Original Article

Molecular study of organic base changes in the IRS gene associated with gestational diabetes

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ABSTRACT

According to recent studies, gene mutations can change the function of insulin and its receptors, so this change can lead to gestational diabetes. Unlike single-gene diseases, getting sick from the mutation allele is affected in a genetic position. Diseases such as type 2 diabetes depend on several gene positions that have a small to medium effect. Arg972 polymorphism in the 1 IRS gene has been shown in diabetic pregnant women or women with gestational hyperglycemia, as well as their infants and its evaluation and association with genetic toxicity in pregnant women. In this study, the frequency of polymorphisms among 50 pregnant women with gestational diabetes was assessed as the patient group and 10 pregnant women without diabetic disorder or pre-diabetes as the control group. Genome DNA was extracted by the kit method and PCR, and sequencing techniques were used to determine individuals' genotypes. The frequency of GG, T / GC, and IRS-1 polymorphism (rs1801278) genotypes was 72% and 28% in the patient group, and 90% and 10% in the control group, respectively.

Keywords: Diabetes Pregnancy, Polymorphism, Sequencing - PCR- IRS-1.

Introduction

Pregnancy is a physiological condition that is associated with metabolic changes. These changes indicate fetal growth and development. Failure to regulate these physiological processes can cause problems, the most common of which is gestational diabetes. Gestational diabetes is a heterogeneous disorder caused by various environmental and genetic factors. Studies have shown that gestational diabetes has some common genotypic and phenotypic characteristics with different types of diabetes, including type one, type two, and juvenile diabetes. Gestational diabetes is temporary diabetes and can occur in any non-diabetic woman during pregnancy. This condition is due to glucose intolerance and, despite its incompatibility with the body, occurs in pregnant women with no history of diabetes. After two tests, if the amount of glucose in the venous blood of a fasting person is higher than 126 mg/dL, the person has diabetes. But this is a simple type of glucose intolerance that needs to be taken care of. This is a common problem in diabetes that (in about 5% of pregnancies) can lead to serious problems for both mother and fetus. This disease usually occurs between the 24th and 28th weeks of pregnancy, and at the same time, the placental lactogen is secreted by the placenta, which reduces insulin sensitivity in the mother. Hormonal changes, along with high weight and family history of diabetes, play a role in this disease. The disease can cause problems such as preterm birth and respiratory problems for both mother and infant. The disease improves after childbirth, but mothers and children are more likely to develop type 2 diabetes at an older age [1]. Type 2 diabetes is the most common type of diabetes, and 90% of diabetics have type 2 diabetes. The prevalence of type 2 diabetes is increasing, and the rate of type 2 diabetes in children has increased almost tenfold. Diabetes is not a simple disease, it's a complex syndrome characterized by high blood sugar. Therefore, diabetic patients are clinically, pathologically, and genetically heterogeneous. Diabetes and pregnancy are so interacting that they can seriously endanger the health of the mother, including diabetic nephropathy, diabetic retinopathy, diabetic neuropathy,

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preeclampsia, infections, increased risk of cesarean section, preterm delivery, and increased postpartum hemorrhage.

Unlike single-gene diseases, getting sick from mutation allele is affected in a genetic position. Diseases such as type 2 diabetes depend on several gene positions that have a small to medium effect. Gestational diabetes is a multifactorial disease in which genes interact not only with each other but also with environmental factors [2].

Insulin activity and secretion may be controlled by genetic variants at different gene positions. Based on the multifactorial model, the susceptibility to the disease can be determined by a combination of multiple genetic variants and environmental factors.

The IRS-1 gene (insulin receptor substrate) is located on chromosome 2 (2q36.3), which has 2 exons. The adapter protein is a signal that is encoded in humans by the IRS-1 gene [3]. The IRS-1 protein is a 131-kDa protein with 1242 amino acids, which includes the convergence of the Christensen plot domain in the N-terminal and the remaining 40bp domain at the bottom of the stream following the C-terminal thickness. It plays a key role in transmitting the signals of insulin and insulin-like growth factor 1 receptors (IGF-1) to the Akt/PI3K intracellular pathways and the MAP kinase Erk pathway. The phosphorylation of IRS-1 tyrosine by the IR insulin receptor introduces several binding sites for proteins that have an SH2 correlation range such as PI3K, Grb-2/Sos complex, SHP2, and PI3K. In addition, Akt kinase is activated by PDK1 through the phosphorylation of T308 and similar sites in PKC. This phosphorylation does not exist in poor tissues of IRS-1. A diabetic cascade seeks to absorb glucose. Signal transmission of IRS-1 may be inhibited by SHP2 in some tissues.Arg972 polymorphism of the IRS-1 gene has been shown in diabetic pregnant women or people with mild gestational hyperglycemia, as well as their infants, and its evaluation and association with genetic toxicity in pregnant women. Diabetic patients have a higher frequency of Gly972Arg IRS-1, and this polymorphism is directly related to insulin resistance and hyperglycemia. In diabetes, hyperglycemia and other related factors produce reactive oxygen species that increase DNA damage. Therefore, the aim of this study was to evaluate Arg972 polymorphism of the IRS-1 gene in pregnant women with diabetes and mild hyperglycemia pregnancy and their infants. In these pregnant women, the level of primary DNA damage has been seen in lymphocytes.

However, effective data show that genetic factors may play a role in gestational diabetes. Relatively few studies have been published on the genetic susceptibility of gestational diabetes. More than 53% of environmental insulin sensitivity and 75% of insulin secretion changes can be explained by genetic components. Previous studies have shown that different genes are linked to the risk of gestational diabetes. Therefore, it was aimed to evaluate and molecular study the organic changes in the 1st IRS gene in relation to gestational diabetes in order to increase the fertility index of the society. Pregnancy is a critical period for appropriate intervention and health measures aimed at reducing the prevalence of type 2 diabetes. The prevalence of gestational diabetes is increasing in many developed countries.

In general, according to the study, many genes are effective in gestational diabetes that their effectiveness varies before pregnancy and during pregnancy, depending on the sex of the fetus and father's genes. In addition, environmental factors (nutrition, weight, lifestyle, mental health condition) have the greatest impact on gestational diabetes and also increase the impact of these genes. Based on the content and the researcher's experience on the importance of gestational diabetes and its prognosis on pregnancy and subsequently on the fetus and due to the effect of genetics on gestational diabetes and the possibility of early detection of people who are at risk, pre-pregnancy screening, prevention and improving lifestyle and nutrition requires further study of the genetics of gestational diabetes to prevent its consequences by early detection. GCK and IRS-1 genes are effective in the cycle that causes gestational diabetes and not enough research have been done on them, and the researcher planned to evaluate the organic changes in these two genes associated with gestational diabetes [4, 5].

Materials and Methods:

Target society: Sampling was performed out of 50 pregnant Iranian women who had referred to Al-Zahra Hospital in Robat Karim with diabetes or pre-diabetes according to Tables 1 and 2, with no history of type 1 or pre-pregnancy diabetes, para-1, who did not smoke, did not drink alcohol, did not use heparin during pregnancy, did not use assisted reproductive techniques or assisted reproductive drugs such as clomiphene and metformin, that were between 18-35 years old.

Also, sampling was performed out of 10 pregnant Iranian women who had referred to Al-Zahra Hospital in Robat Karim without any history of disease and diabetes, as a control group.

Materials used

Materials required for DNA extraction using a blood collection kit from blood sample inside the EDTA tube

1. DNA extraction kit called STAR DNA purification kit from Sinaclon company contains solution A, solution B, solution C.
2. Chloroform
3. 100% ethanol
4. 70% ethanol
5. DNA

Materials required for PCR reaction in IRS-1 polymorphism detection:

2 primers (forward, reverse)

Master Mix

DNA

Distilled water

Materials required for DNA electrophoresis on agarose gel:

Agarose powder

0.5x TBE solution
Distilled water
Safe stain
Loading buffer
DNA ladder
All materials were provided from Sinaclon company.
Reverse and Forward primers were designed to determine the IRS-1 polymorphism by Sanger sequencing and were purchased from Sinaclon.
IRS-1 (Forward): TGGCGAGGTGTCCACGTAGC
IRS-1 (Reverse): CTTCTGTCAGGTGTCCATCC

Method:

5 ml of intravenous blood was taken from the subjects studied, with conscious consent in Fatemeh Al-Zahra Hospital in Robat Karim. The goal was to extract genomic DNA from the blood leukocytes.
Disclosure of PCR products by electrophoresis
Preparation of (Tris-Borate-EDTA) TBE (10X) electrophoresis buffer: The formula of this buffer is: 89 mM Boric acid, 89 mM Tris, 4 mM EDTA
We provided the buffer from Sinaclon Company.
Preparation of TBE 0.5X buffer: We prepared the TBE 0.5X buffer from the 10X buffer and used it for electrophoresis and gel preparation. For this purpose, we added 15 cc of 10X buffer to 285 cc of twice-distilled water.
Preparation of electrophoresis gel: 1.8% Agarose gel was used for electrophoresis of the PCR products. For this purpose, 1.8 g of agarose powder was dissolved in 80 cc of 0.5X TBE buffer and heated until the agarose was completely dissolved in the buffer and a colorless and clear solution was obtained. Then, about 3µl of Safe stain was added to the prepared agarose and the gel solution was poured into the gel tray, which had a suitable comb, and the tray was left to stand for 20 to 30 minutes until the gel changed to solid. Then, the comb was carefully removed from the gel and the agarose gel was ready for electrophoresis.
Method of the preparation of electrophoresis solutions

1. 30% Acrylamide: To prepare 100 ml of this solution, 29 grams of acrylamide powder was mixed with 1 gram of bis-acrylamide powder and the volume increased to 100 ml with distilled water.
2. TBE5X: To prepare 1000 ml of this solution, 54 grams of Tris powder was mixed with 27 grams of boric acid, and 20 ml of EDTA(0/5 M) was added to it and the volume of the solution increased to 1000 ml with distilled water.
3. 10% ammonium persulfate: To prepare 10 ml of this solution, 1 gram of APS powder was added to distilled water to a volume of 10 ml. This solution should be stored in the refrigerator at 4° C.
4. TEMED: This solution is available ready-made, which is very foul-smelling and mutagenic. Therefore, when using it, it should be worked under the extractor hood and the work should be done very quickly.
5. Loading buffer containing formamide: loading buffer was mixed with 10 ml formamide, 200µl EDTA 5 / 0.5M, pH 8, xylene cyanole, and 3mg bromophenol blue.

Gel preparation: 8-5% gel is suitable for electrophoresis on an acrylamide gel, and we used 8% gel in our research. To prepare it in a volume of 32 cc, we did the following:
In this method, the used glasses were first washed with distilled water and cleaned well using alcohol. Then, the glasses were vertically connected to prepare the gel. Then, 8% acrylamide solution was prepared as follows:
15/13 ml of distilled water (ddH_2O), 6/25 ml of acrylamide solution (30%), 350 µl of 10% APS solution (ammonium persulfate), and 5 ml of TBE or 5X Tris-base Boric acid EDTA solution were mixed. Then, 5µl of TEMED was added to 5 ml of this solution. Then the solution was poured between the jars and was used to fill the gaps between the spacers. Then, 10 µl of TEMED was added to the rest of the solution and poured between the glasses and the comb was put inside the gel. It must be careful that there are no air bubbles inside the gel. After closing the gel, the comb and the lower spacer were carefully removed from its inside. The glass containing the gel was tightened by holding the clamp on the tank. It should be noted that there is no air bubble at the bottom of the tank and under the glass. TBE(1X) was used as a buffer. Before loading, the obtained wells were washed well. Then, the product was poured into the wells. In this step, 5 µl of the loading buffer containing the formamide was added to 10 µl of each PCR product. After mixing the buffer with the contents, each sample was added into each well. After that, electrophoresis was run with a voltage of 150V overnight.

Electrophoresis method

The TBE 0.5X buffer was poured into 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 poured into one of the gel wells so that the marker was completely inserted into the well. Then, 5 µl of the PCR product was carefully poured into the gel well. Finally, the tank was connected to the power supply. The power supply was turned on and set to the appropriate voltage. At the end of the electrophoresis time, the power supply was switched off, and with safety, the gel was removed from the tank and placed in the Gel-Doc device, and the multiplied gene was observed. PCR products were stored in the freezer at -20°C for further study.

Table 1: PCR duplication process

Materials used	The amount used
x Taq premix(master mix)2	12/5 ml multiply by the number of samples
Forward primer (10 pmol/ml)	1 ml multiply by the number of samples
Reverse primer (10 pmol/ml)	1 ml multiply by the number of samples

DNA(10-100ng/ml)	1/5 ml multiply by the number of samples
Sterilized distilled water	9 ml multiply by the number of samples

Number of cycles	Time	Temperature (°C)	
30 cycles	3 minutes	94	Initial Denaturation
	35 seconds	94	Denaturation
	35 seconds	61	Annealing
	35 seconds	72	Extension
	7 minutes	72	Final Extension

Interpretation of information

After electrophoresis, several samples of heterozygotes and homozygotes, which were common to all samples of a gel, were selected and sent to Codon Genetics Company for determination of sequence after re-PCR with sterile samplers. Finally, all the results were statistically examined.

Results and Findings

Qualitative and quantitative results of genome extraction of the sample of the studied individuals

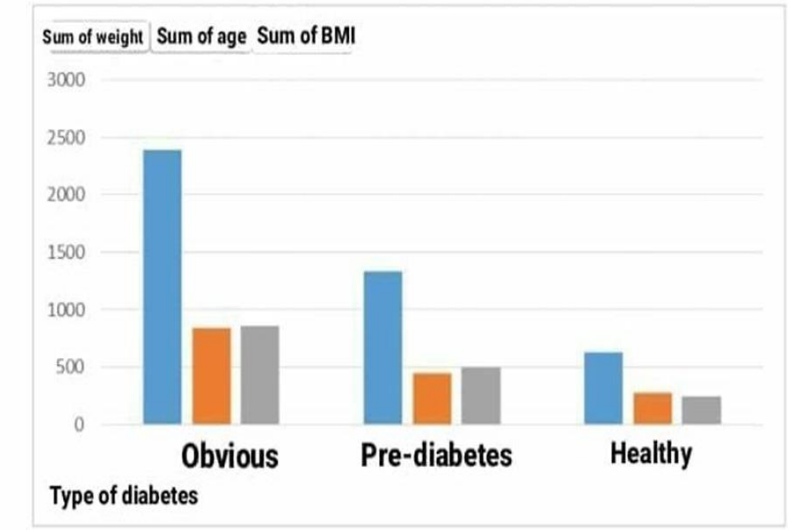

Diagram 1

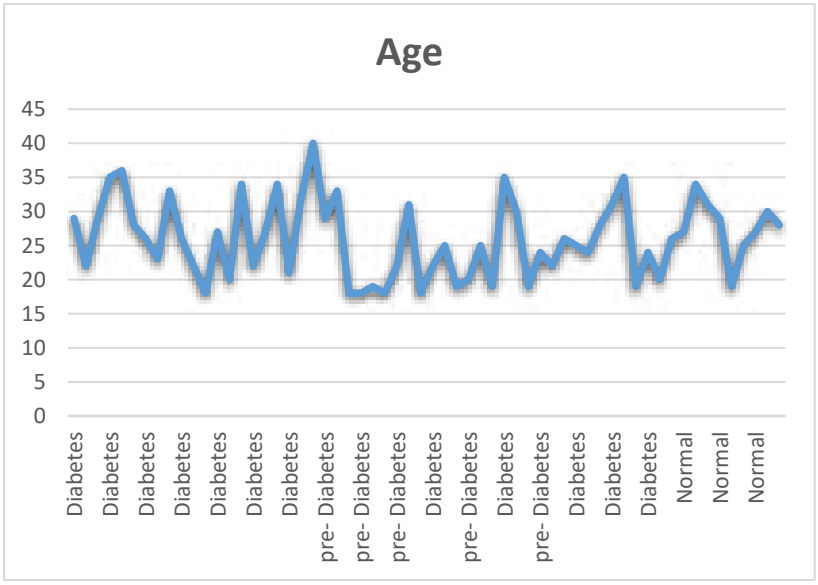

Diagram 2: Age of samples

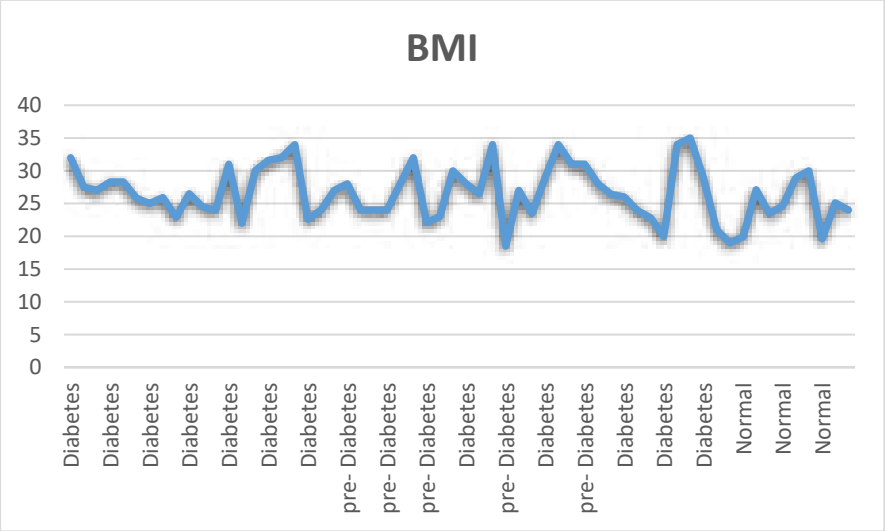

Diagram 3: BMI index

To perform the next steps, DNA was extracted from all diseased and healthy samples using the Star DNA Purification Kit and the PCR process was performed on them. To determine the concentration and to ensure the quality of the abbreviated DNA, 3 µl of DNA and 1 µl of loading buffer were electrophoresed on 1% agarose gel.

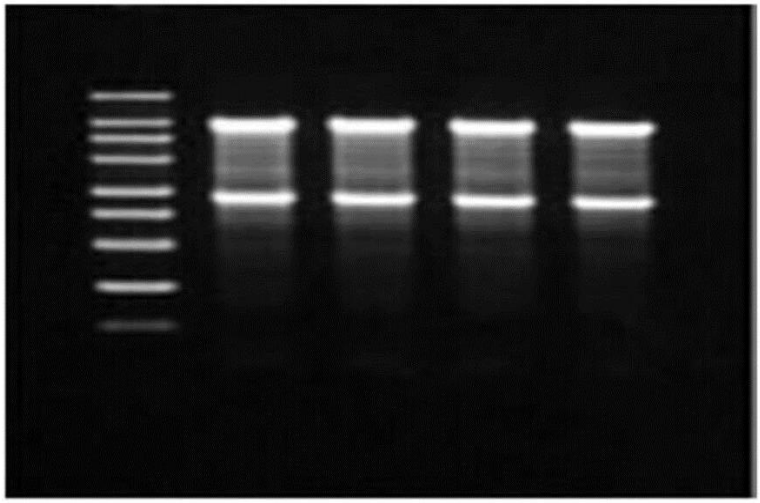

Figure 1: Gene extraction

Standard curve results

According to Corbet's protocol, the manufacturer of the Real-Time PCR device, a suitable standard diagram should have an efficiency of about 95-110% 1.1-0.95). The closer the reproductive efficiency is to 100%, the more accurate and repeatable the test will be. In practice, efforts should be made to achieve a reproduction efficiency of between 95 -110 percent. The low efficiency of the reaction can be due to poor design of the primer or inadequate condition of the reaction. More than 110% of returns can be due to pipette error in diluted series or simultaneous duplication of non-proprietary products such as the primer dimer. In the relative comparison method, the only important point in the standard diagram is that the efficiency of the standard gene used (housekeeping gene) and the gene under study are close to each other.

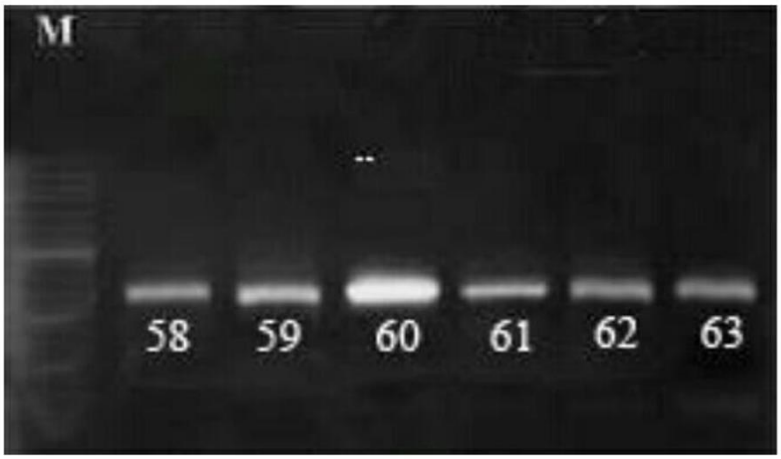

Figure 2: annealing temperature

Results of PCR reaction on samples of healthy and sick people to study IRS-1 gene

The samples were taken to the electrophoresis gel after extraction and examination of the extracted DNA quality and the results were observed as follows. The genetic area was amplified using designed primers and PCR techniques. A comparison of the observed bands with the 50 bp marker showed that in all samples, the observed band corresponded to the expected length.

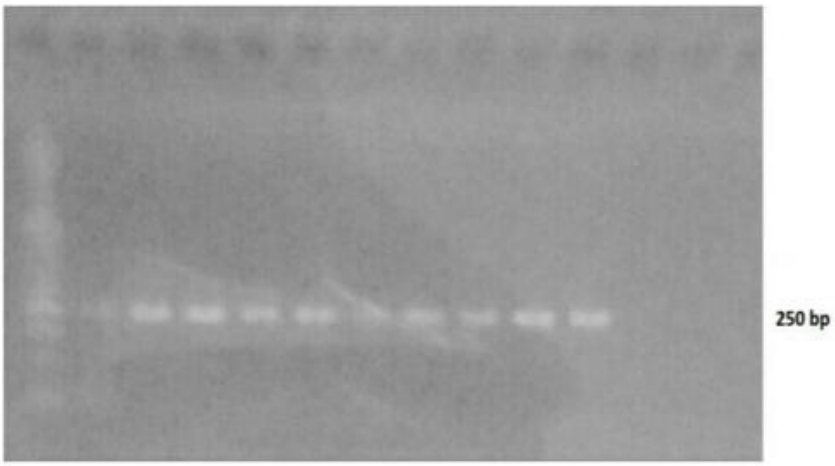

Figure 3: PCR products of the IRS-1 gene of patients

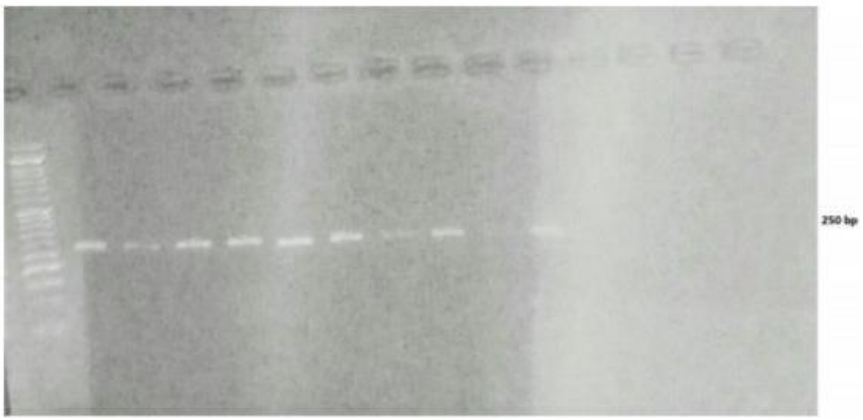

Figure 4: PCR products of healthy individuals' IRS-1 gene

G > C / T

Samples with mutations in IRS-1 polymorphism (rs1801278)

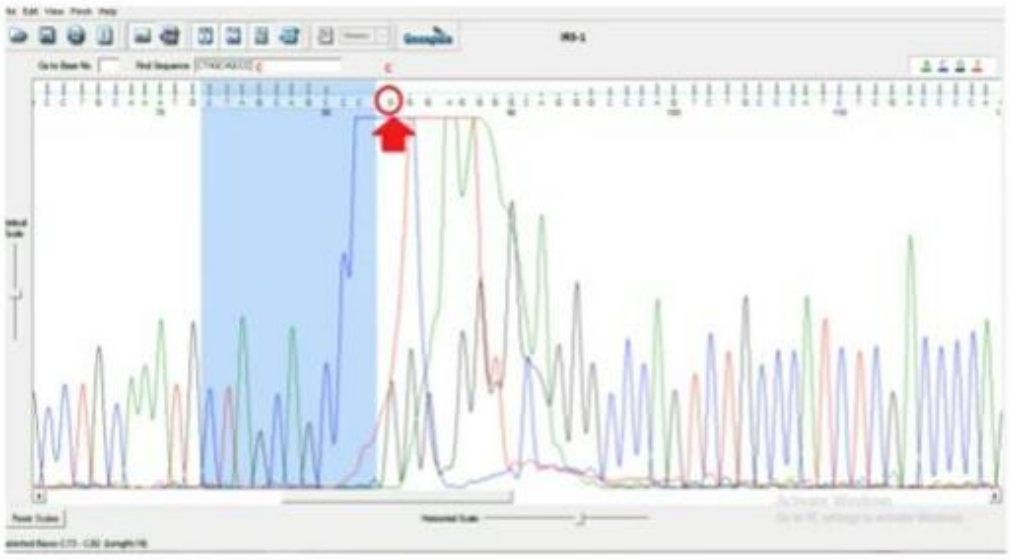

Figure 5: Sample with mutation

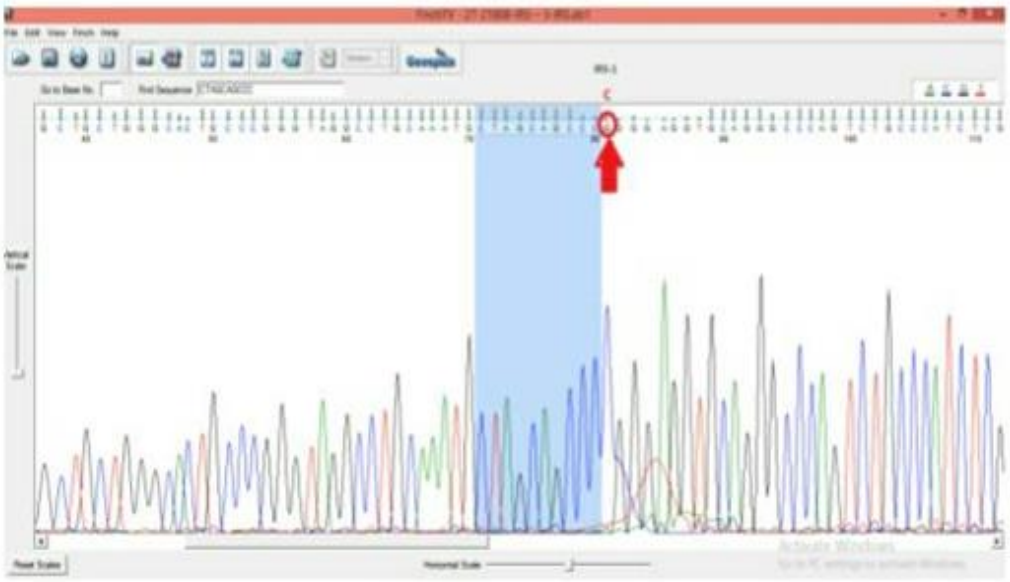

Figure 6: Sample with mutation

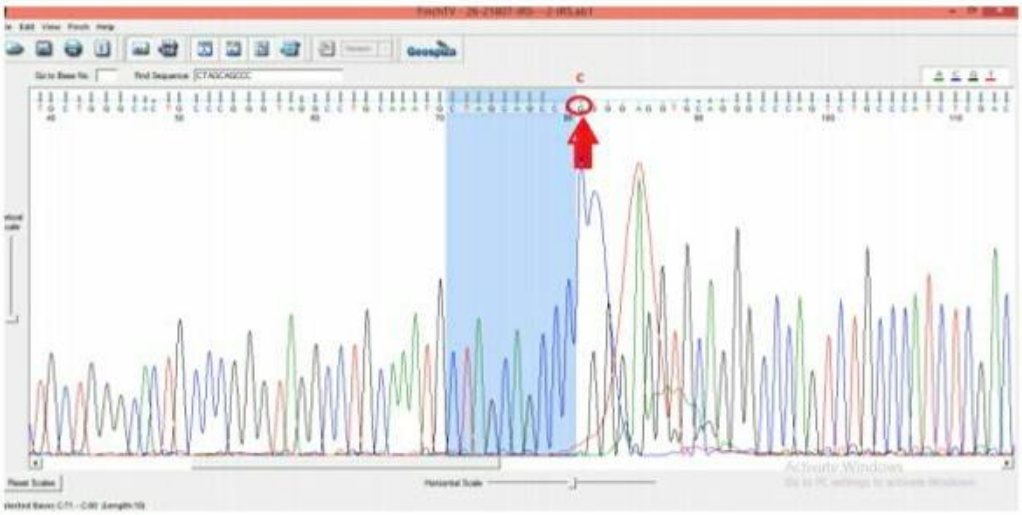

Figure 7: Sample with mutation

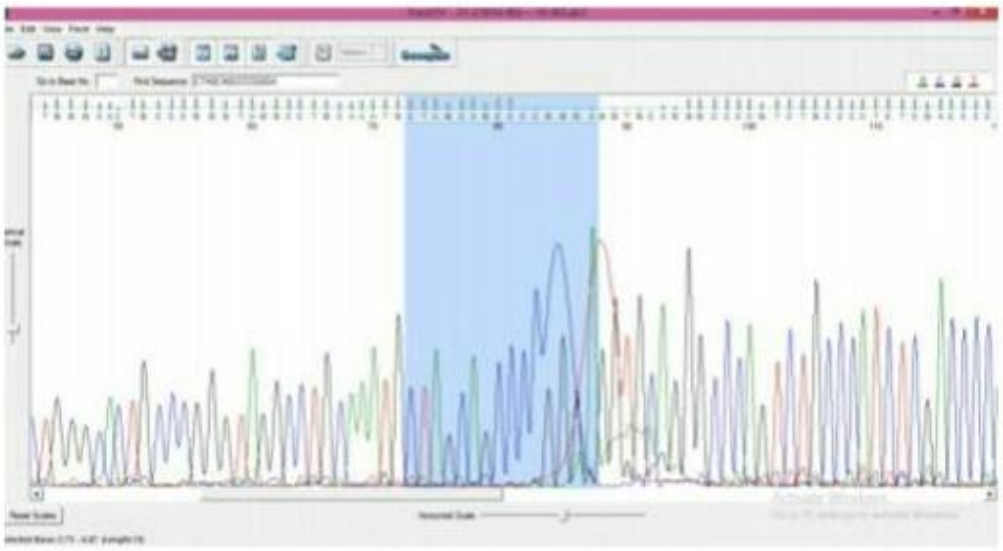

Figure 8: A healthy, mutation-free sample in the (rs1801278) polymorphism of IRS-1

Table 4: The frequency of mutations in the IRS-1 gene

The frequency of IRS-1 mutations	Number of healthy people	Number of people with IRS-1 mutation	whole sample
28%	36	14	Patient group N=50
10%	9	1	Control group N=10

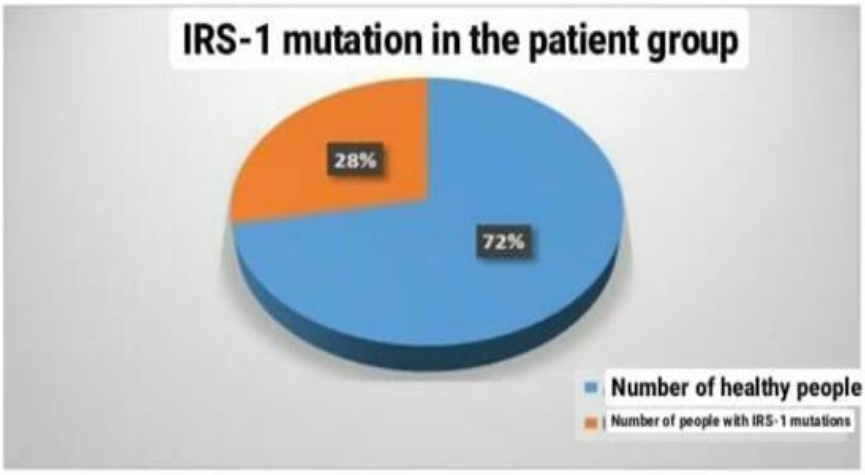

Diagram 4: Motation (IRS-1) in the patient group

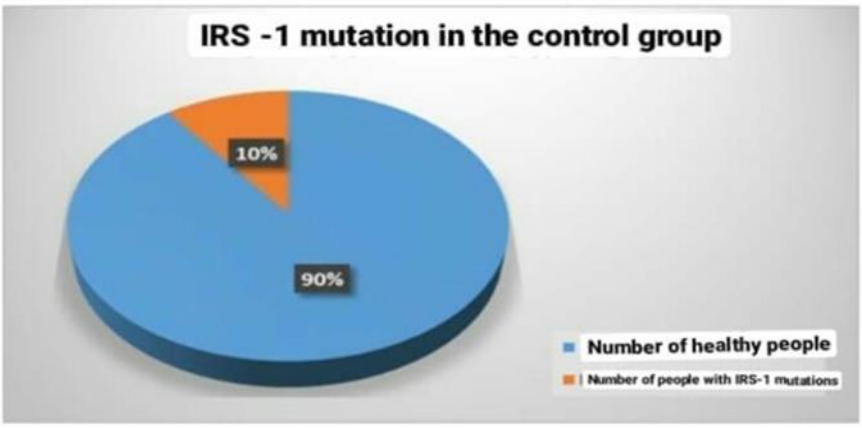

Diagram 5: Healthy group mutation (IRS-1)

Results and Discussion

Physiological changes during a normal pregnancy can lead to gestational diabetes in some people. The first trimester of pregnancy is associated with a change in insulin sensitivity, and as fetal growth increases due to insulin resistance, insulin secretion increases too. Insulin resistance peaks in the third trimester of pregnancy, depending on hormonal changes such as estrogen, progesterone, chorionic human HPL, and somatomammotropin. Various mechanisms have been proposed to explain the pathogenesis of gestational diabetes, the most important of which are: Decreased insulin sensitivity, dysfunction of beta cells and in some cases autoimmune destruction of pancreatic beta cells, the overall prevalence of glucose tolerance disorder is estimated to be between 0 and 36% in different communities. The results of the study showed a different prevalence of gestational diabetes, which largely follows the prevalence of NIDDM1 in the study population. In a study conducted at the Endocrinology and Metabolism Research Center of Tehran University of Medical Sciences on 2416 pregnant women in Tehran, the prevalence of gestational diabetes was about 4.7%. The prevalence of gestational diabetes is partly related to racial differences.

Pregnancy diabetes has many side effects for both mother and fetus in the races that pregnancy diabetes is more common in them. Fetal complications of gestational diabetes include macrosomia, neonatal hypoglycemia, hypocalcemia, jaundice, respiratory distress syndrome, polycythemia, premature infant, miscarriage, and birth defects. Children of mothers with gestational diabetes are more prone to obesity and glucose intolerance. Monitoring blood sugar after a meal along with measuring fasting blood sugar can be effective in reducing the complications of diabetes in pregnancy. Diabetes has many effects on the mother and fetus, and the high prevalence of gestational diabetes indicates the need to design a method for screening, diagnosing, and controlling and treating gestational diabetes.

There are many causes for the pathophysiological justification of the complications of diabetes and pregnancy, the most important of which include high glucose, ketones, and somatomedin inhibitors, all of which also have a synergistic effect. Macrosomia, the most common complication associated with gestational diabetes, occurs in about 20% of cases. In macrosomia, the internal organs, including the liver, heart, pancreatic islets, and adrenal glands, enlarge because hypertrophy and cell hyperplasia occur. One of the most important theories on the causes of fetal complications is maternal hyperglycemia, which causes fetal hyperglycemia and increased insulin secretion by fetal pancreatic beta cells. The result is an increase in the process of anabolism. This theory, which includes maternal hyperglycemia with fetal hyperinsulinism, has been confirmed by clinical studies and has shown that controlling maternal blood sugar reduces the incidence of macrosomia. Of course, macrosomia is not only dependent on maternal blood sugar levels but also factors such as maternal weight gain in pregnancy, the number of births, and gestational age are influential in this process. Maternal high blood sugar is not the only cause of macrosomia, but it is also an important factor. Macrosomia is the cause of asphyxia, neonatal trauma, and cesarean section. Gestational diabetes also appears to increase the risk of developing high blood pressure. However, research in this area is difficult due to the presence of two factors: age and obesity, which are contributing factors to high blood pressure and gestational diabetes. A study by Goldman et al. showed an increase in the incidence of PIH2 and preeclampsia in gestational diabetes compared with the control group. Neonatal hypoglycemia is an important complication of gestational diabetes. Neonatal hypoglycemia is an important complication of gestational diabetes. Maternal hyperglycemia causes fetal pancreatic hyperplasia and predisposes to neonatal hypoglycemia, which has been described by Pedersen and et al. Approximately 24% of infants whose mothers have gestational diabetes may have blood sugar below 30mg/dl during infancy. The treatment and diagnosis of gestational diabetes are effective in reducing neonatal hypoglycemia and reducing the time a baby needs special care. Medical research on the genetics of diabetes will one day help women at risk for gestational diabetes to be aware of the risk of gestational diabetes before pregnancy and to take preventive measures to protect the health of their babies. Some researchers have found that changes in the two genes HKDC1 and BACE2 are associated with sugar and insulin levels in pregnant women, but these two genes are not associated with an increase in sugar and insulin levels outside of pregnancy in people with type 2 diabetes.

Dr. Hayes says that with more research and changes in these two genes and other risk genes, we will one day be able to determine the genetic profile of women's risk of developing gestational diabetes. The findings show the role of the HKDC1 gene in glucose metabolism and the BACE2 gene in insulin secretion during pregnancy compared to the pre- and post-pregnancy period among different races. Using DNA and phenotypic data from 4,000 participants from four different races (Hispanic, Thai, Caribbean, and European) who enrolled in the HAPO study, the researchers came to this conclusion. The study of HAPO or hyperglycemia and its harmful consequences during pregnancy is an international study in several different countries in which pregnant women in different geographical areas with different races and social and geographical conditions participated. Professor Lowe says: The findings of this study could one day help determine the exact and quantitative genetic traits to predict the likelihood of developing gestational diabetes in women.

New research by a large international team of scientists has provided a more complete picture of the genes responsible for type 2 diabetes and pregnancy.

The study found that common alleles, previously identified by researchers, were the most common cause of the disease and varieties that were less common and that scientists assumed played a large role in the disease, did not play such a significant

role. Researchers have been able to identify more than one genetic area that was the site of effective variations of the risk of type 2 diabetes. Most of these common variants are found in all human populations and have already been identified by other extensive genomic studies. In this study, the scientists found a new link between type 2 diabetes and a genetic variation called PAX4, which only exists in people of East Asian descent such as Korea, China, and Singapore. They also showed that a change in the TM6SF2 gene, previously known to be linked to fatty liver disease or hepatic steatosis, would increase the risk of developing type 2 diabetes. The researchers completely identified the genomic sequence of more than 2,600 individuals and the sequence of 13,000 exons (part of the genome that carries the protein codes). This study included people of European, South and East Asian, American, and African descent. In most studies, rare genetic variants certainly affect the risk of developing type 2 diabetes and the results of this study showed that common genetic changes that are common to all populations could better explain the genetic risk of diabetes.

In 2015, Débora, et al. [6] conducted a study entitled "IRS-1 genetic polymorphism and DNA damage in pregnant women with diabetes or mild gestational hyperglycemia." and stated that diabetic patients have a higher frequency of Gly972Arg IRS-1, and this polymorphism is directly related to insulin resistance and hyperglycemia. In diabetes, hyperglycemia, and other related factors including reactive oxygen cause DNA damage. 85 maternal blood samples and umbilical cord blood samples (5-10 ml) were collected and evaluated by PCR- RFLP method to prepare the genotype. The prevalence of Gly/ Arg genotype in pregnant women was statistically significant. In infants, the prevalence of Gly/Arg in the MGH and diabetic groups was significantly higher than in the non-diabetic group. In general, the amount of DNA damage in the diabetic group was higher than the control group. [7]

In 2018, Liu, et al. [8] conducted a study entitled "Investigating the types of genetics that affect gestational diabetes" and reported that the activation of Insulin Receptor Substrate 1 (IRS1) is the main step in the path of insulin signaling. IRS-1 (rs1801278) along with type 2 diabetes in the high-risk population of India was diagnosed. However, people with diabetes in Mexico with a BMI of less than 25, and people with Gly972Arg are at high risk for diabetes. To reveal the role of IRS1 in skinny people with diabetes in Greece and Saudi Arabia, with 148 women with GDM and 107 in the control group, the results showed that G972R polymorphism of IRS-1 gene was strongly associated with increased sensitivity to GDM. However, the prevalence of GLY/Arg genotype in pregnant Brazilian women was not statistically significant.

With early detection of the risk of gestational diabetes before and early in pregnancy, tests for high blood sugar and preventive measures to protect the health of infants will be performed sooner. In order to improve the treatment and prevention of many diseases, it is very important to know the genetic makeup of each person. Careful medical therapies rely on understanding the structures of disease, and our research provides this key information for gestational diabetes. The results of our study showed that many pregnant women are at risk for gestational diabetes with regards to the hundreds or even thousands of genetic variants that are common among different populations. In this study, (rs1801278) polymorphism of the IRS-1 gene was examined in women with gestational diabetes and the control group. In fact, samples from 50 female patients with gestational diabetes and 10 healthy women in terms of (rs1801278) polymorphism of IRS-1 were examined, of whom 72% had GG polymorphism and 28% had GC/T polymorphism. Among the samples of healthy people, 10% had GC/T and 90% had GG.

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