Molecular Evaluation and Study of Organic Base Changes in GCK Gene Related to Gestational Diabetes

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Abstract

Studies in recent years have shown that gene mutations can cause changes in the function of insulin and its receptors, so this change can cause Diabetes during pregnancy and after it. Unlike monogenic diseases, disease incidence is affected by the mutant allele in one gene locus. In diseases similar to type II diabetes, disease incidence depends on several gene loci with a small to moderate impact. The GCK gene encodes a member of the hexokinase family of proteins. Phosphorylated glucose hexokinase to produce 6-phosphate is the first step in most glucose metabolism pathways. Compared to other forms of hexokinase, this enzyme is not inhibited by its product glucose 6-phosphate but remains active when glucose is abundant and leads to diabetic disorder. Thus, this study investigated the frequency of this polymorphism between 50 pregnant women with gestational Diabetes as a patient group and 10 pregnant women without Diabetes or pre-diabetes as a control group. Genomic DNA was extracted by kit method, and PCR and Sequencing techniques were used to determine the genotype of individuals. The frequency of GG and GA genotypes for GCK mutation (rs1799884) was 8% and 92%, respectively, in the patient group and 100% in the control group.

Keywords: *Gestational Diabetes, Polymorphism, GCK, PCR, Sequencing*

Introduction

Pregnancy is a complex metabolic situation. It is associated with significant changes in the hormonal environment and changes in adipokines and inflammatory cytokines. It is also associated with significantly increasing estrogen, progesterone, prolactin, cortisol, placental growth hormone, leptin, and oxidative stress indicators. A reduction in adiponectin from the second trimester intensifies the insulin resistance in the mother to facilitate fuel delivery of the couple to the fetus, playing an essential role in the diabetogenic nature of pregnancy. Gestational Diabetes is a temporary form of Diabetes and can occur in any non-diabetic woman during pregnancy. This state is due to glucose intolerance and appears in pregnant women without a history of Diabetes despite its incompatibility with the body. After two tests, the person is considered diabetic if the glucose level in the fasting person's venous blood is higher than 126 mg/dL. However, this is a simple form of glucose intolerance that needs care. This is a common issue in Diabetes that (in approximately 5% of pregnancies) leads to severe problems for both mother and fetus.

This disease mostly appears between 24 and 28 weeks of pregnancy. Placental lactogen hormone is secreted by the placenta, reducing insulin sensitivity in the mother. Hormonal changes, high weight, and a family history of Diabetes play a role in this disease. According to the American Diabetes Institute, about 4% of women get this form of Diabetes during pregnancy. This disease can cause problems such as the birth of a premature fetus and breathing issues for the mother and the infant. This disease improves after delivery. However, the mother and the child are prone to type II diabetes at older ages (Pappa, 2011). Type II diabetes is the most common type of Diabetes and accounts for 90% of diabetes cases. The prevalence of type II diabetes is continuously increasing. The incidence of type II diabetes in children has increased almost ten times.

Unlike monogenic diseases, the occurrence of the disease is affected by the mutant allele in one gene locus; in diseases similar to type II diabetes, the occurrence of the disease depends on several gene loci that have a small to moderate impact. Gestational Diabetes is a multifactorial disease in which genes interact with each other and environmental factors. Genetic variants in different gene loci control insulin activity and secretion. According to the multifactorial model, disease susceptibility can be determined by combining multiple genetic variants and environmental factors. The genetic predisposition of people does not necessarily cause an obvious syndrome unless they are exposed to special environmental factors.

Several types of glucose-lowering drugs apply their antidiabetic impacts through different mechanisms. Some of these mechanisms are stimulating insulin secretion by drugs of the sulfonylurea family and meglitinides, increasing peripheral absorption of glucose by biguanides and thiazolidinediones, delaying the absorption of carbohydrates from the intestine by alpha-glucosidase inhibitors, reducing hepatic gluconeogenesis by biguanides and thiazolidinediones (Dupuis, 2011), an increase in serum concentration of GLP-1 and reduction of gastric emptying by new peptide analogs such as exenatide and liraglutide, and DPP-4 inhibitors (Saxena et al., 2010). These treatments have disadvantages such as the development of drug resistance, side effects, and even toxicity

due to lack of response. For example, sulfonylureas lose their effectiveness in 44% of patients within six years. None of these glucose-lowering drugs effectively control the increase in blood lipids (Dereke, 2001). There is a need to develop natural plant sources for antidiabetic drugs with the increasing prevalence of Diabetes in the rural population due to the adverse effects of synthetic drugs (Rung et al., 2009).

Additionally, drugs' side effects and interactions, revealed in the human body or during various tests, are significant issues that should be considered (Greeley et al., 2011). The active ingredients in different medicinal plants can reduce blood sugar through different mechanisms. These mechanisms are increasing insulin secretion, activating the glucose catabolism pathway, inhibiting or deactivating the gluconeogenesis pathway, directing glucose into the cell, absorbing free glucose and preventing its binding to proteins, increasing the antioxidant capacity, and preventing damage caused by oxidants produced in different pathways that may be caused by excess glucose and the production of glycine end products or other metabolic pathways, and the prevention of glucose absorption from the intestine (Carmody, 2016).

The GCK (glucokinase) gene is located on chromosome 7 (7p13), which has 15 exons (25). This gene encodes a member of the hexokinase family protein. Phosphorylated glucose hexokinase to produce glucose 6-phosphate is the first step in most glucose metabolism pathways. Compared to other forms of hexokinase, this enzyme is not inhibited by its product glucose 6-phosphate but remains active when glucose is abundant. Using multiple and alternative plasma promoters of this gene leads to distinct protein isoforms that show tissue-specific expression in the pancreas and liver. This enzyme plays a significant role in glucose-stimulated insulin secretion in the pancreas. However, this enzyme is vital in absorbing glucose and converting it to glycogen in the liver.

Mutations in this gene, which changes the enzyme's activity, are associated with various types of Diabetes and hypoglycemia (Chakera, 2012). In gestational Diabetes, insulin resistance causes the body to release insulin by the pancreas, which can cause weight gain. The risk of GDM increases with increasing BMI of pregnant women. Glucokinase is an enzyme that phosphorylates glucose and detects glucose secretion by the liver. Glucokinase regulatory protein regulates the adaptation of blood sugar absorption and excretion. This polymorphism may be responsible for affecting the concentration of glucose or triglycerides in the imbalance of connection with other genes (Beltcheva, 2014).

Royal Devon and EXETER NHS FOUNDATION TRUST molecular genetics group conducted a study entitled "Management of pregnancy in patients with hyperglycemia caused by various types of diseases in the glucokinase gene (GCK)." This cross-sectional study was conducted on Glucokinase, an enzyme found in pancreatic beta cells. The study method was PCR. The size of the fetus, and thus, the complications related to the size of the fetus, are highly dependent on whether the child inherits GCK or not. If the child inherits this type of gene, it will be 600 grams heavier than normal. Therefore, the GCK status of the newborn has a higher impact on the outcomes of the fetus than the mother (Watanabe 2011). Lisa R. Letourneau et al. (2018) conducted a study entitled "Management and Pregnancy Outcomes of diabetic pregnant women with GCK-MODY in the United States of America."

A cross-sectional study conducted by Redcap in pregnant women over 18 years old showed that the mean birth weight of infants with GCK was higher at all times than mothers treated with insulin. Finally, the unavoidable rate compared to the rate of the US population was 15-20%. The results revealed that GCK plays a significant role in gestational Diabetes and its prognosis (34). Nannan Wu, Ying Fu, Simo Liu, Dong Zhao, et al. (2018) conducted a study entitled "Investigation of genetic types that affect gestational diabetes". The results revealed that the G972R polymorphism of the IRS-1 gene is strongly associated with the increased susceptibility to GDM. However, the prevalence of the Gly / Arg genotype in the group of Brazilian pregnant women was not statistically significant (Laura 2016). Rudland, V. L (2016) conducted a study entitled "Identification of Glucokinase in monogenetic gestational diabetes mellitus, new abortion prevention criteria, and HbA1c Utility" by Rudland, V. L et al. in 2016. Glucokinase monogenic diabetes (GCK-onset diabetes of the young (MODY)) should be differentiated from gestational diabetes mellitus (GDM). New pregnancy screening (NSC) criteria were proposed to identify women for GCK testing. They tested NSC and HbA1c in a multinational group of GDM by Big Dye Terminator version 3.1 Cycle Sequencing Kit and investigated the predicted referrals for GCK testing. Four out of the 31 diabetic pregnant women had the GCK gene. HbA1c before delivery was not higher in people with GCK-MODY (Rudland, V. L. 2016).

Due to the high prevalence of gestational Diabetes and the personal and social problems resulting from it and its prognosis on the mother and the infant, and given the level of genetic impact on gestational Diabetes and the possibility of early detection of people at risk, pre-pregnancy screening, prevention, and modification of the lifestyle and nutrition of people at risk to treat chronic disorders of the mother during pregnancy, and to ensure the health of the mother and the infant, it is necessary to conduct more studies in the field of genetics of gestational Diabetes to prevent its complications and consequences by early diagnosis. According to the collected information, no study has been conducted in Iran so far. Hence, the present study evaluates and investigates the

molecular changes of the organic base in the GCK gene regarding gestational Diabetes.

Materials and Methods

The present study is applied in terms of purpose and descriptive in terms of method. Sampling was done from 50 Iranian pregnant women who were referred to Fatemeh Al-Zahra Robat Karim Hospital with overt Diabetes or pre-diabetes according to Tables 1 and 2, without a history of type 1 and type II diabetes before pregnancy, para 1 (not having a first pregnancy or history of pregnancy that is more than 22 weeks), not using tobacco and drugs, not using alcoholic beverages, age range of 18 to 35 years, not using heparin during pregnancy, not using assisted reproductive methods (IVF, IUI, Microinjection, or surrogate uterus) or assisted reproductive drugs such as clomiphene and metformin. Furthermore, 10 Iranian pregnant women referred to Fatemeh Al-Zahra Rabat Karim Hospital without any history of illness and Diabetes was used as a sample control group.

From fifty samples of Iranian pregnant women suffering from gestational Diabetes, two ccs of venous blood were collected in an EDTA tube and stored in appropriate laboratory conditions. Ten samples from healthy Iranian pregnant women without any history of disease with the same volume of 2 cc of venous blood stored in an EDTA tube were kept. In this study, based on the reviewed articles and NCBI website and Oligo, MEGA, and Finch Tv software, the specificity and creation of secondary structures were examined by Oligo analyzer and Primer Blast NCBI and ordered to SinaClon Company for synthesis.

SinaClon Company prepared the primer used in this study. The forward and reverse primers for determining GCK polymorphism were designed by the Sanger sequencing method and purchased from SinaClon Company. SinaClon Company prepared the primer used in this study. The forward and reverse primers for determining GCK polymorphism were designed by the y Sanger sequencing method and purchased from SinaClon Company.

GCK (Forward): TGCATGGCAGCTCTAATGAC

GCK (Reverse): TTAGGCTGCAGGTGACTGTG

First, 500 µl of blood sample was added to a 1.5 cc Eppendorf microtube, and 500 µl of solution A was added to it and vortexed for 5 seconds to extract DNA from the peripheral blood sample. Then, it was centrifuged at 3000 rpm for 5 minutes. Then, the supernatant was discarded, and 500 µl of solution A was added to the sediment and vortexed for 5 seconds. It was centrifuged again for 5 minutes at 3000 rpm, and the supernatant was discarded. Then, 450 µl of solution B was added to the sediment and vortexed for 2-3 seconds. Then, 50 µl of solution C was added to them and vortexed for 2-3 seconds. Finally, 750 µl of chloroform was added to the resulting solution and centrifuged for 6 minutes at 6000 rpm. Then, 500 µl of the supernatant phase was removed, and a new 1.5 cc microtube was poured (our solution had three phases at this stage). Then, we added one cc of 100% ethanol to the solution and shook it to mix, and it was centrifuged for 7 minutes at 11,200 rpm. The supernatant solution was discarded again and mixed with 800 cc of 70% ethanol, shortly vortexed for 2 seconds, and finally centrifuged at 7000 rpm for 6 minutes. The supernatant solution was discarded and left to evaporate for 15 minutes with the lid open at room temperature. Finally, 50 µl of distilled water was added to the primary compound. TBE 0.5 X buffer in the required amount was poured inside the electrophoresis tank, and a tray containing 1.8% agarose gel was placed inside the tank. With the help of a sampler, 3-4 µl of the marker was carefully and gently poured into one of the gel wells so the marker was completely inserted into the well. Then, 5 µl of the PCR product was gently and carefully poured into the gel well. Finally, the tank was connected to the electric power source. Then, we turned on the power supply and set it to the appropriate voltage. After the end of the electrophoresis time, the electric power source was turned off, and the gel was removed from the tank. Then, it was placed in the Gel-Doc device, and the amplified gene was observed. The PCR products were stored in a freezer at -20 degrees Celsius for further studies. Table 1 presents the PCR amplification process for the GCK gene.

Table 1: PCR amplification process for GCK gene

Materials used	Amount of materials used
2x taq premix(master mix)	12.5 ml multiplied by the number of samples
Forward primer (10 pmol/ml)	1 ml multiplied by the number of samples
Reverse primer (10 pmol/ml)	1 ml multiplied by the number of samples
DNA(10-100ng/ml)	1.5 ml multiplied by the number of samples
	9 ml multiplied by the number of samples

	Temperature C °	Time	Number of cycles

Initial Denaturation	94	3 minutes	35 cycles
Denaturation	94	30 seconds	
Annealing	60	30 seconds	
Extension	72	30 seconds	
Final Extension	72	5 minutes	

After electrophoresis of the samples on agarose gel, some heterozygous (with two specific bands) and homozygous (single band) samples, common to all samples of the same gel, were selected. After PCR again with sterile head samplers, they were sent to Codon Genetics Company for sequencing. Finally, all the results were analyzed statistically.

Results

Table 2 presents the specifications of the samples.

Table 2: Specifications of the samples

Type of Diabetes	BMI	Weight	Abortion	Number of pregnancies	Age	Sample
Overt	32	78	0	1	29	1
Overt	27.5	80	1	2	22	2
Overt	27	85	1	2	29	3
Overt	28.3	86	2	3	35	4
Overt	28.3	90	2	3	36	5
Overt	25.8	68	0	1	28	6
Overt	25	78	0	1	26	7
Pre-diabetes	25.9	75	0	1	23	8
Overt	22.9	55	0	1	33	9
Overt	26.5	75	0	1	26	10
Pre-diabetes	24.5	63	0	1	22	11
Prediabetes	24	65	0	1	18	12
Overt	31	88	1	2	27	13
Prediabetes	22	61	0	1	20	14
Overt	30	86	3	4	34	15
Overt	31.6	84	0	1	22	16
Overt	32	88	2	3	27	17
Overt	34	85	0	1	34	18
Overt	22.6	51	1	1	21	19
Prediabetes	24	65	0	1	32	20
Overt	27	85	1	2	40	21
Prediabetes	28	70	1	2	29	22
Prediabetes	24	60	0	1	33	23
Overt	24	67	0	1	18	24
Prediabetes	24	65	0	1	18	25

Prediabetes	28	71	0	1	19	26
Prediabetes	32	86	0	1	18	27
Pre-diabetes	22.1	54	0	1	22	28
Prediabetes	23	68	1	2	31	29
Prediabetes	30	85	0	1	18	30
Overt	28	69	0	1	22	31
Overt	26.5	72	0	1	25	32
Prediabetes	34	98	0	1	19	33
Pre-diabetes	18.5	54	0	1	20	34
Prediabetes	27	71	0	1	25	35
Overt	23.5	68	0	1	19	36
Overt	29	75	0	1	35	37
Overt	34	97	0	1	30	38
Overt	31	84	1	2	19	39
Pre-diabetes	31	89	0	1	24	40
Overt	28	65	2	3	22	41
Pre-diabetes	26.4	54	0	1	26	42
Overt	26	82	0	1	25	43
Pre-diabetes	23.9	74	0	1	24	44
Overt	22.8	58	2	3	28	45
Overt	20	52	1	2	31	46
Overt	34	99	.	1	35	47
Overt	35	94	1	2	19	48
Overt	29	74	0	1	24	49
Overt	21	68	2	3	20	50
Healthy	19	56	0	1	26	51
Healthy	19.9	54	0	1	27	52
Healthy	27.1	62	0	1	34	53
Healthy	23.5	60	0	1	31	54
Healthy	24.6	65	0	1	29	55
Healthy	28.9	69	0	1	19	56
Healthy	30	79	0	1	25	57
Healthy	19.6	51	0	1	27	58
Healthy	25.1	68	0	1	30	59

Healthy	24	65	0	1	28	60

The following results were observed after extracting and examining the quality of the extracted DNA and the samples were taken to gel electrophoresis. The target gene region was amplified using designed primers and PCR techniques. A comparison of the observed bands with the bp50 marker showed that the observed band corresponds to the expected length in all samples.

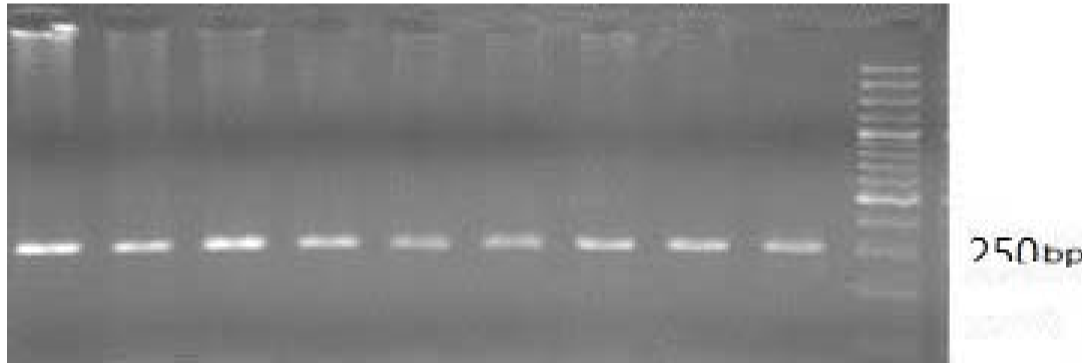

Figure 1: PCR products of patients with GCK gene

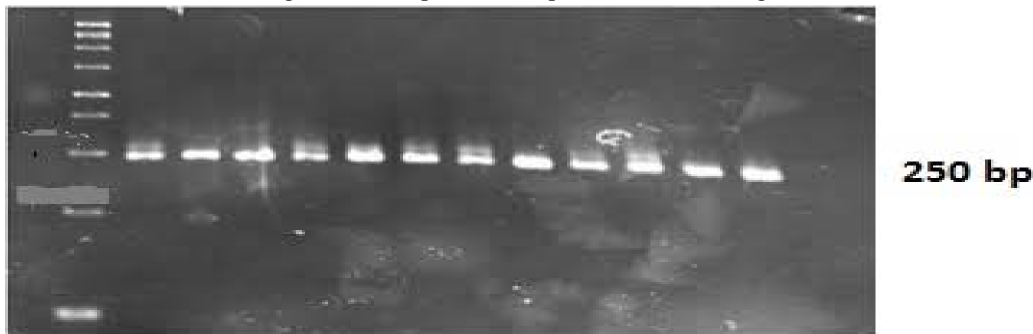

Figure 2: PCR products of healthy people with GCK genes

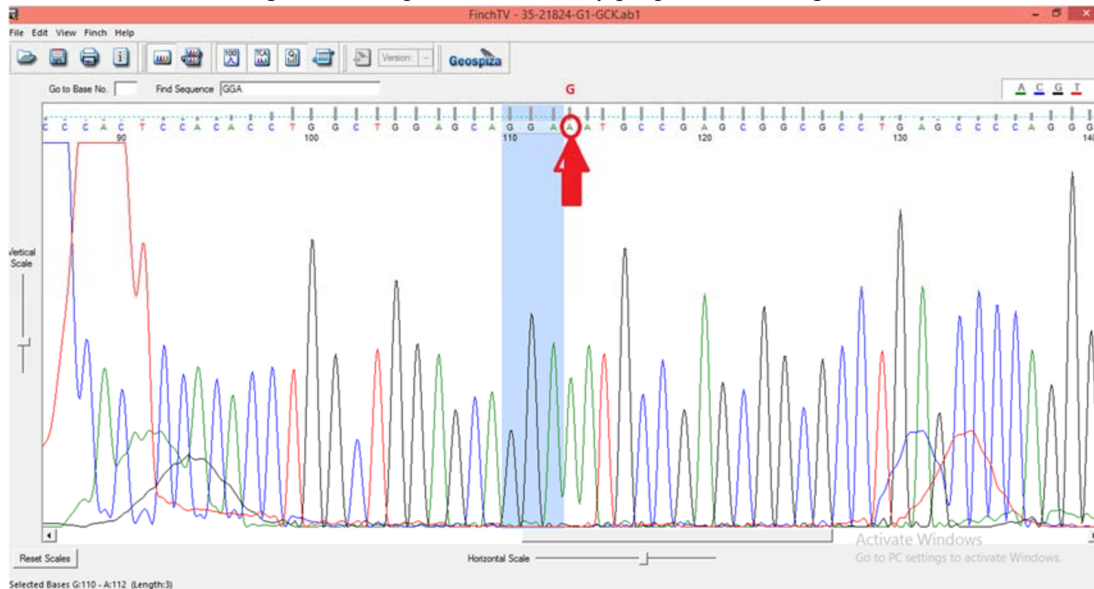

Figure 3: Samples with mutation in GCK polymorphism (Rs1799884)

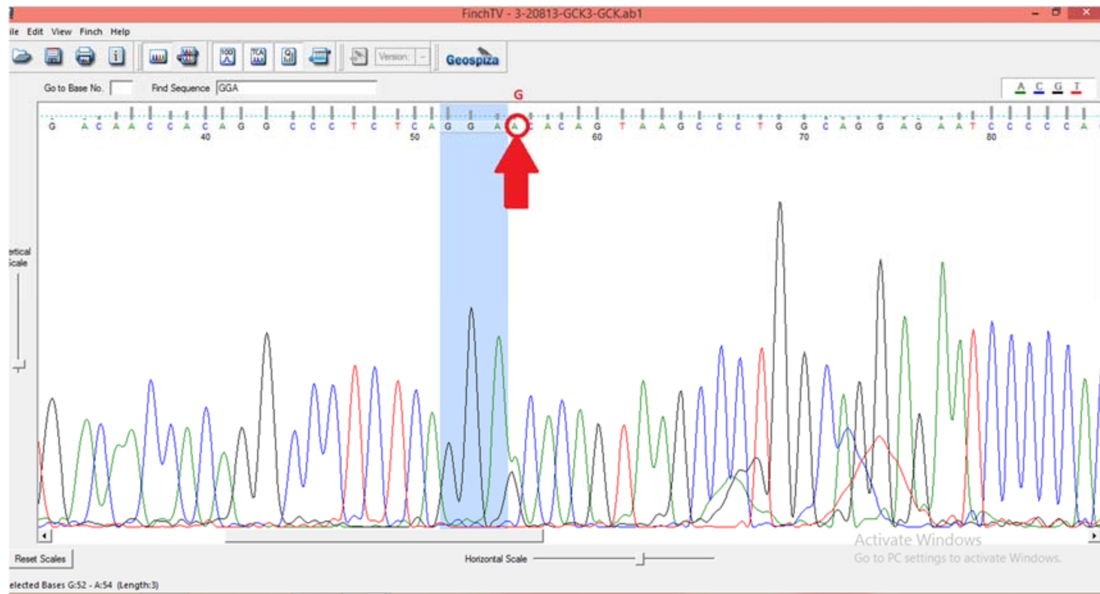

Figure 4: Samples with mutation in GCK polymorphism (Rs1799884)

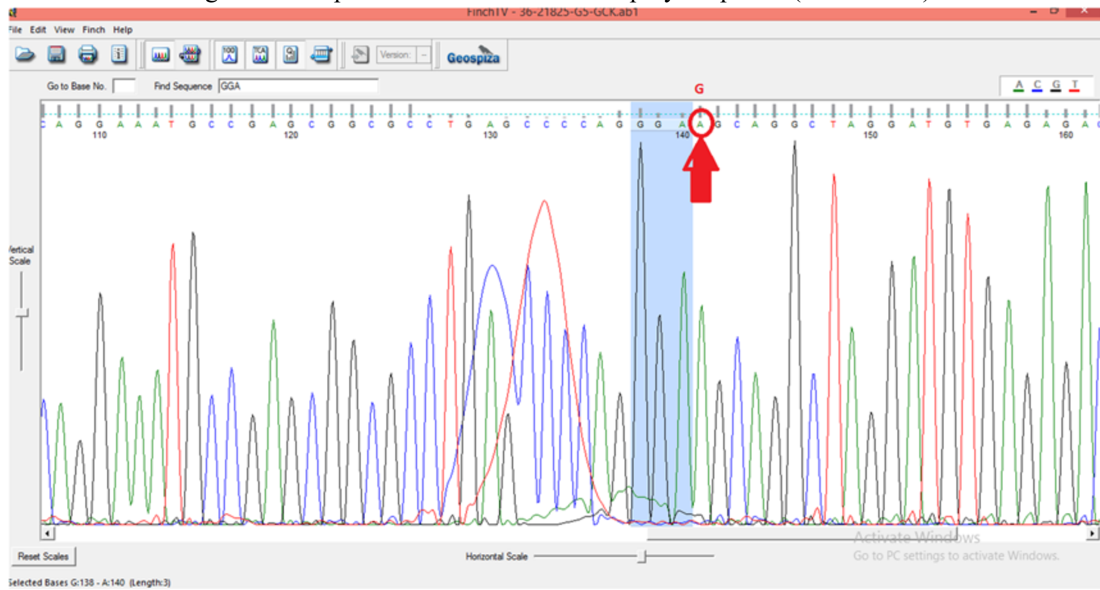

Figure 5: Samples with mutation in GCK polymorphism (Rs1799884)

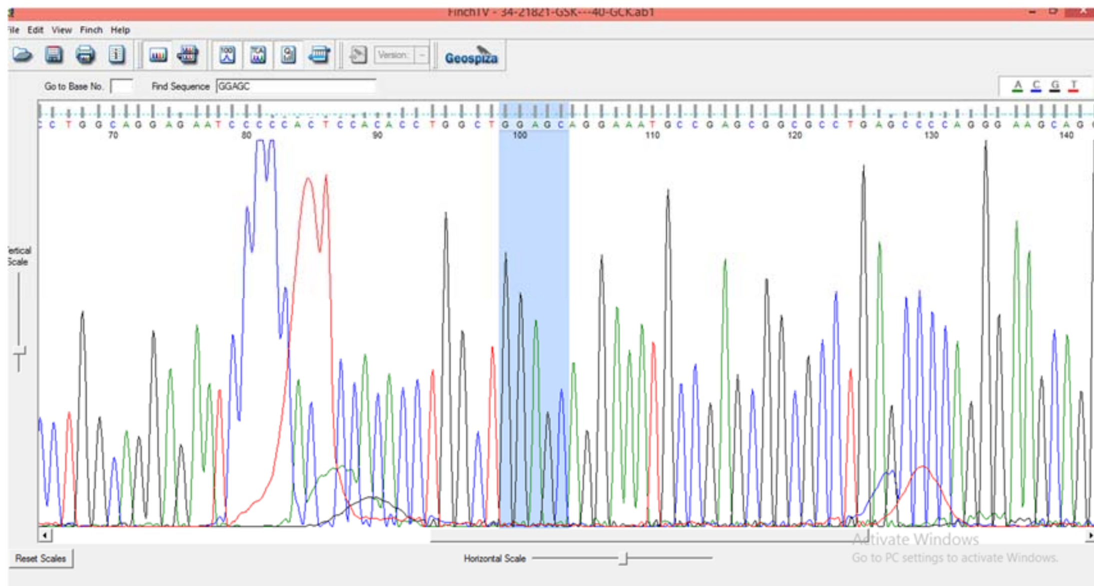

Figure 6: A healthy sample without mutation in GCK polymorphism (rs1799884)

Table 3: Number of people with GCK mutation

Total sample	Number of people with GCK mutation GA	Number of healthy people GG	Frequency of GCK mutation
Patient group n= 50	4	46	8%
Control group n=10	Zero	10	Zero

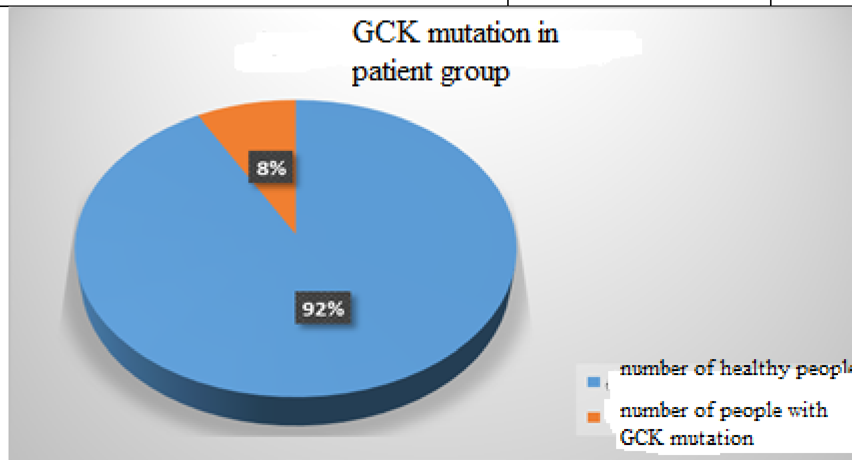

Figure 7- The frequency of mutation in patients

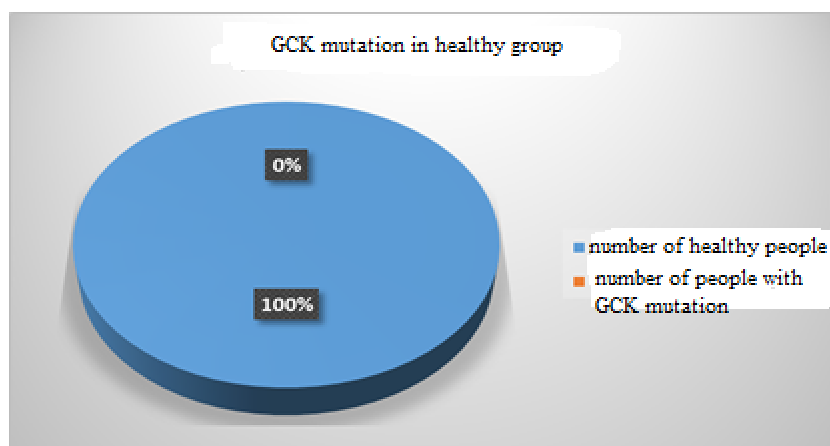

Figure 7- The frequency of mutation in healthy people

Discussion

Medical studies on the genetics of Diabetes will help women at risk of gestational Diabetes to be aware of their risk of gestational Diabetes before pregnancy and take preventive measures to protect the health of their infants. Some researchers found that changes in HKDC1 and BACE2 genes are related to sugar and insulin levels in pregnant women. However, these two genes are unrelated to increased sugar and insulin levels outside pregnancy in people with type II diabetes. Hayes states, "We will be able to determine the genetic profile of women's risk of developing gestational diabetes with more research and changes in these two genes and other risk genes." The results of this study report the role of the HKDC1 gene in glucose metabolism and the BACE2 gene in insulin secretion during pregnancy compared to the period before and after pregnancy among different races. The researchers obtained these results using DNA and phenotypic information of more than 4 thousand participants from 4 different races (Hispanic, Thai, Afro-Caribbean, and European) who were enrolled in the HAPO study. The study of HAPO or hyperglycemia and its harmful results during pregnancy was international research in several different countries. In this study, pregnant women from different geographic regions with different races and socio-geographical statuses participated. Lowe stated that the study's results can help to accurately and quantitatively determine genetic traits to predict the possibility of developing gestational Diabetes in women.

New studies by a large international group of scientists provided a more complete picture of the genes responsible for type II diabetes and pregnancy. The study revealed that the common alleles that were previously identified by researchers are the most common cause of this disease, and the less common variety and scientists assumed that they play a crucial role in developing this disease did not play a significant role. GCK (glucokinase) is located on chromosome 7 (13p7), which has 15 exons (Poirier 2017). This gene encodes a member of the hexokinase family of proteins. Phosphorylated glucose hexokinase to produce glucose 6-phosphate is the first step in most glucose metabolism pathways. Compared to other forms of hexokinase, this enzyme is not inhibited by its product glucose 6-phosphate but remains active when glucose is abundant. Using multiple and alternative plasma promoters of this gene leads to distinct protein isoforms that show tissue-specific expression in the pancreas and liver. This enzyme plays a significant role in glucose-stimulated insulin secretion in the pancreas. However, this enzyme is crucial in absorbing glucose and converting it to glycogen in the liver. Mutations in this gene, which alters enzyme activity, are associated with various types of Diabetes and hypoglycemia. In gestational Diabetes, insulin resistance causes the body to release insulin by the pancreas, which can cause weight gain. The risk of GDM increases with increasing BMI of pregnant women. Glucokinase is an enzyme that phosphorylates glucose and detects glucose secretion by the liver. Glucokinase regulatory protein regulates the adaptation of blood sugar absorption and excretion. This polymorphism may affect glucose or triglyceride concentration in the linkage imbalance with other genes.

Conclusion

The results indicate that many pregnant women are at risk of gestational Diabetes due to hundreds or even thousands of genetic variants that are common among different populations. The subjects were examined regarding GCK polymorphism mutation (rs1799884). Among female patients, 92% had GG polymorphism, and 8% had GA polymorphism. However, among healthy subjects, 0% had GA, and 100% had GG. Based on the results, it is recommended to examine the expression level of other genes related to Diabetes and gestational Diabetes in addition to the investigated GCK gene. Also, it is recommended to examine HbA1c in addition to examining fasting glucose and OGTT.

Conflict of interest:

None.

Financial support:
None.
Ethics statement:
None.

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