Association of HaeIII single nucleotide polymorphisms in the SLC2A1 gene with risk of diabetic nephropathy; evidence from Kurdish patients with type 2 diabetes mellitus

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Summary

Aims: Given the growing rate of patients with type 2 diabetes mellitus, uncovering the effects of gene polymorphism on diabetes pathogenesis has attracted a lot of attention. Because glucose transporter 1 is involved in glucose uptake, the polymorphism of this gene may be an important risk factor in type 2 diabetes mellitus or in the progression of diabetes complications such as diabetic nephropathy. As far as the authors are concerned, this study is the first one aiming at evaluating the probable effects of solute carrier family 2 facilitated glucose transporter member 1 (SLC2A1) HaeIII polymorphism on clinical and laboratory outcomes of Kurdish patients with type 2 diabetes mellitus.

Methods: This study was conducted involving 126 diabetic nephropathy patients and 150 diabetic patients without renal involvement. Serum levels of Cystatin C, fasting blood glucose, creatinine and urinary albumin; levels of glycated hemoglobin and estimated glomerular filtration rate were measured. Moreover, the Hae III polymorphism of SLC2A1 gene was determined by PCR-restriction fragment length polymorphism (RFLP).

Results: The rate of CC genotype was higher (37%) in patients with diabetic nephropathy compared with controls. There were a significant correlation between the CC genotype and risk of diabetic nephropathy. There were significant correlations between genotypes, serum Cystatin C and estimated glomerular filtration rate in patients with diabetic nephropathy.

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Conclusions: The results demonstrated the high frequency of C allele of SLC2A1 Haelll in Kurdish patients with diabetic nephropathy. It was also found that this polymorphism is a significant risk factor for diabetic nephropathy. The effect of this polymorphism on clinical and laboratory characteristics of diabetic nephropathy patients was significant.

Introduction

Affecting millions of people worldwide, type 2 diabetes mellitus (T2DM) is a highly increasing complex endocrine and metabolic disorder that have led to high rates of morbidity and mortality. T2DM accounts for over 90% of diabetes and is recognized as a global epidemic by the World Health Organization. Though the etiology of T2DM is poorly understood, risk factors that may affect the development of this complex metabolic disease include ethnicity, lifestyle and genetic factors. T2DM is associated with micro and macrovascular complications, among which diabetic nephropathy (DN) is the most serious microvascular complication of diabetes mellitus and is one of the most important leading causes of the end stage renal failure in developing countries. DN was found to be present in 12–23% of individuals with T2DM. Besides, the prevalence of DN in Iranian patients with T2DM is 14–26%. Higher levels of glucose uptake by glomerular mesangial cells have been introduced as the main cause of nephron damage in DN. The main histological event associated with DN is Glomerular mesangial enlargement due to glomerulosclerosis and accumulation of extracellular matrix proteins. Besides, the glucose transporter 1 (Glut1), the main glucose carrier in mesangial cells, is increased in renal cortex, and higher glucose concentrations over-express the Glut1 protein concentration. This positive feedback accelerates glucose uptake in mesangial cells, activation of protein kinase C isozymes and polyol pathway, and accumulation of fibronectin. Furthermore, it has been shown that DN has a direct correlation with ethnic and genetic background.

Due to the high prevalence of T2DM, identifying the genes or genetic loci associated with the risk or protection of T2DM is important for understanding the mechanisms underlying the disease and for providing patients with the potential benefits of personalized prevention and treatment programs. As far as the authors are concerned, to date, over 56 susceptibility loci have been identified for T2DM. However, from this rapidly growing list of candidate genes, only a few have consistently been associated with the disease. In addition, these susceptibility loci have been identified predominantly in Caucasian populations. Although several single-nucleotide polymorphisms (SNPs) have been studied as susceptibility loci for T2DM in Iranian individuals, the genes that confer susceptibility to this condition remain unknown.

Glut1, also known as solute carrier family 2 facilitated glucose transporter member 1 (SLC2A1), is located at 1p34.2; its cDNA length is about 3.7 kb, with a core promoter region and 10 exons ranging in size from 97 to 1866 bp and the coding region accounts for <10% of the total. The SLC2A1 encodes the membrane transport protein Glut1, a carrier protein that maintains the glucose concentration at about 5 mM and is responsible for the low-level of basal glucose uptake required to sustain respiration in all cells, especially in erythrocytes.

Previous investigations have examined SLC2A1 as a candidate gene that might confer susceptibility to DN. Moreover, several SLC2A1 gene SNPs including Xba1 (rs841853), Enh2-1 (rs841847), Enh2-2 (rs841848), HaeIII (rs1385129) and HpyCH4V (rs710218) have been studied. The results obtained from those studies, however, have generated considerable controversy. Many studies have been conducted on the effects of SLC2A1 SNPs on protein function among patients with different ethnicities; however, they have yielded discrepant results. One possible reason for the conflicting results may be genetic heterogeneity in different populations or clinical heterogeneity in different studies. In addition, to the best of the authors’ knowledge, there is little, if any, information about the SLC2A1 gene obtained from Kurdish population. Therefore, this study attempted to assess the possible association of Haelll SLC2A1 polymorphism with the development of nephropathy in Kurdish patients with T2DM.

Materials and methods

Patients

A total of 380 patients (with disease duration >10 years) were diagnosed with T2DM, according to the World Health Organization diagnostic criteria for diabetes mellitus. All studied subjects were admitted to the Diabetes outpatient center of Sanandaj Tohid Hospital (Kurdistan, Iran). Among these, 126 individuals (28 males and 98 females) having an average age of 51.27 ± 11.32 (within the age range of 38–78), with two 24 h micro-albuminuria levels >30 mg/24 h, were considered to have DN. The study also involved 254 T2DM patients without DN (130 males and 124 females) as control group with an average age of 47.2 ± 9.7 (within the age range of 30–67), without a known history of T2DM at least for the past 10 years. In addition, patients with a history of urinary tract infection, hematuria, nephritis and other conditions of renal disease were excluded from the study. All individuals were from Kurdistan, a province in western Iran with a population consisting of Kurds. Written informed consent was obtained from all patients and the study was approved by the ethics committee of Kurdistan University of Medical Sciences.

Collection of specimens

Fasting blood samples were collected and sera were separated. The whole blood was employed for measuring total glycosylated hemoglobin (HbA1c) and DNA analysis. Moreover, serum samples were used for determining creatinine and Cystatin C using commercial kits, according to the manufacturer’s instructions. Glomerular filtration rate (estimated glomerular filtration rate (eGFR)) was estimated according to Modification of Diet in Renal Disease formula. In addition, 24-h urine specimen was used for urinary albumin assay based on standard sandwich enzyme-linked immuno-sorbent assay technology. All specimens were stored at –70°C, pending simultaneous analysis.

DNA extraction

Genomic DNA was extracted from the whole blood using DNA Extraction Kit (DNP) (CinnaGen Inc, Tehran, Iran) according to the manufacturer’s instructions. Blood samples were incubated...
with lysis buffer, then DNA was selectively precipitated. Next, the insoluble DNA was washed and desalted by wash buffer and was stored at $-20^\circ$C, pending simultaneous analysis.

**PCR-RFLP analysis**

The HaeIII SLC2A1 SNP (g20882C>T) was identified using PCR restriction fragment length polymorphism. The PCR reaction was performed in a final volume of 25 μl using PCR Master Mix kit (CinnaGen Inc, Tehran, Iran), and 10 pmol of each primer with final concentration of 400 nM, and 100 ng DNA. Primer pair was used to amplify a fragment of 177 bp of exon 2-SLC2A1 gene. SLC2A1-Exon 2 forward primer was 5'-CTCCCA GACACGCCTATACAGT-3' and SLC2A1-Exon 2 reverse primer was 5'-GTTGGCCTTAGACACCCAGC-3'. The PCR condition was: 5 min at 95°C (initial denaturation), followed by 45 cycles of 95°C for 45 s (denaturation), 60°C for 30 s (annealing) and 72°C for 45 s using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). In each PCR run, samples with no DNA template were used as negative controls. Amplified DNA fragments (177 bp) were cut by restriction enzyme BshFI (Jena Bioscience, Germany) for 15 min at 37°C. The genotypes were identified by electrophoresis of DNA fragments generated after digestion (two bands: 109 and 68 bp for 20882 CC, one band: 177 bp for 20882 TT and three bands: 177, 109 and 68 bp for heterozygous 20882 CT genotype).

**Statistical analysis**

Chi-squared test was employed to evaluate whether the alleles or genotype frequencies differ between cases and controls. For $2 \times 2$ contingency tables, the odds ratio (OR) and its confidence interval were calculated to determine if the association was statistically significant at nominal 5% level. Moreover, the potential correlations between cystatin C, eGFR, HbA1c, urinary micro-albumin, fasting blood glucose (FBG) and creatinine were investigated using non-parametric Mann–Whitney test. In all performed hypothesis tests, a P value < 0.05 was considered as statistically significant. Wherever appropriate, results were presented in tables as Mean ± SD, if normality assumption was met; otherwise Median ± the interquartile range (IQR) was used.

**Results**

In the first part of the study, 126 DN patients and 254 patients without DN at nucleotide 20882 of the SLC2A1 gene were genotyped to assess its potential association with DN. Genotype distribution in both T2DM patients with and without DN showed significant deviation from a Hardy-Weinberg distribution ($P = 0.000$). The CC genotype was detected more frequently in DN patients than in patients without DN, and the difference was significant (37% vs. 8%, $P = 0.004$). Interestingly, it was found that the frequency of carriers of at least one T-allele at position 20882 of the SLC2A1 gene (carriers of 20882 TT and 20882 CT genotypes) was significantly higher in patients without DN as compared with DN patients (92% vs. 62%, $P = 0.05$). The 20882 CC and 20882 CT genotypes were associated with DN in T2DM patients (OR = 6.3905, 95% CI = 3.2595–12.5291, Z = 5.4, $P = 0.0001$). A summary of the genotyping results is presented in Figure 1. Furthermore, there were no statistically significant association between studied SNP and age and gender of individuals.

Furthermore, DN patients had higher HbA1c, urinary micro-albumin, serum creatinine, serum Cystatin C and eGFR when compared with patients without DN (see Table 1). As shown in Table 1, a noticeable increase in Cystatin C, serum creatinin, urinary micro-albumin and eGFR was observed in patients with

![Figure 1](image-url). Prevalence of SLC2A1 HaeIII genotypes in diabetic patients with and without DN. According to the plot, the prevalence of HaeIII SLC2A1 gene polymorphism was statistically significant: *Between the CC genotypes in DN patients with no DN controls. **Between TT genotypes in DN patients with no DN controls. ***Between CC and TT genotypes in DN patients with no DN controls. **** Between CC and TT genotypes in DN patients.
DN compared with other patients (P = 0.05). On the other hand, the difference between DN patients and patients without DN with regard to HbA1c and FBG was not significant.

Table 2 illustrates the values of laboratory markers in different study genotype groups. As shown in this table, there were significant differences regarding serum Cystatin C and eGFR between genotypes; in addition, the concentration of these markers was significantly higher in CC genotype (median (IQR) was 640 (810) for Cystatin C and 58 (16) mg/dl for eGFR) compared with TT genotype (median (IQR) was 570 (670) ng/ml for Cystatin C and 89 (24) mg/dl for eGFR) was). However, the mean (IQR) concentration of FBG, HbA1c, creatinine and microalbumin was 570 (670) ng/ml for Cystatin C and 58 (16) mg/dl for eGFR). Since CT genotype had low values, it was removed from statistical analysis. Data are presented as median (IQR). Similar letters (i.e. a, b, c and d) in each column represent significant difference (P < 0.05) between groups.

Table 1. Laboratory characteristics of study groups

<table>
<thead>
<tr>
<th></th>
<th>FBG (mg/dl)</th>
<th>HbA1c (% of total hemoglobin)</th>
<th>Microalbumin (mg/24h)</th>
<th>Cr (mg/dl)</th>
<th>CysC (ng/ml)</th>
<th>eGFR (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DN (n = 150)</td>
<td>126.5 ± 30.4</td>
<td>5.9 ± 1.3</td>
<td>19.48 ± 8.79a</td>
<td>0.93 ± 0.26b</td>
<td>1060.4 ± 435.97a</td>
<td>112 (33)a</td>
</tr>
<tr>
<td>DN (n = 126)</td>
<td>130.2 ± 29.3</td>
<td>6.1 ± 2.1</td>
<td>184.04 ± 60.56a</td>
<td>1.54 ± 0.24b</td>
<td>1657.79 ± 243.9c</td>
<td>85 (29)d</td>
</tr>
</tbody>
</table>

FBG, Fasting blood glucose; HbA1c, Glycated hemoglobin; Cr, Creatinine; CysC, Cystatin C, eGFR, estimated Glomerular filtration rate. Data are presented as mean ± SD, except for eGFR which is presented as median (IQR). Similar letters (i.e. a, b, c and d) in each column represent significant difference (P < 0.05) between groups.

Table 2. Laboratory characteristics of patients with regard to genotypes

<table>
<thead>
<tr>
<th></th>
<th>FBG (mg/dl)</th>
<th>HbA1c (% of total hemoglobin)</th>
<th>Microalbumin (mg/24h)</th>
<th>Cr (mg/dl)</th>
<th>CysC (ng/ml)</th>
<th>eGFR (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>160 (82)</td>
<td>5.3 (1.5)</td>
<td>175.42 (23.5)</td>
<td>1.27 (0.2)</td>
<td>570 (670)a</td>
<td>89 (24)b</td>
</tr>
<tr>
<td>CC</td>
<td>163 (78)</td>
<td>5.4 (0.8)</td>
<td>194.12 (35.04)</td>
<td>1.35 (0.8)</td>
<td>640 (810)a</td>
<td>58 (16)</td>
</tr>
</tbody>
</table>

HbA1c, Glycated hemoglobin; Cr, Creatinine; CysC, Cystatin C. Since CT genotype had low values, it was removed from statistical analysis. Data are presented as median (IQR). Similar letters (i.e. a and b) in each column represent significant difference (P < 0.05) between groups.

Discussion

In this study, we focused on SLC2A1 HaeIII polymorphism which has been less studied in previous assessments. For the first time in Iran and especially in Kurdish region, our study was carried on T2DM Kurdish patients. There are several studies concerning the role of genetic polymorphism in SLC2A1 gene in diabetic patients, especially patients with T2DM.10,24,28 Among all SNPs of this gene, less attention has been focused on SLC2A1 HaeIII polymorphism of this locus may result in higher risk of diabetes or this polymorphism. In contrary, another study reported a significantly higher susceptibility to DN for homozygote HaeIII SNP compared with CC + CT genotypes while the OR was 13.1.25 In a recent study, Marques et al.10 evaluated the impacts of Glut1 polymorphisms on susceptibility to DN in Brazilian patients with type 1 diabetes. They analysed four common Glut1 gene polymorphisms (rs3820589, rs1385129, rs841847 and rs841848) in 452 patients. They showed that there is a negative significant correlation between rs3820589 and DN. They could not find any association between rs1385129 (HaeIII) and progression of renal disease in there population. The results obtained by this study are in line with the Ng study but reject the results of the latters. The most frequent genotype observed for SLC2A1 HaeIII polymorphism in the patients of this study was CC. Moreover, it was found in this study that the C allele increases the risk of DN. In this study, the frequency of CC genotype was higher in DN group, and this genotype had a great correlation with risk of DN. The HaeIII polymorphism of SLC2A1 results in affectless alanine substitution at location 15 with Ala. This polymorphism located on exon 2 of SLC2A1 gene which is a transmembrane domain with an alpha-helix structure. Higher rate of C allele as it is shown in present study may result in higher up-take in glucose to mesangial cells and this mechanism may accelerate the development of DN. Furthermore, In line with previous studies,10,29,30 we could not find any correlation between HaeIII polymorphism with age and gender of studied subjects.

It has also been found that the distribution of SLC2A1 polymorphism is significantly influenced by ethnicity.10 A significant difference has been reported regarding the frequency of the SLC2A1 rs841853 SNP between different ethnic groups.10 In a recent meta-analysis carried out by Du et al.10 it has been showed that the SLC2A1 rs841853 polymorphism may confer increased susceptibility to T2DM in Asians. However, there is no currently available strong evidence supporting the association of this genetic variation with T2DM in Caucasians, Blacks or the overall population.10 Given that there are a few studies, as far as the authors are concerned, that have investigated the relationship between HaeIII polymorphism and risk of diabetes, there cannot be a correct interpretation of the relation between ethnicity and HaeIII polymorphism.
Glut1 has been widely studied both at molecular and functional levels. It is a membrane-embedded protein that mediates the transfer of glucose from the surrounding medium into the cells. Glut1 is translocated after insulin stimulation of adipocytes, which may contribute to the action of insulin on glucose uptake. Genetic changes in glucose transporter, especially Glut1 in glomerular cells occur early in diabetes. These changes play a pathogenic role in the development of extracellular matrix (ECM) expansion and perhaps other features of DN. It appears that at least some diabetic patients may be predisposed to nephropathy because of polymorphisms in their SLC2A1 genes.

On the other hand, the results of the current study clearly showed that C allele of SLC2A1 HaeIII polymorphism associated with higher Cystatin C and eGFR, two powerful markers of kidney damage. In line with previous studies, this study also indicated that HaeIII polymorphism of SLC2A1 might increase the risk of DN. However, there was no any relationship between HaeIII polymorphism and HbA1c, or between FBG and microalbuminuria.

In conclusion, the findings of this study revealed the high frequency of CC genotype of SLC2A1 HaeIII polymorphism in Kurdish patients with DN compared with diabetic patients without DN. Furthermore, a strong correlation was found between this polymorphism and clinical and laboratory characteristics in T2DM patients with DN compared with patients without DN. Finally, it can be concluded from the findings of this piece of research that the C allele of HaeIII SNP of SLC2A1 gene is a powerful risk factor for the progression of DN in Kurdish patients with T2DM.

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References


