

Association of *GCK* Gene Promoter Polymorphism and their Role in its mRNA Expression among Type 2 Diabetes Mellitus Patients

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ABSTRACT

Introduction: Glucokinase (*GCK*) gene alteration or inactivation leads to Maturity-Onset Diabetes of the Young (MODY) and Neonatal Diabetes. Alterations in *GCK* gene has been shown to be associated with increased risk of type 2 diabetes, hyperglycaemia and impaired beta cell function.

Aim: To evaluate the *GCK* (-30G/A, rs1799884) alterations in Type 2 Diabetes Mellitus (T2DM) patients.

Materials and Methods: After patients' selection, blood samples were taken and all biochemical parameters were analysed by auto-analyser using serum. Whole blood samples were used to extract DNA and RNA for genotyping of -30G/A polymorphism using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) as well as *GCK* mRNA expression using quantitative real time PCR.

Results: Several parameters such as fasting sugar, serum uric acid, Low Density Lipoprotein (LDL), High Density Lipoprotein (HDL), Triglyceride (TG), Cholesterol, high-sensitivity C-Reactive Protein (hs-CRP) were analysed among T2DM cases and healthy controls and differences among them were found to be significantly associated ($p < 0.0001$). It was observed that patients who were smoking among T2DM cases, were found to be associated with

increased fasting sugar ($p = 0.001$), postprandial sugar ($p < 0.0001$), HbA1c ($p = 0.003$), blood urea ($p = 0.01$), cholesterol ($p = 0.02$) compared to those who were not smokers. T2DM patients who were alcoholic, showed increased LDL ($p = 0.006$) and cholesterol ($p = 0.01$) compared to non-alcoholic T2DM patients. T2DM cases which were reported hypertensive, showed increased hs-CRP ($p = 0.02$) and cholesterol ($p = 0.02$), compared to non-hypertensive T2DM cases and differences among them were found to be significantly associated. Significant differences were observed in distribution of *GCK* -30G/A genotypes among T2DM cases and healthy controls ($p < 0.0001$). Odds ratio was calculated and it was observed that the heterozygous GA genotype had 4.66 odds ratio while odds ratio for mutant AA genotype was 35.98 in reference to wild type. Patients with homozygous GG showed 0.25 mean fold decreased *GCK* mRNA expression while heterozygous GA and mutant AA genotype showed more than 0.17 and 0.16 fold decreased. *GCK* mRNA was observed and differences among them was found to be significant ($p < 0.0001$).

Conclusion: Study revealed that *GCK* heterozygous GA and mutant AA genotypes were associated with T2DM risk and decreased expression may be responsible for disease progression and worsening of the disease.

Keywords: Gene expression, Maturity onset diabetes of the young, Neonatal diabetes, Polymorphism

INTRODUCTION

The Type 2 Diabetes Mellitus (T2DM) is very multifaceted metabolic disease, characterised by increased blood glucose level, resistance to insulin, impaired secretion of insulin due to defect in pancreatic beta cell and elevated hepatic glucose secretion. The actual reason of progression of T2DM and its development have not been fully analysed yet. Evidences suggested that several genetic and environmental factors are involved. Although the genetic contribution to T2DM has been recognised, large-scale Genome-Wide Association Studies (GWAS) confirmed 56 established susceptible loci in human [1-3].

GCK is a key regulator of phosphorylation of glucose, and in the first rate-determining step in the pathway of glycolysis. It also regulates metabolism of glucose in liver and glucose stimulated secretion of insulin from β -cells of pancreas [4, 5]. Activating mutation in *GCK* results in persistent hyperinsulinemic hypoglycemia while inactivation of mutations in *GCK* causes neonatal diabetes mellitus and Maturity Onset Diabetes of the Young (MODY) [6-8]. Furthermore, genetic variant rs1799884 in the β -cells specific promoters of *GCK* in pancreas has been linked with increased risk of T2DM, hyperglycemia and reduced function of β -cells [9, 10].

In particular, various Single Nucleotide Polymorphisms (SNPs) in *GCK* gene are linked with reduced regulation of glucose and increased risk of T2DM [11,12]. Insulin mediated *GCK* activation supports utilisation of glucose through activating important enzymes required in lipogenesis and glycogenesis processes, like Fatty Acid Synthase (FSN) and Insulin Receptor Substrate-1 (IRS-1). Due to Genetic Variation, *GCK* transcription decreases and *GCK* activity also reduce which results in reduced sensing of glucose in liver cells, decreased secretion of insulin from β -cells and ultimately diabetes mellitus [13]. Haeusler RA et al., reported that expression of *GCK* in liver is inhibited by >60% in people with diabetes mellitus in comparison with people without diabetes [14]. Higuchi N et al., stated that nutritional elements like amino acids may enhance the expression of *GCK* in hepatic cells [15].

The present study intends to investigate the association of SNP within the *GCK* gene and mRNA expression among T2DM patients.

MATERIALS AND METHODS

Study Design and Duration

The current study was a case-control study in which 200 samples, including 100 T2DM patients and 100 control subjects were included. All the subjects were analysed for a period of two years. i.e. December 2017 to July 2019.

The study has been conducted only after the due clearance and approval from the Ethics committee of GKV (vide Proposal No. 14/08/2015/GKV/IEC/2015).

Inclusion and Exclusion criteria: Samples of primary cases of acute nature of T2DM were included and samples of chronic cases of T2DM were excluded in this study. Only T2DM samples were collected and mixed cases samples or type 1 diabetes mellitus samples were excluded. The samples were selected between the age of 18-50 years for both male and female. Samples below 18 years of age and above 50 years of age were excluded from this study.

Patient's Selection and Blood Sample Collection

Total 200 study subjects were included in present study. Out of 200 study subjects, 100 were T2DM and 100 healthy subjects. Three mL of blood sample were drawn in EDTA vials only after the informed consent being obtained from patients and healthy subjects. In present study formula used for calculating the sample size is given below and adjusted the power accordingly.

$$N = Z^2 P(1-P)/d^2$$

N is the sample size,

Z is the value from the standard normal distribution reflecting the confidence level that will be used (Z=1.96 for 95%CI)

P is expected proportion in population based on previous studies or pilot studies.

Power is 80%

d is precision (corresponding to effect size)

Calculated sample size came out to be 150

All the samples were stored in -80°C for further processing.

DNA Extraction, RNA Extraction and cDNA Synthesis

Three mL of blood samples were processed by RBCs lysis buffer to collect the WBCs and stored in phosphate buffer and few parts were processed for DNA extraction by salting out method. RNA was extracted by Trizol reagent according to the manufacturer's protocol (AMRESCO, USA) from T2DM cases as well as from healthy control samples stored in phosphate buffer at -80°C. Quality of total RNA was analysed by Nanodrop and cDNA synthesis which was done by using 100 ng total RNA following manufacturer's protocol (Verso, Thermo Scientific, USA). Briefly, 100 ng of total RNA, 5X cDNA synthesis buffer, dNTPs (5 mM each), RT enhancer, Verso enzyme mix and random hexamers (400 ng/μL) in the total volume of 20 μL incubated for 60 minutes at 42°C, and after synthesis cDNA was stored at -20°C for expression study.

Genotyping of GCK Gene

Extracted DNA was used to study the GCK -30G/A promoter polymorphism using Allele specific PCR method. The primer sequence for wild type forward sequence was, FW: 5'-ACAACCACAGGCCCTCTCAGGAG-3', forward mutant sequence was FM: 5'-ACAACCACAGGCCCTCTCAGGAA-3' and common reverse for both was CR: 5'-TTTGAGTAATTAGGCTGCAG-3'. After protocol standardization, the programme was set for amplification at 94°C for 10 minutes, denaturation at 94°C for 40 seconds, annealing at 62°C for 40 seconds, extension at 72°C for 40 seconds for 40 cycle, final extension for 5 minutes at 72°C and hold was 4°C for 10 minutes, respectively. After amplification, agarose gel electrophoresis was done to visualise the 155 bp band of amplified product under UV light.

mRNA Expression of GCK Gene

Quantitative real time PCR was performed by using Sybgreen mastermix, cDNA was synthesised from 100 ng of total RNA. The primer sequences for GCK mRNA expression were as: forward primer 5'-ACCAAGCGGTATCAGCATGTG-3'; reverse primer 5'-TGGACTTCTCTGTGATTGGCA-3' and internal control GAPDH

forward primer 5'-TGTGGGCATCAATGGATTTGG-3' and reverse primer 5'-ACACCATGTATTCCGGGTCAAT-3'. The parameters for qRT-PCR assay were as follows: 95°C for 5 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and hold temperature on 4°C. QRT-PCR was performed in triplicate with Applied Biosystems 7500 PCR System (Applied Biosystems, USA).

STATISTICAL ANALYSIS

All the data recorded in excel and Statistical Package for the Social Sciences (SPSS) 19, graph pad prism were used to analyse the data. Chi-square and Fisher's-exact test were used to compare the genotypes among the groups for qualitative data. Hardy-Weinberg equation was used to calculate the allele frequency among T2DM cases and healthy controls. Mann-Whitney U test and Kruskal-Wallis test were used to compare the group for statistical significance in quantitative data. The p-value <0.05 were taken as statistically significant.

RESULTS

Demographic Characteristic of T2DM Patients and Healthy Controls

Patients in age group ≤50 years were 37 and >50 years were 63, while healthy controls in age group ≤50 years were 35 and >50 years were 65. Among T2DM cases, (74 males, 26 females) while in healthy controls (75 males and 25 females). More details are depicted in [Table/Fig-1].

Variables	T2DM cases n (%)	Healthy controls n (%)
Age (Years)		
≤50 years	37 (37)	35 (35)
>50 years	63 (63)	65 (65)
Gender		
Males	74 (74)	75 (75)
Females	26 (26)	25 (25)
Smoking status		
Yes	69 (69)	65 (65)
No	31 (31)	35 (35)
Alcoholism		
Yes	53 (53)	55 (55)
No	47 (47)	45 (45)
Hypertension		
Yes	27 (27)	20 (20)
No	73 (73)	80 (80)

[Table/Fig-1]: Demographic characteristics of T2DM patients and healthy controls.

Biochemical Parameters among T2DM Cases and Healthy Controls

Several parameters such as fasting sugar, serum uric acid, LDL, HDL, TG, Cholesterol, hs-CRP were analysed among T2DM cases and healthy controls and differences among them were found to be significantly associated (p<0.0001, [Table/Fig-2]). All the biochemical parameters were increased whereas HDL was decreased in T2DM cases.

Association of Risk Factors among T2DM Cases

All the biochemical parameters among T2DM cases were analysed concerning smoking, alcoholism, and hypertension [Table/Fig-3]. It was observed that patients who were smoking were found to be associated with increased fasting sugar (p=0.001), postprandial sugar (p<0.0001), HbA1c (p=0.003), blood urea (p=0.01), cholesterol (p=0.02) compared to those who were not smokers. T2DM patients who were alcoholic showed increased LDL (p=0.006) and cholesterol (p=0.01) compared to non-alcoholic T2DM patients and differences among them were found to be significantly associated.

Variables	T2DM cases (Mean±SD)	Healthy controls (Mean±SD)	p-value
Fasting sugar	156.4±49.28	87.92±9.26	<0.0001
Serum uric acid	5.71±2.11	4.20±0.74	<0.0001
LDL	224.4±73.76	87.28±36.83	<0.0001
HDL	30.39±6.49	39.50±9.06	<0.0001
TG	411.3±100.4	148.4±59.74	<0.0001
Cholesterol	311.3±44.43	153.9±46.47	<0.0001
hs-CRP	6.55±1.85	2.12±1.85	<0.0001

[Table/Fig-2]: Comparison of biochemical parameters between Type 2 Diabetes Mellitus (T2DM) and healthy controls.
LDL: Low density lipoprotein; HDL: High density lipoprotein; TG-Triglyceride; hs-CRP: high-sensitivity C-reactive protein

Variables	Smoking		p-value
	Yes	No	
Fasting sugar (mg/dL)	167.5±52.25	134.8±23.60	0.001
Post prandial sugar (mg/dL)	235.4±65.7	172.3±19.53	<0.0001
HbA1c (%)	8.71±1.71	7.75±0.78	0.003
Blood urea (mg/dL)	16.62±6.20	14.6±4.60	0.01
Creatinine (µmol/L)	1.03±0.41	1.16±0.45	0.05
Serum uric acid (mg/dL)	5.82±2.39	5.46±1.28	0.75
Albumin (g/dL)	4.52±1.20	4.44±1.38	0.45
Globulin (g/dL)	3.13±0.98	3.36±0.89	0.25
Total protein (g/dL)	7.64±1.31	7.82±1.58	0.56
hs-CRP (mg/L)	6.69±1.79	6.22±1.95	0.24
LDL (mg/dL)	229.3±72.03	213.5±77.57	0.37
HDL (mg/dL)	29.80±6.86	31.71±5.43	0.25
TG (mg/dL)	409.2±91.11	416.0±120.1	0.94
Cholesterol (mg/dL)	318.6±40.76	294.8±48.42	0.02

Variables	Alcoholism		p-value
	Yes	No	
Fasting glucose (mg/dL)	159.6±49.67	154.9±45.77	0.62
Post prandial glucose (mg/dL)	219.3±61.51	211.9±64.11	0.56
HbA1c (%)	8.26±1.50	8.59±1.59	0.29
Blood urea (mg/dL)	15.81±6.72	15.85±4.72	0.97
Creatinine (µmol/L)	1.08±0.50	1.06±0.32	0.73
Serum uric acid (mg/dL)	5.61±2.14	5.82±2.09	0.45
Albumin (g/dL)	4.38±1.23	4.63±1.27	0.46
Globulin (g/dL)	3.28±1.06	3.10±0.81	0.98
Total protein (g/dL)	7.68±1.21	7.72±1.58	0.98
hs-CRP (mg/L)	6.48±2.01	6.62±1.66	0.51
LDL (mg/L)	246.3±74.84	205.0±67.73	0.006
HDL (mg/dL)	29.72±6.97	31.15±5.88	0.34
TG (mg/dL)	425.8±93.24	407.3±107.1	0.57
Cholesterol (mg/dL)	321.2±39.47	301.0±47.39	0.01

Variables	Hypertension		p-value
	Yes	No	
Fasting glucose (mg/dL)	163.6±47.07	155.1±48.03	0.42
Post prandial glucose (mg/dL)	230.1±56.84	210.5±64.39	0.16
HbA1c (%)	8.44±1.77	8.40±1.46	0.91
Blood urea (mg/dL)	15.11±4.64	16.10±6.25	0.60
Creatinine (µmol/L)	1.02±0.45	1.09±0.42	0.27
Serum uric acid (mg/dL)	6.11±2.59	5.56±1.90	0.47
Albumin (g/dL)	4.41±1.32	4.53±1.23	0.69
Globulin (g/dL)	3.30±1.08	3.16±0.90	0.93
Total protein (g/dL)	7.73±1.39	7.69±1.40	0.97
hs-CRP (mg/L)	7.18±2.18	6.31±1.66	0.02
LDL (mg/L)	228.1±73.35	214.5±75.35	0.16

HDL (mg/L)	31.03±5.36	28.67±8.74	0.41
TG (mg/L)	416.3±104.5	397.7±88.62	0.53
Cholesterol (mg/L)	329.3±31	304.6±46.91	0.02

[Table/Fig-3]: Association of risk factors with biochemical parameters among T2DM cases.

T2DM cases who were reported hypertensive showed increased hs-CRP (p=0.02) and cholesterol (p=0.02) compared to non-hypertensive T2DM cases and differences among them were found to be significantly associated.

GCK -30G/A Genotype Distribution among T2DM Cases and Healthy Controls

Significant differences were observed in distribution of GCK -30G/A genotypes among T2DM cases and healthy controls (p<0.0001). In T2DM cases homozygous GG, heterozygous GA, mutant homozygous AA was (42%), (49%), (9%), respectively, while in healthy controls it was (80%), (20%) and (0%), respectively. Risk allele A among T2DM cases was 0.34 while in healthy controls it was 0.10. However, G allele in T2DM was 0.67 while in healthy controls it was 0.90 [Table/Fig-4].

Variables	GG (%)	GA (%)	AA (%)	p-value	G allele	A allele
T2DM cases	42 (42%)	49 (49%)	9 (9%)	<0.0001	0.67	0.34
Healthy controls	80 (80%)	20 (20%)	00 (0)		0.90	0.10

[Table/Fig-4]: Distribution of GCK genotypes among T2DM cases and healthy controls.

GCK -30G/A Genotype and Risk among T2DM Cases

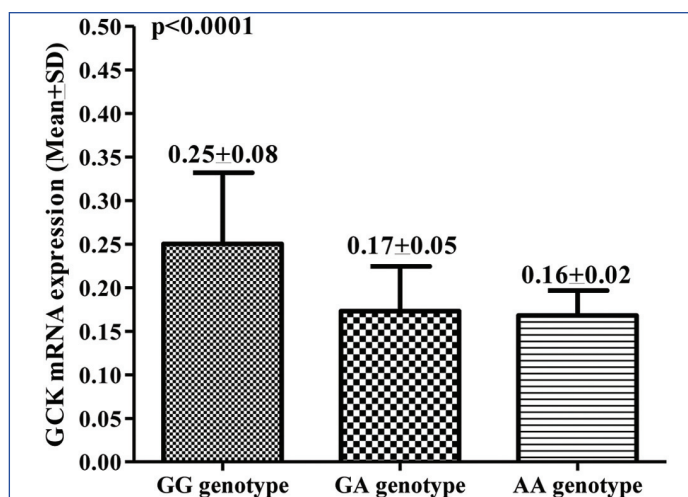
Odds ratio was calculated and observed that the heterozygous GA genotype had 4.66 odds ratio while odds ratio for mutant AA genotype was 35.98 in reference to wild type genotype [Table/Fig-5].

Genotypes	T2DM cases	Healthy controls	Odds ratio
GG	42 (42%)	80 (80%)	Ref
GA	49 (49%)	20 (20%)	4.66 (2.46-8.85)
AA	9 (9%)	0 (0%)	35.98 (2.04-633.4)

[Table/Fig-5]: Risk association of GCK -30G/A genotype among T2DM cases.

Association of GCK mRNA Expression with -30G/A Genotypes

In T2DM cases, overall 0.20 (Mean±SD, 0.20±0.07) mean fold change increased expression was observed compared to healthy controls. However, significant difference was observed in expression pattern with respect to genotypes. GCK genotype patients with homozygous GG showed 0.25 mean fold decreased GCK mRNA expression while heterozygous GA and mutant AA genotype showed more than 0.17 and 0.16 mean fold decreased GCK mRNA, respectively and differences among them were found to be significant (p<0.0001, [Table/Fig-6]).



[Table/Fig-6]: GCK mRNA expression among homozygous, heterozygous and mutant.

DISCUSSION

Glucokinase (*GCK*) on the control of systemic blood glucose homeostasis that is summarised as the *GCK* glucose sensor paradigm. In pancreatic beta-cells, *GCK* serves as a sensor of blood glucose that regulates insulin secretion. In the liver, *GCK* controls the glucose disposal that promotes glycogen and triglyceride synthesis [16]. Many activating and inactivating *GCK* mutants have been characterised in patients [17]. Several studies have identified environmental factors, including physical inactivity and caloric excess, which contribute to the pathogenesis of type 2 diabetes and the metabolic syndrome [18]. Likewise, there is much evidence to demonstrate that genetics plays an important role in these diseases [19,20].

Authors found an association of *GCK* promoter polymorphism which was linked with T2DM. Distributions of biochemical parameters were also significantly different between T2DM cases and association of risk factor were also found to be associated with biochemical parameters among T2DM. A significant difference was found in *GCK*-30G/A genotype and allele frequency distribution among T2DM cases and healthy controls. Risk allele A was higher in T2DM cases as compared to healthy controls. Odds ratio also showed that risk was associated with heterozygous GA and mutant AA genotypes among T2DM cases. With respect to genotype, decreased *GCK* mRNA expression was found in T2DM cases and significant difference was observed among genotypes in T2DM cases.

Inactivation of mutations in *GCK* causes neonatal diabetes mellitus and MODY [21, 22]. Genetic variant rs1799884 in the β -cells specific promoters of *GCK* in pancreas has linked with increased risk of T2DM, hyperglycemia and reduced function of β -cells [23,24]. It has been said that *GCK* -30G/A has been conclusively associated with fasting glucose in European population [25]. *GCK*-30G/A polymorphism of *GCK* influences susceptibility to phenotypes of impaired glucose regulation. Recent studies support the influence of genetic and environmental factors on T2DM susceptibility. A twin study on Danish population observed the significant high heritability for reduced glucose tolerance in T2DM patients [26]. Muller YL et al., and Rosta K et al., reported that several SNP's in *GCK* gene are linked with reduced regulation of glucose and increased risk of T2DM [11,12].

Several studies reported that activity of *GCK* decreases in T2DM [27,28]. *GCK* gene mutation causes MODY. Therefore, post-transcriptional or transcriptional effects causes reduction in activity of *GCK*. Under normal conditions, Fork head box O (FOXO) is inactivated by insulin, through AKT mediated phosphorylation and nuclear exclusion. Hence, common reason for extreme production of glucose during diabetes mellitus is improper hepatic FOXO process activation, because of insulin resistance. Suppressing *GCK* and promoting G6PC are two important characteristics of production of glucose by FOXO pathway [29,30].

Present study is significant in unmasking the underlying role of *GCK* gene which plays an important role in T2DM. This gene showed a strong association with T2DM. So, further studies to find the association of *GCK* gene and its effects can be performed in other populations. More studies with more clinicopathological and anthropogenic parameters in different ethnic populations can provide the preventive measures for T2DM patients.

Limitation(s)

There are few limitations of present study. Firstly, the sample size of the present study was small. Hence, this study need to confirm on larger sample size. Secondly, it cannot be excluded that the variation in a variety of diet and medications may have affected present study findings.

CONCLUSION(S)

Present study concluded that risk was found to be associated with the *GCK* gene -30G/A nucleotide alteration. Decreased *GCK* mRNA

expression was observed and associated with -30G/A genotypes which may be responsible for the worsening of the disease.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: Sep 23, 2019
- Manual Googling: Aug 26, 2020
- iThenticate Software: Sep 28, 2020 (9%)

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- Financial or Other Competing Interests: As declared above
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
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