilluminator as shown in (Figure 1).

Restriction fragment length polymorphism (RFLP) analysis was performed in 15µl reaction mixture containing 3µl best quality water, 1.5µl (10X) buffer, 0.5µl (10U/µl) *Csp6I* restriction enzymes (Thermo Scientific) and 10µl of amplified product. The mixture was incubated for 4 hrs at 37°C in dry water bath. Digested PCR products were separated by electrophoresis on 2.5% agarose gel. Gels were stained with ethidium bromide and observed under UV transilluminator. Three genotypes GG, GA, AA were observed. Wild type GG homozygote of SNP rs945504 showed two bands of 277bp and 100bp, and GA heterozygote showed three bands of 377bp, 277bp and 100bp, while AA mutant homozygote showed single band of 377bp (Figure 2).

Statistical analysis

The statistical differences in clinical parameters between T2DM and control groups were assessed by Student's t-test and data are presented as means ±SD. One way ANOVA was used to evaluate the association of clinical parameters with genotype distribution in cases as well as controls. The genotype and allele frequency distribution of rs945508 polymorphisms in control subjects and T2DM cases were compared by chi-square ($\chi^{2)}$ test. Differences were considered to be significant at P <0.05. Logistic regression analyses were used to evaluate the link between T2DM as dependent variable and potential clinical parameters.

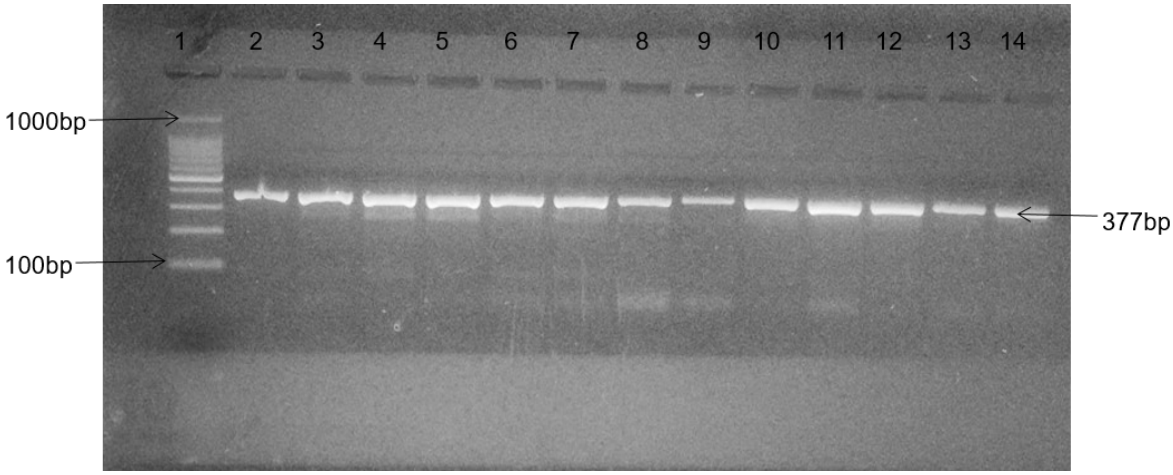

Figure 1: Amplified products (377bp) of ARHGEF11 gene fragment containing SNP rs945508 in 1.5% agarose gel. Lane 1 shows 100-1000bp DNA Ladder, lanes 2 to 14 show amplified products of 377bp.

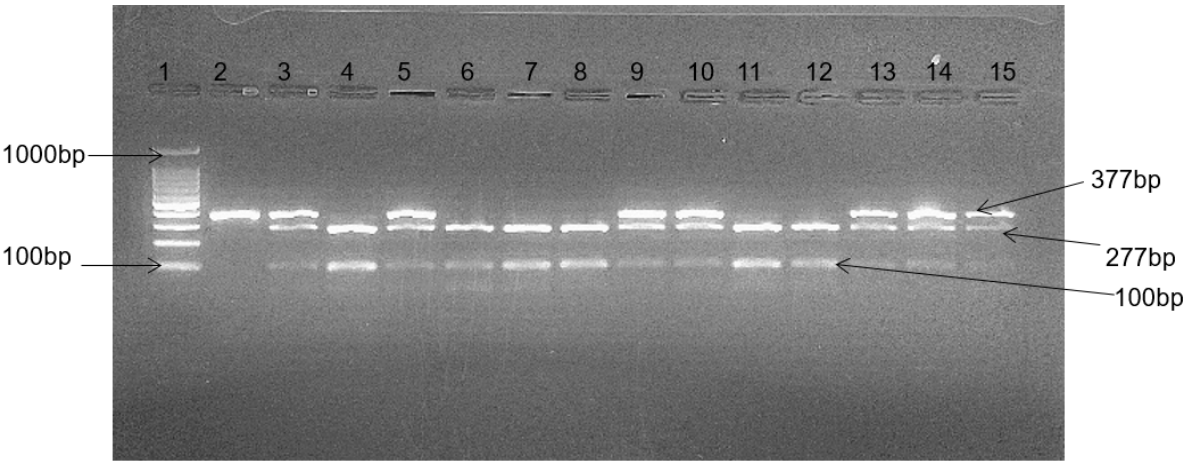

Figure 2: RFLP analysis of the ARHGEF11 gene amplified products at SNP rs945508 in 2.5% agarose gel. Lane 1 shows 100-1000 bp DNA ladder, lanes 2 to 15 shows digestive pattern of 377 bp products upon digestion by *Csp6I* restriction enzyme. Lane 2 shows homozygote mutant AA genotype, lanes 3, 5,9,10,13,14,15 shows heterozygote GA genotype and lanes 4,6,7,8,11,12 show wild type homozygote GG genotype.

RESULTS

Clinical characteristic analysis of study subjects

Clinical characteristic of the T2DM patients (n=168) and healthy controls (102) are already published in our previous communication [17]. Statistically significant differences could not be observed between T2DM cases and controls with regards to age, BMI, SBP, DBP and TG (p> 0.05). However clinical characteristic like FPG, HbA1c%, LDL-C, CHL, insulin, HOMA-IR were significant higher in T2DM cases as compared to control subjects (p<0.05) and clinical characteristic like HDL-C, HOMA-β were significant higher in control subjects as compared toT2DM cases (p<0.05).

Genotypes and allele frequency distribution

The genotype and allelic frequency distribution of ARHGEF11 gene at SNP rs945508 polymorphism is shown in Table 1 and significant differences could not be observed in T2DM cases and control subjects (χ2=1.13, p= 0.567). Frequency of minor allele (A) was found to be higher in T2DM cases as compared to control subjects (χ2=0.96, OR (95% CI) =1.21 (0.82-1.80), p= 0.327).

Clinical characteristic analysis of genotypes at SNP rs945508

The relationship between genotypes distribution at SNP rs945508 and clinical characteristic analysis has been shown in Table 2 and again significant association between clinical parameter and genotype distribution could not be observed in T2DM cases, while in control subjects, TG and HDL-C parameters show the significant association with genotype distribution (p=0.013 and 0.014) respectively.

Regression analysis

Logistic regression analysis was carried out using SPSS software. When T2DM is taken as dependent variable and sex and SNP rs945508 were taken as categorical independent variable and HbA1c%, HDL-C, Total CHL, Insulin and HOMA-IR were taken as continuous independent variables (Table 3). It was observed that HbA1c%, HDL-C, Total CHL, Insulin, HOMA-IR were found to be independently associated with risk of development of T2DM.

Discussion

A very few studies in different ethnic population reported the association of ARHGEF11 gene polymorphism at SNP rs945508 with T2DM. A total 39 exon variants in ARHGEF11 gene were identified including 2 nonsynonymous (G1456S and R1467H), 2 synonymous (S694S and N1207N), 3 in the 5'-flanking region, 4 in the 5'-untranslated region, 1 in the 3'-untranslated region [18]. In the present study, R1467H variant in exon was not found associated with T2DM (χ2=1.13, p= 0.567) and minor allele (A) frequency is 25.0% in the control subjects. In Amish population R1467H variant was also found to be linked with T2DM (P = 0.04; OR 0.66 [95% CI 0.44–0.98])[18]. While in Pima Indian, German Caucasian, Korean and Chinese population R1467H variant was associated with higher risk of T2DM respectively (P = 0.01; OR 3.39 [95% CI (1.29–8.93]), (P = 0.004; OR 1.43[95% CI 1.12-1.87]), (P= 0.042; OR 5.24 [95% CI 1.06-25.83]), (P = 0.024) [19-21, 16].

In our study, TG and HDL-C parameter in controls subjects show the significant association with genotype distribution (p=0.013 and 0.014) respectively. HbA1c%, HDL-C, Total CHL, Insulin, HOMA-IR were found to be independently associated with increased T2DM risk as observed by regression analysis.

Table 1: Genotype and alleles frequency of SNP rs945508

SNP (rs945508)	T2DM Case	Control subjects	χ^2	OR (95% CI)	p-value
GG	86 (51.2%)	59 (57.8%)			
GA	67 (39.9%)	35 (34.3%)			
AA	15 (8.9%)	8 (7.8%)	1.13		0.567
Alleles					
G	239 (71.1%)	153 (75.0%)			
A	97 (28.9%)	51 (25.0%)	0.96	1.21 (0.82-1.80)	0.327
Additive					
GG	86	59			
GA	67	35	1.03	1.31 (0.77-2.22)	0.310
AA	15	8	0.29	1.28 (0.51-3.22)	0.590
Dominant					
GG Recessive	86	59	1.13	1.30 (0.79-2.14)	0.287
AA	15	08	0.1	0.861 (0.35-2.12)	0.751
Heterozygous					
GA	67	35	0.84	0.787 (0.47-1.31)	0.359

SNP single nucleotide polymorphism; χ^2 chi square; OR odd ratio; CI confidence interval; p-value <0.05 statistically significant.

Table 2: Analysis of clinical characteristic in association with genotype distribution of ARHGEF11 gene at SNP rs945508

Parameter	T2DM Case			Control subjects		
	GG	GA+AA	p-value	GG	GA+AA	p-value
BMI (kg/ m^2)	25.33±3.76	25.57±4.30	0.699	24.60±4.82	24.36±3.47	0.777
SBP (mmHg)	133.87±15.21	132.54±17.85	0.604	133.61±12.53	133.39±12.79	0.920
DBP (mmHg)	88.38±13.92	87.65±13.74	0.729	85.96±9.13	85.67±10.63	0.887
FPG (mg/dl)	186.15±57	179.87±68.59	0.522	102.45±10.73	104.02±9.58	0.448
HbA1c %	7.63±1.11	7.36±1.24	0.130	5.44±0.42	5.50±0.49	0.508
TG (mg/dl)	163.37±36.25	162.01±38.44	0.806	144.40±39.88	166.53±49.32	0.013*
HDL-C	50.27±11.51	48.61±12.46	0.372	57.26±9.47	52.84±7.86	0.014*
LDL-C	110.85±43.90	102.89±38.14	0.211	92.25±24.97	95.95±25.87	0.468
Total CHL	193.79±47.19	183.56±37.39	0.122	178.40±25.78	182.09±28.50	0.494
Insulin (IU/ml)	7.28±6.33	6.14±4.28	0.175	4.60±2.92	5.46±4.13	0.222
HOMA-IR index	3.62±4.52	2.67±2.04	0.084	1.17±0.78	1.43±1.15	0.195

CONCLUSION

Significant difference could not be observed in genotypic distribution among T2DM cases and control subjects, however clinical parameters viz., FPG, HbA1c%, LDL-C, total CHL, Insulin, HOMA-IR were significantly higher in T2DM cases. Studies with larger sample size among various ethnicities are still required so as to reach to definite conclusion.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest**.**

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