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International Journal of Current Research
Vol. 4, Issue, 03, pp.107-111, March, 2012

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

α2 –HEREMANS SCHMID GLYCOPROTEIN (AHSG) GENE Thr256Ser POLYMORPHISM IN TYPE 2 DIABETES AMONG SOUTH INDIANS

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ARTICLE INFO

Article History:
Received 15th December, 2011
Received in revised form
13th January, 2011
Accepted 29th February, 2011
Published online 31st March, 2012

Key words:
Type 2 Diabetes,
α2 -Heremans Schmid Glycoprotein Gene,
Single nucleotide polymorphism.

ABSTRACT

Alpha 2-Heremans-Schmid glycoprotein (AHSG) is a negative acute-phase reactant and extra osseous calcification inhibitor. Decreased serum concentration of AHSG is independently related to insulin resistance. To identify the single nucleotide polymorphisms (SNPs) of the alpha2-Heremans-Schmid glycoprotein (AHSG) gene and assess their association with Type 2 diabetes in South Indian population. The study included 110 normal healthy volunteers, (68 M: 42 F, mean age 50 ± 7.97 yrs) and 101 T2DM subjects (65 M: 36 F, mean age 52.33 ± 11.12 yrs) of MV hospital for diabetes, Chennai, India. Anthropometric and biochemical parameters were measured. Polymerase Chain Reaction was carried out in the genomic DNAs using primers 5'-GTA AGG CAA CAC TCA GTG A-3' and 5'-TCA TCA CTG CCA TGT CTA G-3'. PCR-RFLP was performed with the amplicon of 731 bp; using the restriction enzyme *SacI* that cleaves between T and C (GAGCT*C) and produces 709 and 22 bps bands in agarose gel electrophoresis. Results from our study shows that 98 (89.09 %), 12 (10.91%) and no Controls had, CC (Thr/Thr), CG (Thr/Ser) and G/G (Ser/Ser) polymorphism respectively. 68 (67.32 %), 32 (31.68 %) and 1(0.99%) T2DM patients had CC (Thr/Thr), CG (Thr/Ser) and GG (Ser/Ser) polymorphism respectively. This is statistically significant ($p< 0.001$). AHSG gene Thr256Ser SNP was significantly associated ($p< 0.001$) with T2DM among south Indian population.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is characterized by insufficiency of insulin secretion and insulin resistance on target tissues (1). Both genes and the environment contribute to the development of the disease (2). Insulin mediates its actions through phosphorylation of the insulin receptor (IR) (3). Alpha 2-Heremans-Schmid glycoprotein (AHSG) also called Ba-α2-glycoprotein was renamed as α2HS-glycoprotein (4). It is an abundant 49-kDa plasma protein synthesized predominantly in the liver. The AHSG gene is located at chromosome 3q27, a susceptibility locus for type 2 diabetes and the metabolic syndrome (5). AHSG is also known as fetuin-A to distinguish it from the product of the adjacent paralogous gene, FETUB (fetuin-B) (6). AHSG is a multifunctional protein with diverse biological functions that include the regulation of calcium homeostasis (5). A possible role for AHSG in influencing genetic susceptibility to type 2 diabetes was first suggested by in-vitro work demonstrating that AHSG inhibited in a dose-dependent manner, the insulin-stimulated tyrosine kinase activity of the insulin receptor, insulin receptor autophosphorylation, and insulin substrate 1 phosphorylation (6). In humans, serum AHSG levels have been reported to be significantly higher in patients with gestational diabetes than in healthy pregnant women and to be correlated with indirect measures of insulin resistance (7). A single nucleotide polymorphism (SNP) in the promoter region of AHSG was reported to be associated with insulin-mediated inhibition of lipolysis and stimulation of lipogenesis in adipocytes (8). These observations indicated that AHSG may play a physiological role in the regulation of insulin signalling and energy homeostasis. Human AHSG is a positional candidate gene for type 2 diabetes and the metabolic syndrome (MetS) (9). The prevalence of Diabetes is rapidly rising in India and all over the globe at an alarming rate .The epidemic of type2 diabetes accounts for more than 90 per cent of all diabetes cases (10).

The prevalence of diabetes in south India (Chennai) during 1988 was 8.2 %. Mohan *et al.,* (2007) reported about 13.5 % diabetic prevalence in Chennai during 2001 and they also documented a high prevalence (14.3 %) during 2006. The International Diabetes Federation (IDF) estimates the total number of diabetic subjects to be around 1,439 millions in South East Asia and this is further set to rise to 1,788 millions by the year 2030 (13). It has been suggested that the association of SNP in AHSG gene (Thr256Ser) may be involved in the pathogenesis of type 2 diabetes (2). The presence of AHSG gene polymorphism and its disease association have been studied in other populations including French (5), Japanese (10), Danish (9), Swedish (15) and

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European-Americans[16]. There is paucity of data regarding the association of AHSG gene Thr256Ser SNP in type 2 diabetes among South Indian population. Therefore this study was carried out to determine the association of Thr256Ser SNP in the AHSG gene among south Indian population.

MATERIALS AND METHODS

The study was undertaken on the subjects attending outpatient department of M.V.Hospital for Diabetes & Diabetes Research Centre, Royapuram, Chennai. 211 patients were included in the study and categorized in two groups. In which hundred and one (101) T2DM subjects (diagnosed as per World Health Organization criteria) (18) were included in the study. Other Hundred and ten (110) subjects, healthy volunteers who were not having diabetes served as control. All samples were subjected to routine clinical and laboratory examinations. Clinical, biochemical and anthropometrical evaluation 5 ml of Venous Blood samples were drawn in a vacutainer (BD) tube containing EDTA, after overnight fasting. 300 µl blood was utilised for the isolation of DNA. Plasma glucose, urea, creatinine, serum total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides were measured by Hitachi 912 auto analyzer (Mannheim, Germany) using commercial kit. HbA1c was measured by high- performance liquid chromatography using Bio-rad Turbo variant II auto analyzer.

The study was conducted in Department of Biochemistry & Molecular Genetics, at M.V.Hospital for Diabetes & Diabetes Research Centre, Royapuram, Chennai, Tamil Nadu, India. Height and body weight were recorded and the BMI was calculated according to the formula: weight in kilograms/ square of height in meters (18). The isolation of DNA from the peripheral blood cells was performed by using Phenol - chloroform extraction method. DNA was stored at -20c for future use. The stored DNA was subjected to PCR amplification by using Eppendorf Master Cycler (Germany). Genomic DNA (~ 50ng) was incubated in a total reaction volume of 20µl containing equal concentration of the forward primer 5'- GTA AGG CAA CAC TCA GTG A - 3' and reverse primer 5'- TCA TCA CTG CCA TGT CTAG -3'(~80 Pico moles) (Ocimum bio-solutions, India), 200 µM deoxynucleotide triphosphate, 10X PCR buffer pH 8.3 containing Magnesium chloride ($MgCl_2$) 15 mM and 1.5 units of *Taq* DNA polymerase (Genetbio). DNA was initially denatured at 95°C for 5 min prior to amplication. PCR amplication was accomplished using 35 cycles consisting of 1 min denaturation at 95°C, 45 sec annealing at 58°C, and 1 min extension at 72°C. The final extension included a 5 min at 72°C.

The amplified products were subjected to restriction digestion using *Sac I* enzyme. The reaction was performed in a total volume of 10 µl consisting of 5 µl amplicon, Tris-HCl buffer, (100mM KCl , 1mM DTT, 1 mM EDTA, pH 7.4 at 25ºC) and 2 units of *Sac I* enzyme (GAGCT*C) (Fermentas Life sciences, USA) . Samples were then digested for overnight at 37°C and the digested PCR products were subjected to electrophoresis using 1.5 % agarose gel stained with ethidium bromide (Etbr). The bands were seen under UV light using gel documentation system (Biotech, India). Presence of bands in the electrophoresis, corresponding to following genotypes, Thr/Ser, Ser/Ser and Thr/Thr were looked for. Three bands corresponding to 731 bp, 709 bp and 22 bp, indicate the presence of Thr/Ser polymorphism whereas two bands at 709 bp and 22 bp indicate Ser/Ser and only one band at 731 bp shows the presence of Thr/Thr i.e. no mutation (Fig 1). Ethics The study was approved by the ethics committee of Diabetic Research Centre (DRC), M.V hospital for diabetes WHO Collaboration Centre for Research, Education and Training in diabetes, Royapuram, Chennai, India 600013.

Statistical analysis: Biochemical parameters (fasting blood sugar, postprandial blood sugar, urea, creatinine, HbA1c, triglyceride and total cholesterol, etc) and BMI of all the subjects were expressed as mean ± SD. The statistical analysis was carried out by using Social Package for Statistical Sciences (SPSS) version 10 and primer of biostatistics software version 5.0was used to analyze the data obtained. Pearson chi-square test was performed to find the statistical significance between the genotypes, and the gene frequency was calculated by allele counting. Unpaired Student's t-test was used to find association between variables. A level of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Comparison of clinical characteristics of South Indian subjects (Table 1). There was no significant difference in age, BMI, Total-Cholesterol, Triglyceride, HDL- Cholesterol, LDL-Cholesterol, VLDL- Cholesterol, Urea and creatinine among two groups and there was a significant association of fasting blood sugar in type 2 diabetic subjects when compared to the control subjects. Among 101 T2DM subjects, homozygous wild type (CC), heterozygous (CG) and homozygous genotype mutant (GG) were 68 (67.3 %), 32 (31.7 %) and 1 (0.99 %) respectively (Table 2). The genotype frequencies in control subjects were, 98 (89.09 %) and 12 (10.91 %) in homozygous wild and heterozygous mutant respectively; homozygous mutant (GG) was none (0%). X^2 test =13.59, degree of freedom = 1, ($p < 0.001$). Comparison of biochemical parameters between these two different groups indicates no significant association with AHSG polymorphism (Table 3). In vitro DNA amplification of AHSG gene using the specific primers resulted in 731 bp DNA product. On digestion of the amplified fragment (amplicon) with *Sac I* restriction enzyme, DNA fragments of 731 bp (CC) or 709 bp and 22 bp (GG) or 731 bp, 709 bp and 22bp (CG) were observed. Thus each of the samples revealed one of the three different electrophoretic patterns (Fig 1).

To our knowledge this is the first study that reports AHSG gene Thr256Ser SNP in T2DM in south Indian population. In the present study, we found that there was significant association of AHSG gene Thr256Ser single nucleotide polymorphism with T2DM among south Indian population. But this polymorphism had no significant association with age, postprandial blood sugar, urea, creatinine, HbA1c, triglyceride, total cholesterol and BMI but significantly associated with fasting blood sugar. The association of AHSG gene Thr256Ser SNP was studied in French [5], Japanese [10], Swedish [15] and European-Americans [16] with respect to T2DM, obesity, cardiovascular disease and dyslipidemia, whereas we have studied the association only with T2DM in south Indian population.

Table 1. Comparison of Clinical characteristics of south Indian subjects

Characteristics	Control subjects Mean ± SD (n =110)	Type 2 DM subjects Mean ± SD (n =101)	P -Value
Age (yrs)	53.2 ± 9.41	52.3 ± 12.6	0.560 (NS)
Body mass index (wt(kg)/m^2)	23.32 ± 2.2	23.52 ± 6.6	0.764 (NS)
Fasting blood sugar (mg/dl)	95.95 ± 17.1	155.7 ± 47.63	0.001 (S)
T-Cholesterol (mg/dl)	180.8 ± 32.2	183.1 ± 40.87	0.649 (NS)
Triglyceride (mg/dl)	140.52 ± 82.2	152.2 ± 70.53	0.271 (NS)
HDL-cholesterol (mg/dl)	43.01 ± 8.7	42.7 ± 13.94	0.845 (NS)
LDL-cholesterol (mg/dl)	116.07 ± 28	120.8 ± 38.31	0.304 (NS)
VLDL-cholesterol (mg/dl)	22.02 ± 11.1	23.39 ± 24.47	0.596 (NS)
Urea (mg/dl)	24.8 ± 6.09	25.6 ± 11.9	0.535 (NS)
Creatinine (mg/dl)	0.7 ± 0.18	0.7 ± 0.33	1.000 (NS)

NS – Not significant, p<0.05 – statistically significant
Data are expressed as mean ± SD and Median. T-cholesterol- total cholesterol, HDL-cholesterol- High density lipoprotein cholesterol, LDL-low density lipoprotein, VLDL- very low density lipoprotein.

Table 2 .Frequencies of genotypes in study subjects

Genotypes	Control (n=110)	T2DM (n=101)	Test of Association	
			p value	Odds ratio
CC	98	68		
CG	12	32	0.001	3.96
GG	0	1		
Total Genotypes	110	101		
Allele Frequencies				
Allele	Control	T2DM	Test of Association	
			p value	Odds ratio
C	208	168		
G	12	34	0.001	12.88
Total Allele	220	202		

Table 2 *shows* the number of study subjects, distribution of genotype (CC, CG & GG), and allelic frequencies (C, G), Chi Square, p value and odd ratio both control and type 2 diabetes study subjects.

Table 3. Comparison of biochemical parameters between non-polymorphic Individuals (CC) and polymorphic individuals (CG & GG) in T2DM in south Indian Population

Parameters	CC Vs CG and GG p-value
BMI	0.819
Fasting plasma glucose (mg/dl)	0.750
Postprandial plasma glucose (mg/dl)	0.308
HbA1c (%)	0.692
Total Cholesterol (mg/dl)	0.318
Triglyceride (mg/dl)	0.657

Statistical data between variables of polymorphic with non-polymorphic individuals.
The variables of T2DM showing non significant association.
NS – Non significant, p<0.05 – statistically significant.

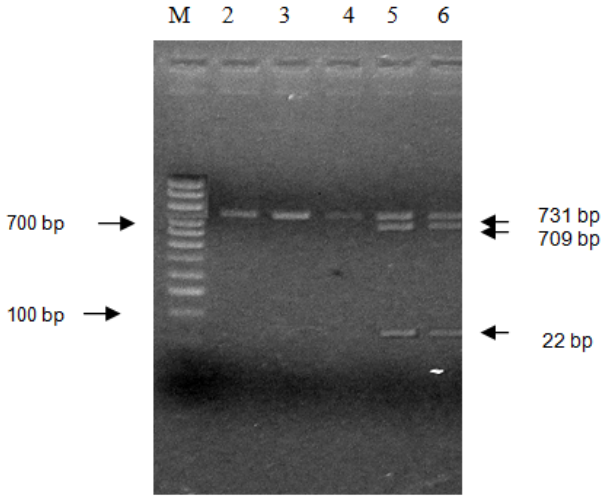

Fig 1. Restriction band pattern of T256S polymorphism in the exon 7 of the AHSG gene
Lane 1 M- molecular wt. marker-100 bp DNA ladder.
Lanes 2, 3 & 4 showing the CC (Thr/Thr)-homozygotes for 256 Thr allele (WILD) showing bands at 731 bp.
Lanes 5 and 6 showing the CG (Thr/Ser)-heterozygotes for 256 Thr/Ser allele (heterozygous) showing bands at 731, 709 bp and 22 bp.

As per Mari Inoue *et al* [10] genotype frequencies were CC-59.5 %, CG-39.6 % and GG-0.0 % of AHSG gene Thr256Ser SNP in Japanese population, whereas in the present study genotype frequencies were CC-67.3%, CG-31.7 % and GG-1%. The genotype frequencies as in Japanese population are nearly same in heterozygous mutant CG and homozygous wild CC in south Indian T2DM population are compared to that of Japanese population. The homozygous mutant GG genotype was completely absent in Japanese population but it was at least 1 % in the present study. Allison *et al* [16] reported the negative association of Thr256Ser polymorphism in T2DM and body mass index in European-Americans, whereas we found statistically significant association in T2DM. There was no significant association in BMI among south Indian T2DM as was seen in Allison *et al.* Cathrina Lavebratt [15] *et al* reported significant association of this polymorphism with BMI in Swedish population (p= 0.027), This may be due to ethnic difference. Afshan siddiq *et al* [5] found a non significant association with T2DM (p=0.09), whereas we found statistical association with T2DM in south Indian population. In biochemical parameters (plasma glucose, Triglyceride and cholesterol) no significant association was seen in French population. Similar finding was seen in south Indian population also. The study results varied between south Indian and French, Japanese and European-American due to the difference in population, genetic diversity and sample size. The conformation of Thr256Ser SNP in AHSG gene in Indian population can be strengthened by only including more number of samples. These studies had reported different percentage of association of this polymorphism with T2DM without any statistical significance, while the present study had found association, statistically significant of this polymorphism with T2DM of south Indian population.

The present study has been carried out in limited number of samples in T2DM; hence the findings have to be validated by including more number of samples. The association of AHSG gene Thr256Ser SNP may have effect on T2DM due to the susceptibility locus of AHSG gene on chromosome 3q27 as it is a positional candidate gene for T2DM. In conclusion, the findings of this study revealed that AHSG gene T256S mutation was significantly associated with T2DM in south Indian population. However, our genetic association study is underpowered to arrive at definitive conclusions because of the small number of study subjects. Further studies based on a larger population are required to confirm this possibility. In addition, association between the polymorphism and clinical phenotypes including insulin resistance, atherosclerosis, etc may also be investigated.

Acknowledgements

This work was supported by Biochemistry staffs of M.V.Hospital for Diabetes, Royapuram, Chennai-600013.

Duality of interest

The author declares that there is no duality of interest associated with this manuscript.

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