Gli-similar 3 (GLIS3) rs7020763 (C>G) polymorphism in patients with type 2 diabetes mellitus

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Abstract

Type 2 diabetes mellitus (T2DM) is a heterogeneous group of metabolic disorders characterized by the incapability of pancreatic beta cells to increase insulin secretion to compensate for insulin resistance in the peripheral tissues. T2DM is a multifactorial disease including several environmental factors with the presence of genetic predisposition. The transcription factor GLI-Similar 3 (GLIS3) has an important role in the development, survival and proliferation of pancreatic beta-cells and insulin gene expression regulation. Accordingly, genome-wide association studies have shown that GLIS3 gene polymorphism may confer risk to type 2 diabetes mellitus T2DM. The present study intended to investigate the association between GLIS3 rs7020673 gene polymorphism and type 2 diabetes mellitus and its impact on glycemic control among Egyptian population. This study was conducted on 100 Egyptian patients diagnosed as T2DM patients and 100 age- and sex-matched non-diabetic normal controls. All subjects underwent full history taking, thorough clinical examination, routine laboratory investigations including fasting blood glucose (FBG), fasting insulin and hemoglobin A1c (HbA1c). Detection of rs7020673 polymorphism of GLIS3 gene was done by real-time polymerase chain reaction (PCR) and verified by sanger sequencing. Genotype and allele frequencies of rs7020673 did not differ between case and control groups. Regarding the heterozygous mutant genotype (GC), it was statistically less frequently distributed in diabetic patients (53%) versus controls (67%). Therefore, it can be considered as a negative risk factor for T2DM (OR: 0.5098, 95% CI (0.2827-0.9193), (P< 0.05). In conclusion, our study indicated that the GC mutant genotype may be proposed as a negative risk factor for T2DM in Egyptian population.

Keywords: Type 2 diabetes mellitus (T2DM), Polymorphisms, GLIS3, Sanger sequencing.

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Introduction

Type 2 diabetes mellitus (T2DM) is a multifactorial metabolic disease characterized by impaired glucose hemostasis that is primarily caused by lack of response of peripheral tissues to insulin and/or insufficient production/secretion of insulin by β cells of pancreas. Environmental and genetic variations are key risk factors for T2DM.¹,²

According to the recent report (Diabetes Atlas edition 2021) of International Diabetes
Federation (IDF),³ approximately 537 million people around the world are suffering from diabetes, with 90% of these individuals having T2DM. Egypt is in the World’s 8th place in terms of diabetes incidence, affecting up to 10.9 million people. By the year 2045, Egypt is expected to be in the 6th place, with about 19.9 million diabetic patients, due to the rapidly increasing and aging population, which will represent the highest incidence in the Middle East and North Africa region.³

T2DM risk is strongly heritable. Genetic studies offer a powerful approach for better screening and treatment of diseases by identifying alterations at molecular level associated with physiological trait. Recent genome-wide association studies (GWAS) in different ethnic population around the world have identified hundreds of T2DM susceptible genomic variants, although translating these findings into clinical practice is still challenging.⁴

GLI-Similar 3 (GLIS3) protein is a member of the GLIS subfamily of Krüppel-like zinc finger transcription factors that functions as an activator or repressor of gene expression. GLIS3 plays a critical role in pancreatic cell lineage specification, particularly in the development of mature beta cells. GLIS3 is also important to beta cell mass maintenance and regulation of insulin expression in adults. GWAS in humans have found an association between common variants in GLIS3 and type 1 diabetes mellitus (T1DM), T2DM, and gestational diabetes.⁵

The present study aimed to investigate the potential association between the GLIS3 (rs7020763) gene polymorphism and T2DM and its impact on glycemic control, as measured by hemoglobin A1c (HbA1c) level, among Egyptian population.

**Patients and Methods**

**Study participants**

This is a case-control study, conducted at the Main Laboratory, Clinical Pathology Department, Ain Shams University Hospitals. Subjects enrolled in the study were divided into two groups. Group I, included 100 T2DM patients recruited from the Endocrinology Clinic, Ain Shams University Hospitals and diagnosed according to the International Diabetes Federation (IDF) criteria. These include several situations: a fasting plasma glucose (FPG) level of 126 mg/dL or higher on more than one occasion (fasting is defined as no caloric intake for at least 8 hours), a 2-hour plasma glucose level of 200 mg/dL or higher after a 75-g oral glucose load on more than one occasion, or HbA1c level of 6.5% or higher, or a random plasma glucose level of 200 mg/dL or higher with classic symptoms of hyperglycemia. Subjects with any of the following conditions were excluded from the study, chronic kidney disease, malignancy, pregnancy, and hypothyroidism. Group II, a control group, included 100 age- and sex- matched nondiabetic normal subjects.

**Sampling**

Venous blood samples (10 ml) were withdrawn by venipuncture from each subject after an overnight fast of 8 hours and divided as follows: 5 ml of venous blood were put into an EDTA vacutainer then stored at −20 °C until DNA extraction and detection of gene polymorphism. Aliquots (2 ml) venous blood were placed into another EDTA vacutainer tube for analyzing HbA1c. For glucose and insulin assessment, aliquots (3 ml) of venous blood were collected into yellow-topped serum separator vacutainer tubes, allowed to clot at room temperature for 15–30 min prior to centrifugation at 1000 xg for 10 min for serum separation.

**Laboratory analyses**

All subjects included in this study underwent full history taking, thorough clinical examination with special emphasis on measurements of body weight and height for calculation of body mass index (BMI, calculated as person’s weight in kilograms divided by the square of height in meters), routine laboratory investigations. Such investigations included fasting blood glucose (FBG) assayed on an automatic blood chemistry analyzer (AU680 Beckman coulter, Inc. 250s. Kraemer Blvd. Brea, CA92821, USA.), according to the manufacturer’s instructions. Fasting insulin, assayed using a non-competitive sandwich enzyme linked immunoassay (ELISA) commercial kit (supplied by Glory Science, USA),
according to the manufacturer’s instructions. The HbA1c was measured by a commercial turbidimetric inhibition immunooassay using kit (Roche/Hitachi Cobas® c501 System, Roche Diagnostics International Ltd., Switzerland), according to the manufacturer’s instructions. The Homeostatic Model Assessment of Insulin Resistance (HOMA IR) was calculated (fasting insulin in mIU/L x fasting glucose in mg/dL)/405). The detection of rs7020673 polymorphism of GLIS3 gene was performed by real-time polymerase chain reaction (PCR).

Genotyping

Genomic DNA was extracted from peripheral blood by using a DNA purification mini kit and a genotyping master mix, both supplied by ThermoFisher® (Thermo Scientific, 168 Third Avenue Waltham, MA USA 02451), according to the manufacturer’s instructions. The GLIS3 rs7020673 single nucleotide polymorphism (SNP) was analyzed by real time PCR on DTlite real time PCR System (DNA-Technology, Varshavskoe shosse (high-way), 1252h, Bld. 5, fl. 1, office 12, Moscow, 117587 Russia) using the readymade genotyping assay kit supplied by ThermoFisher® (Thermo Scientific, 168 Third Avenue Waltham, MA USA 02451) containing sequence-specific forward and reverse primers and two fluorescents (VIC/FAM) labeled TaqMan probes, according to the manufacturer’s instructions for distinguishing between the two alleles of rs7020673.

The presence or the absence of the SNP and the allelic discrimination was established according to the type of the emitted fluorescence of either of the reporter dyes or both at the same time as in Table 1.

<table>
<thead>
<tr>
<th>Fluorescence Signals</th>
<th>Sequence Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIC-dye fluorescence only</td>
<td>Homozygosity for Allele 1 i.e. (Wild genotype; CC)</td>
</tr>
<tr>
<td>FAM-dye fluorescence only</td>
<td>Homozygosity for Allele 2 i.e. (Mutant genotype; GG)</td>
</tr>
<tr>
<td>Both VIC- and FAM-dye fluorescence</td>
<td>Allele 1- Allele 2 heterozygosity i.e. (heterozygous genotype; CG)</td>
</tr>
</tbody>
</table>

Validation of random samples

To obtain exact nucleotide sequencing, two samples were randomly selected and sequenced by the Sanger sequencing using an automated ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). The real time PCR end products of selected samples were subjected to amplification by specific primers (Forward primer: AGGACTGGCCATGTCACTTT and Reverse primer: GGACCATGCTCTCTAGACCT) mediated conventional PCR method applied by Thermo scientific, this PCR amplification yielded 464 bp products.

The 464 bp PCR products were purified by QIA quick Gel Extraction Kit (QIAGEN, Strasse 1, 40724 Hilden, Germany), according to the manufacturer’s instructions then sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo scientific; Singapore), according to the manufacturer’s instructions.

The sequence product was purified using a commercial kit (Centri-step Spin Columns Kit, Invitrogen, ThermoFisher®, USA) then the purified sequence product was subjected to sequencing by capillary electrophoresis using an automated ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. Then data analysis was done using https://blast.ncbi.nlm.nih.gov/Blast.cgi website, the results of sequencing yielded a case with homozygous mutant genotype GG and another case with heterozygous GC as shown in Figure 2 (A and B).

Statistical analysis

Data analysis was done using IBM SPSS statistics (V. 25.0, IBM Corp., USA, 2017-2018). For comparing qualitative data in different groups, Chi Squared test/ Fisher exact test was used. Wilcoxon rank sum test and Kruskal Wallis test were used for comparing of non-parametric data between two groups and three genotypes,
respectively. Measurement of association of data between different groups was assessed by means of Odds ratio test. A $P$ value < 0.05 was considered significant.

**Figure 2 (A).** Detection of GLIS3 homozygous GG mutation using Sanger Sequencing.

**Figure 2 (B).** Detection of GLIS3 heterozygous GC mutation using Sanger Sequencing.

**Results**

The descriptive and comparative statistics of the various studied parameters between diabetic patients (Group I) and controls (Group II) are shown in Table 2. There was a statistically significant increase in the HbA1c%, fasting glucose, fasting insulin, and HOMA-IR in diabetic patients (Group I) compared with controls (Group II) ($P < 0.001$ for all). In addition, there was a statistically significant increase in the Body Mass Index (BMI) in group I compared with group II ($P=0.043$).
Table 2. Descriptive and Comparative Statistics of the various Studied Parameters between Diabetic Patients (Group I) versus Controls (Group II).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diabetic Patients (Group I) (n=100)</th>
<th>Healthy Controls (Group II) (n=100)</th>
<th>*P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median (IQR)</td>
<td>median (IQR)</td>
<td></td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>29 (27 – 31)</td>
<td>28.05 (25.7 – 30.95)</td>
<td>0.043</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.1 (7.2 – 9.775)</td>
<td>5.3 (5.025 – 5.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting Glucose (mg/dL)</td>
<td>189 (155 – 243)</td>
<td>88 (83 – 92)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting Insulin (μlU/ml)</td>
<td>15 (12 – 20)</td>
<td>7 (5 – 10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA.IR</td>
<td>7.66 (4.96 – 10.79)</td>
<td>1.54 (1.027 – 2.11)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*P<0.05: Significant; Z: Wilcoxon’s Rank-Sum Test; HbA1c: Glycated hemoglobin A1c; BMI: Body mass index; HOMA.IR: Homeostatic Model Assessment of Insulin Resistance.

There was no difference between the genotypic and allelic frequencies of the studied GLIS3 rs7020673 C>G polymorphism in the diabetic patients (Group I) compared with controls (Group II) Table (3).

However, the heterozygous mutant genotype (GC), was less frequently distributed in Group I (53%) versus Group II (67%), (OR: 0.5098, 95% CI (0.2827-0.9193), (P < 0.05) as shown in Table 4.

Table 3. Genotypic and Allelic Frequency of GLIS3 rs7020673 C>G Polymorphism in Diabetic Patients (Group I) in comparison to Controls (Group II).

<table>
<thead>
<tr>
<th>GLIS3 rs 7020673</th>
<th>Diabetic Patients (n=100)</th>
<th>Controls (n=100)</th>
<th>*P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC genotype (%)</td>
<td>2 (2%)</td>
<td>4 (4%)</td>
<td></td>
</tr>
<tr>
<td>GC genotype (%)</td>
<td>53 (53%)</td>
<td>67 (67%)</td>
<td>NS</td>
</tr>
<tr>
<td>GG genotype (%)</td>
<td>45 (45%)</td>
<td>29 (29%)</td>
<td></td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C allele (%)</td>
<td>57 (28.5%)</td>
<td>75 (37.5%)</td>
<td>NS</td>
</tr>
<tr>
<td>G allele (%)</td>
<td>143 (71.5%)</td>
<td>125 (62.5%)</td>
<td></td>
</tr>
</tbody>
</table>

*P > 0.05 is not significant (NS). X²: Chi-Square Test.

Table 4. Association between GLIS3 rs7020673 Genotypes in Diabetic patients (Group I) versus controls (Group II).

<table>
<thead>
<tr>
<th>GLIS3 rs7020673 Genotypes in (Group I) versus (Group II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>P value</td>
</tr>
<tr>
<td>OR</td>
</tr>
<tr>
<td>(95% CI)</td>
</tr>
</tbody>
</table>

OR: Odds ratio; 95% CI: 95% confidence interval; *P > 0.05 is not significant (NS). X²: Chi square test;
Comparative statistics between the studied GLIS3 rs7020673 genotypes among diabetic patients in respect to different demographic and laboratory findings are shown in Table 5 and Figure 3. There was no difference between frequencies of GLIS3 genotypes CC, GG and GC concerning age, BMI, HbA1c%, fasting glucose, fasting insulin, and HOMA-IR.

**Table 5.** Comparative Statistics between the GLIS3 rs7020673 Genotypes among Diabetic Patients in Respect to Studied Demographic and Laboratory Findings.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CC (n=2)</th>
<th>GC (n=53)</th>
<th>GG (n=45)</th>
<th>*P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Median</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>46.5</td>
<td>52 (46 – 59.5)</td>
<td>53 (46 – 57.5)</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>28.2</td>
<td>29 (27 – 31)</td>
<td>29 (27.3 – 31.25)</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.9</td>
<td>8.2 (7.05 – 9.85)</td>
<td>8.1 (7.25 – 9.75)</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting Glucose (mg/dl)</td>
<td>124</td>
<td>190 (156 – 266)</td>
<td>189 (157 – 242)</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting Insulin (μlU/ml)</td>
<td>18</td>
<td>15 (12 – 18)</td>
<td>17 (13 – 22)</td>
<td>NS</td>
</tr>
<tr>
<td>HOMA.IR</td>
<td>5.49</td>
<td>6.92 (4.68 – 9.96)</td>
<td>9.148 (5.44 – 11.37)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*P > 0.05 is not significant (NS), Kruskal-Wallis Test; HbA1c: Glycated hemoglobin A1c; BMI: Body mass index; HOMA.IR: Homeostatic Model Assessment of Insulin Resistance.

**Figure 3.** Relation between GLIS3 rs7020673 genotypes and HBA1c percentage.

**Discussion**

GLI-similar 3 (GLIS3) protein is a transcription factor that plays a key role in the development and maintenance of pancreatic beta cells as well as in the regulation of insulin gene expression, insulin secretion and β cell survival. Importantly, GWAS have linked single nucleotide polymorphisms in GLIS3 to increased risk for T1DM as well as T2DM in multiple populations. The present study aimed to investigate the potential association between the GLIS3 (rs7020763) gene polymorphism and T2DM and its impact on glycemic control as estimated by HbA1c. Although a non-significant statistical difference regarding the GLIS3 rs7020673 genotypes frequencies (CC, GC, and GG) was found in diabetic patients as compared to the controls, the mutant genotype (GC) versus (GG) was significantly higher in the control group.
than the patients’ group (P < 0.05). Therefore, GC genotype could be proposed as a negative risk factor for T2DM.

These findings were in accordance with that reported by Durate et al., 2017 on the Brazilian population who investigated the association of GLIS3 rs7020673 and rs10758593 polymorphisms with T1DM that showed no association in GLIS3 rs7020763 genotypes between diabetic (T1DM) and the control group, but the frequency of haplotypes with ≥3 minor alleles of these SNPs was increased in T1DM patients compared to controls. Additionally, Durate et al., 2021 meta-analysis revealed that GLIS3 SNPs rs7020673 and rs10758593 did not show any significant association with T1DM but stated that this result should be interpreted with caution considering the small number of individuals in the included studies and small number of studies included for each SNP.

In the present study as regards the allelic frequencies, in spite that the GLIS3 rs7020673 C allele was more distributed among the controls when compared with diabetic patients (37.5% versus 28.5%), it did not reach a statistically significant difference. On the other hand, the G allele as compared to C allele was more frequently distributed among diabetic patients’ group when compared with the controls (71.5 % versus 62.5%), however, no statistically significant difference was observed.

Data of the present study revealed that GC genotype and C allele were more frequently distributed in controls when compared with diabetic patients. This finding may provide evidence that C allele has a protection effect against development of diabetes. Further studies with larger sample size are required to reveal the validity of such observation.

In addition, several studies examined the association between the rs7020673 SNP and T1DM. In this context, Steck et al., 2014 were not able to find any association between the rs7020673 SNP and T1DM in a non-Hispanic prospective cohort from the USA, but they showed that the C allele was associated with protection against the development of islet autoimmunity (IA) after adjustment for co-variables. However, in contrast to these findings, Winkler et al., 2014 demonstrated that the C allele of the rs7020763 SNP was associated with risk for T1DM in children. They found that combination of GLIS3 rs7020673 and other eight SNPs in different genes significantly improved the prediction of T1DM compared with the provided high-risk by HLA DR/DQ alone. In addition, Kiani et al., 2015 found a statistically significance association of GLIS3 rs7020673 (C allele) with T1DM in Pakistani population.

Additionally, Durate et al., 2017 stated that C allele of GLIS3 rs7020673 and A allele of rs10758593 were significantly associated with risk of T1DM only when combined in haplotypes, suggesting that these SNPs might interact in the susceptibility for the disease. On the other hand, a previous study done by Barrette et al., 2009 performed a GWAS combined with a meta-analysis of two previously published studies from European populations (Great Britain and Denmark), showed that G allele of GLIS3 rs7020673 SNP was associated with protection for T1DM in both screening and replication samples.

Interestingly, there are different GLIS3 polymorphisms are known to be associated with T2DM (e.g., rs7041847 and rs7430200). Studies of these SNPs showed that the A allele of the rs7430200 and rs7041847 conferred risk for T2DM. The aforementioned studies demonstrated the key role of GLIS3 in beta cell development, maintenance and function, and strongly point GLIS3 as a convincing example of a gene predisposing for both T1DM and T2DM. In fact, the GLIS3 region is the only locus showing association with both forms of diabetes, glucose metabolism traits.

The conflict and variability in the results seen in the different association studies may arise due to the differences in several genetic factors and ethnic stratification that affects the GLIS3 gene polymorphism, in addition to the interaction with the environmental factors, variation in study design and sample size. Therefore, further large, multi-ethnic studies with higher sample size are needed to clarify the association.

In conclusion, our study of GLIS3 (C>G) polymorphism and T2DM showed that GC mutant genotype was significantly higher in the
control group than the patients’ group and thus could be considered as a negative risk factor for T2DM in Egyptian population. Moreover, no association was detected between GLIS3 rs7020673 (C>G) polymorphism and glycemic control as estimated by HbA1c levels in the T2DM patients.

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Author Contributions
ESE; designed and approved the whole research protocol. WES; contributed to the protocol design, revised laboratory work, and approved the final paper version to be published. MMM; supervised sample collection according to inclusion criteria, revised clinical data, diagnosis, and patient classification. WAY; monitored data collection process and the laboratory work, interpreted the data, and critically revised the paper. LEM; collected the samples and patient’s clinical data, carried out the laboratory work and analysed it, carried out statistical analysis and wrote the manuscript. All authors have read and approved the manuscript.

Declaration of Conflicting Interests
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Ethical approval
The study protocol was reviewed and approved by the Researcher Ethics Committee of the Faculty of Medicine, Ain shams University (approval number FMASU MD 157/2016).

Informed consent
Verbal informed consents were obtained from all participants before enrolment in the study.

References
