Investigation of *PAX3* and *PVT1* polymorphisms of genes in patients with type 2 diabetes in Iran by Tetra ARMS-PCR method

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Abstract

Background and hypothesis Evidence from genetic epidemiology suggests that type 2 diabetes has a strong genetic basis. The aim of this study is to study the polymorphisms of PAX3 and PVTI genes in type 2 diabetes patients in Iran Tetra ARMS-PCR Cat Method .

Research Method Then 10 blood samples were collected from type 2 diabetes patients in Milad Hospital in Tehran and stored in the refrigerator. 10 control samples were considered and demographic information was collected for all subjects. DNA extraction was performed using a special kit and the quality of the extracted DNA samples was checked by agaron gel electrophoresis. T-ARMS PCR technique was used to identify the genetic polymorphisms associated with (3) rs2305619 and (2648875) PVT1) using designed primers and were placed on electrophoresis for genetic and allelic distribution, then using the program SPSS version 23 for Chi-Deva test to check the quantitative data in these two groups after verification Check the validity of the data and then use the Mann-Wedney test .

The results of the examination of the repeat fragments in the present study on agarose gel showed that in (2305619) A3 (1(2648875) .Polymorphisms as well as the presence of formed bands indicate the presence of A and G alleles in both women Statistical calculations showed that the polymorphisms of the PAX3 and PVTI genes and the disease. There was no statistically significant association with type 2 diabetes in the population included in the study (0.05). The highest relative genetic frequencies were seen in the AA and GG genotypes in PVTIS PAX3, respectively, which had the greatest role in type 2 diabetes and excluded gender and body mass index, while cholesterol level, fasting blood sugar and triglycerides . HBAIC had a significant effect on type 2 diabetes and was reported as significant , m 1005.

Conclusion of polymorphisms (2305619) X3 and (12648875) PVT1 in type 2 diabetes

The absence of cholesterol, fasting blood sugar, triglycerides, etc. in the study population has an effective and important role .HbA1C showed a significant association between polymorphism and disease onset .

Keywords: *Polymorphism Type 2 diabetes Demographics Diabetes PVTI PAX3 Tetra ARMS-PCR*

INTRODUCTION

Type 2 diabetes (T2D) is one of the major life-threatening diseases globally, accounting for 4.2 million deaths worldwide in 2019 as assessed by International Diabetes Federation (IDF) consortium. According to the IDF report, among 20 countries belongs to the Middle East and North Africa (MENA) region, Iran is ranked third with the highest number of adults (5.4 million) who suffered from diabetes. The prevalence of diabetes in Iran's adult population was 11.4% in 2014, estimating 9.2 million Iranian individuals will have diabetes by the year 2030.

Type 2 diabetes is a common multifactorial metabolic disease, resulting from both genetic and non-genetic (environmental) factors. The heritability of T2D ranges from 20 to 80%, suggesting the considerable role of genetic factors in the development of T2D; the heritable component of the disease is polygenic where many genes and their variants contribute to an enhanced risk of T2D development. The advent of high-throughput genotyping technologies has created a significant breakthrough in understanding the underlying genetic components of complex diseases, including T2D. A large number of common and low-frequency T2D susceptibility variants have been characterized by the genome-wide association studies (GWAS) and the whole-genome sequencing. Most of these variants are located near genes that were previously known to be involved in diabetes pathogenesis, such as TCF7L2, CDKAL1, CDKN1C, and IGF2BP2. Among them, TCF7L2 is responsible for the largest proportion of the T2D-associated variance in the various ethnic groups. TCF7L2 encodes a transcription factor played a central role in the Wnt signaling pathway to regulate glucose homeostasis. Since not all individuals are equally affected by type 2 diabetes through the unhealthy lifestyle and some are more sensitive than others, the corresponding genetic variants can lead to the population disparities in the T2D prevalence (1).

Diabetes can be classified into the following general categories: Type 1 diabetes (due to autoimmune β-cell destruction, usually leading to absolute insulin deficiency, including latent autoimmune diabetes of adulthood). Type 2 diabetes (due to a progressive loss of adequate β-cell insulin secretion frequently on the background of insulin resistance)

Specific types of diabetes due to other causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young), diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation). Gestational diabetes mellitus (diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation). This section reviews most common forms of diabetes but is not comprehensive. For additional information, see the American Diabetes Association (ADA) position statement “Diagnosis and Classification of Diabetes Mellitus” (2).

Type 1 diabetes and type 2 diabetes are heterogeneous diseases in which clinical presentation and disease progression may vary considerably. Classification is important for determining therapy, but some individuals cannot be clearly classified as having type 1 or type 2 diabetes at the time of diagnosis. The traditional paradigms of type 2 diabetes occurring only in adults and type 1 diabetes only in children are no longer accurate, as both diseases occur in both age-groups. Children with type 1 diabetes typically present with the hallmark symptoms of polyuria/polydipsia, and

approximately one-third present with diabetic ketoacidosis (DKA). The onset of type 1 diabetes may be more variable in adults; they may not present with the classic symptoms seen in children and may experience temporary remission from the need for insulin (3–5). Occasionally, patients with type 2 diabetes may present with DKA (6), particularly ethnic and racial minorities (7). It is important for the provider to realize that classification of diabetes type is not always straightforward at presentation and that misdiagnosis is common (e.g., adults with type 1 diabetes misdiagnosed as having type 2 diabetes; individuals with maturity-onset diabetes of the young [MODY] misdiagnosed as having type 1 diabetes, etc.). Although difficulties in distinguishing diabetes type may occur in all age-groups at onset, the diagnosis becomes more obvious over time in people with β-cell deficiency.

In both type 1 and type 2 diabetes, various genetic and environmental factors can result in the progressive loss of β-cell mass and/or function that manifests clinically as hyperglycemia. Once hyperglycemia occurs, patients with all forms of diabetes are at risk for developing the same chronic complications, although rates of progression may differ. The identification of individualized therapies for diabetes in the future will require better characterization of the many paths to β-cell demise or dysfunction (8). Across the globe many groups are working on combining clinical, pathophysiological, and genetic characteristics to more precisely define the subsets of diabetes currently clustered into the type 1 diabetes versus type 2 diabetes nomenclature with the goal of optimizing treatment approaches. Many of these studies show great promise and may soon be incorporated into the diabetes classification system (9).

Characterization of the underlying pathophysiology is more precisely developed in type 1 diabetes than in type 2 diabetes. It is now clear from studies of first-degree relatives of patients with type 1 diabetes that the persistent presence of two or more islet autoantibodies is a near certain predictor of clinical hyperglycemia and diabetes. The rate of progression is dependent on the age at first detection of autoantibody, number of autoantibodies, autoantibody specificity, and autoantibody titer. Glucose and A1C levels rise well before the clinical onset of diabetes, making diagnosis feasible well before the onset of DKA. Three distinct stages of type 1 diabetes can be identified (Table 2.1) and serve as a framework for future research and regulatory decision-making (8,10). There is debate as to whether slowly progressive autoimmune diabetes with an adult onset should be termed latent autoimmune diabetes in adults (LADA) or type 1 diabetes. The clinical priority is awareness that slow autoimmune β-cell destruction can occur in adults leading to a long duration of marginal insulin secretory capacity. For the purpose of this classification, all forms of diabetes mediated by autoimmune β-cell destruction are included under the rubric of type 1 diabetes. Use of the term LADA is common and acceptable in clinical practice and has the practical impact of heightening awareness of a population of adults likely to develop overt autoimmune β-cell destruction (11), thus accelerating insulin initiation prior to deterioration of glucose control or development of DKA (4,12). The paths to β-cell demise and dysfunction are less well defined in type 2 diabetes, but deficient β-cell insulin secretion, frequently in the setting of insulin resistance, appears to be the common denominator. Type 2 diabetes is associated with insulin secretory defects related to inflammation and metabolic stress among other contributors, including genetic factors. Future classification schemes for diabetes will likely focus on the pathophysiology of the underlying β-cell dysfunction (13).

Fasting and 2-Hour Plasma Glucose

The FPG and 2-h PG may be used to diagnose diabetes. The concordance between the FPG and 2-h PG tests is imperfect, as is the concordance between A1C and either glucose-based test. Compared with FPG and A1C cut points, the 2-h PG value diagnoses more people with prediabetes and diabetes (14). In people in whom there is discordance between A1C values and glucose values, FPG and 2-h PG are more accurate (15).

A1C

Recommendations

1. To avoid misdiagnosis or missed diagnosis, the A1C test should be performed using a method that is certified by the NGSP and standardized to the Diabetes Control and Complications Trial (DCCT) assay.

2. Marked discordance between measured A1C and plasma glucose levels should raise the possibility of A1C assay interference and consideration of using an assay without interference or plasma blood glucose criteria to diagnose diabetes.

3. In conditions associated with an altered relationship between A1C and glycemia, such as hemoglobinopathies including sickle cell disease, pregnancy (second and third trimesters and the postpartum period), glucose-6-phosphate dehydrogenase deficiency, HIV, hemodialysis, recent blood loss or transfusion, or erythropoietin therapy, only plasma blood glucose criteria should be used to diagnose diabetes.

The A1C test should be performed using a method that is certified by the NGSP (www.ngsp.org) and standardized or traceable to the Diabetes Control and Complications Trial (DCCT) reference assay. Although point-of-care A1C assays may be NGSP certified and cleared by the U.S. Food and Drug Administration (FDA) for use in monitoring glycemic control in people with diabetes in both Clinical Laboratory Improvement Amendments (CLIA)-regulated and CLIA-waived settings, only those point-of-care A1C assays that are also cleared by the FDA for use in the diagnosis of diabetes should be used for this purpose, and only in the clinical settings for which they are cleared. As discussed in Section 6 “Glycemic Targets” (https://doi.org/10.2337/dc21-S006), point-of-care A1C assays may be more generally applied for assessment of glycemic control in the clinic.

A1C has several advantages compared with FPG and OGTT, including greater convenience (fasting not required), greater preanalytical stability, and less day-to-day perturbations during stress, changes in diet, or illness. However, these advantages may be offset by the lower sensitivity of A1C at the designated cut point, greater cost, limited availability of A1C testing in certain regions of the developing world, and the imperfect correlation between A1C and average glucose in certain individuals. The A1C test, with a diagnostic threshold of ≥6.5% (48 mmol/mol), diagnoses only 30% of the diabetes cases identified collectively using A1C, FPG, or 2-h PG, according to National Health and Nutrition Examination Survey (NHANES) data (16).

When using A1C to diagnose diabetes, it is important to recognize that A1C is an indirect measure of average blood glucose levels and to take other factors into consideration that may impact

hemoglobin glycation independently of glycemia, such as hemodialysis, pregnancy, HIV treatment (17), age, race/ethnicity, pregnancy status, genetic background, and anemia/hemoglobinopathies.

Age

The epidemiologic studies that formed the basis for recommending A1C to diagnose diabetes included only adult populations (16). However, recent ADA clinical guidance concluded that A1C, FPG, or 2-h PG can be used to test for prediabetes or type 2 diabetes in children and adolescents (18).

Race/Ethnicity/Hemoglobinopathies

Hemoglobin variants can interfere with the measurement of A1C, although most assays in use in the U.S. are unaffected by the most common variants. Marked discrepancies between measured A1C and plasma glucose levels should prompt consideration that the A1C assay may not be reliable for that individual. For patients with a hemoglobin variant but normal red blood cell turnover, such as those with the sickle cell trait, an A1C assay without interference from hemoglobin variants should be used. An updated list of A1C assays with interferences is available at www.ngsp.org/interf.asp.

African Americans heterozygous for the common hemoglobin variant HbS may have, for any given level of mean glycemia, lower A1C by about 0.3% compared with those without the trait

(19). Another genetic variant, X-linked glucose-6-phosphate dehydrogenase G202A, carried by 11% of African Americans, was associated with a decrease in A1C of about 0.8% in homozygous men and 0.7% in homozygous women compared with those without the variant (20).

Even in the absence of hemoglobin variants, A1C levels may vary with race/ethnicity independently of glycemia (21). For example, African Americans may have higher A1C levels than non-Hispanic Whites with similar fasting and postglucose load glucose levels (22). Though conflicting data exists, African Americans may also have higher levels of fructosamine and glycated albumin and lower levels of 1,5-anhydroglucitol, suggesting that their glycemic burden (particularly postprandially) may be higher (23). Similarly, A1C levels may be higher for a given mean glucose concentration when measured with continuous glucose monitoring (24). Despite these and other reported differences, the association of A1C with risk for complications appears to be similar in African Americans and non-Hispanic Whites (25).

Other Conditions Altering the Relationship of A1C and Glycemia

In conditions associated with increased red blood cell turnover, such as sickle cell disease, pregnancy (second and third trimesters), glucose-6-phosphate dehydrogenase deficiency (26), hemodialysis, recent blood loss or transfusion, or erythropoietin therapy, only plasma blood glucose criteria should be used to diagnose diabetes (27). A1C is less reliable than blood glucose measurement in other conditions such as the postpartum state (28), HIV treated with certain protease inhibitors (PIs) and nucleoside reverse transcriptase inhibitors (NRTIs), and iron-deficient anemia (29).

Confirming the Diagnosis

Unless there is a clear clinical diagnosis (e.g., patient in a hyperglycemic crisis or with classic symptoms of hyperglycemia and a random plasma glucose ≥200 mg/dL [11.1 mmol/L]), diagnosis requires two abnormal test results, either from the same sample (30) or in two separate test samples. If using two separate test samples, it is recommended that the second test, which may either be a repeat of the initial test or a different test, be performed without delay. For example, if the A1C is 7.0% (53 mmol/mol) and a repeat result is 6.8% (51 mmol/mol), the diagnosis of diabetes is confirmed. If two different tests (such as A1C and FPG) are both above the diagnostic threshold when analyzed from the same sample or in two different test samples, this also confirms the diagnosis. On the other hand, if a patient has discordant results from two different tests, then the test result that is above the diagnostic cut point should be repeated, with careful consideration of the possibility of A1C assay interference. The diagnosis is made on the basis of the confirmed test. For example, if a patient meets the diabetes criterion of the A1C (two results ≥6.5% [48 mmol/mol]) but not FPG (<126 mg/dL [7.0 mmol/L]), that person should nevertheless be considered to have diabetes.

Each of the tests has preanalytic and analytic variability, so it is possible that a test yielding an abnormal result (i.e., above the diagnostic threshold), when repeated, will produce a value below the diagnostic cut point. This scenario is likely for FPG and 2-h PG if the glucose samples remain at room temperature and are not centrifuged promptly. Because of the potential for preanalytic variability, it is critical that samples for plasma glucose be spun and separated immediately after they are drawn. If patients have test results near the margins of the diagnostic threshold, the health care professional should discuss signs and symptoms with the patient and repeat the test in 3–6 months.

Diagnosis

In a patient with classic symptoms, measurement of plasma glucose is sufficient to diagnose diabetes (symptoms of hyperglycemia or hyperglycemic crisis plus a random plasma glucose ≥200 mg/dL [11.1 mmol/L]). In these cases, knowing the plasma glucose level is critical because, in addition to confirming that symptoms are due to diabetes, it will inform management decisions. Some providers may also want to know the A1C to determine the chronicity of the hyperglycemia.

Type 2 Diabetes

Type 2 diabetes, previously referred to as “noninsulin-dependent diabetes” or “adult-onset diabetes,” accounts for 90–95% of all diabetes. This form encompasses individuals who have relative (rather than absolute) insulin deficiency and have peripheral insulin resistance. At least initially, and often throughout their lifetime, these individuals may not need insulin treatment to survive. There are various causes of type 2 diabetes. Although the specific etiologies are not known, autoimmune destruction of β-cells does not occur, and patients do not have any of the other known causes of diabetes. Most, but not all, patients with type 2 diabetes have overweight or obesity. Excess weight itself causes some degree of insulin resistance. Patients who do not have obesity or overweight by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region. DKA seldom occurs spontaneously in type 2 diabetes; when seen, it usually arises in association with the stress of another illness such as

infection, myocardial infarction, or with the use of certain drugs (e.g., corticosteroids, atypical antipsychotics, and sodium–glucose cotransporter 2 inhibitors) (31). Type 2 diabetes frequently goes undiagnosed for many years because hyperglycemia develops gradually and, at earlier stages, is often not severe enough for the patient to notice the classic diabetes symptoms caused by hyperglycemia. Nevertheless, even undiagnosed patients are at increased risk of developing macrovascular and microvascular complications. Patients with type 2 diabetes may have insulin levels that appear normal or elevated, yet the failure to normalize blood glucose reflects a relative defect in glucose-stimulated insulin secretion. Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. Insulin resistance may improve with weight reduction, exercise, and/or pharmacologic treatment of hyperglycemia but is seldom restored to normal. Recent interventions with intensive diet and exercise or surgical weight loss have led to diabetes remission (32).

The risk of developing type 2 diabetes increases with age, obesity, and lack of physical activity. It occurs more frequently in women with prior gestational diabetes mellitus (GDM), with hypertension or dyslipidemia, with polycystic ovary syndrome, and in certain racial/ethnic subgroups (African American, American Indian, Hispanic/Latino, and Asian American). It is often associated with a strong genetic predisposition or family history in first-degree relatives (more so than type 1 diabetes). However, the genetics of type 2 diabetes is poorly understood and under intense investigation in this era of precision medicine (13). In adults without traditional risk factors for type 2 diabetes and/or younger age, consider islet autoantibody testing (e.g., GAD65 autoantibodies) to exclude the diagnosis of type 1 diabetes.

Age

Age is a major risk factor for diabetes. Testing should begin at no later than age 45 years for all patients. Screening should be considered in adults of any age with overweight or obesity and one or more risk factors for diabetes.

BMI and Ethnicity

In general, BMI ≥25 kg/m2 is a risk factor for diabetes. However, data suggest that the BMI cut point should be lower for the Asian American population (33). The BMI cut points fall consistently between 23 and 24 kg/m2 (sensitivity of 80%) for nearly all Asian American subgroups (with levels slightly lower for Japanese Americans). This makes a rounded cut point of 23 kg/m2 practical. An argument can be made to push the BMI cut point to lower than 23 kg/m2 in favor of increased sensitivity; however, this would lead to an unacceptably low specificity (13.1%). Data from WHO also suggests that a BMI of ≥23 kg/m2 should be used to define increased risk in Asian Americans. The finding that one-third to one-half of diabetes in Asian Americans is undiagnosed suggests that testing is not occurring at lower BMI thresholds (34). Evidence also suggests that other populations may benefit from lower BMI cut points. For example, in a large multiethnic cohort study, for an equivalent incidence rate of diabetes, a BMI of 30 kg/m2 in non-Hispanic Whites was equivalent to a BMI of 26 kg/m2 in African Americans (35).

Medications

Certain medications, such as glucocorticoids, thiazide diuretics, some HIV medications (23), and atypical antipsychotics (36), are known to increase the risk of diabetes and should be considered when deciding whether to screen.

HIV

Individuals with HIV are at higher risk for developing prediabetes and diabetes on antiretroviral (ARV) therapies, so a screening protocol is recommended. The A1C test may underestimate glycemia in people with HIV; it is not recommended for diagnosis and may present challenges for monitoring (24). In those with prediabetes, weight loss through healthy nutrition and physical activity may reduce the progression toward diabetes. Among patients with HIV and diabetes, preventive health care using an approach used in patients without HIV is critical to reduce the risks of microvascular and macrovascular complications. Diabetes risk is increased with certain PIs and NRTIs. New-onset diabetes is estimated to occur in more than 5% of patients infected with HIV on PIs, whereas more than 15% may have prediabetes. PIs are associated with insulin resistance and may also lead to apoptosis of pancreatic β-cells. NRTIs also affect fat distribution (both lipohypertrophy and lipoatrophy), which is associated with insulin resistance. For patients with HIV and ARV-associated hyperglycemia, it may be appropriate to consider discontinuing the problematic ARV agents if safe and effective alternatives are available (37). Before making ARV substitutions, carefully consider the possible effect on HIV virological control and the potential adverse effects of new ARV agents. In some cases, antihyperglycemic agents may still be necessary.

Testing Interval

The appropriate interval between screening tests is not known. The rationale for the 3-year interval is that with this interval, the number of false-positive tests that require confirmatory testing will be reduced and individuals with false-negative tests will be retested before substantial time elapses and complications develop (38). In especially high-risk individuals, particularly with weight gain, shorter intervals between screening may be useful.

Community Screening

Ideally, testing should be carried out within a health care setting because of the need for follow-up and treatment. Community screening outside a health care setting is generally not recommended because people with positive tests may not seek, or have access to, appropriate follow-up testing and care. However, in specific situations where an adequate referral system is established beforehand for positive tests, community screening may be considered. Community testing may also be poorly targeted; i.e., it may fail to reach the groups most at risk and inappropriately test those at very low risk or even those who have already been diagnosed (39).

Screening in Dental Practices

Because periodontal disease is associated with diabetes, the utility of screening in a dental setting and referral to primary care as a means to improve the diagnosis of prediabetes and diabetes has been explored, with one study estimating that 30% of patients ≥30 years of age seen in general dental practices had dysglycemia (40). A similar study in 1,150 dental patients >40 years old in

India reported 20.69% and 14.60% meeting criteria for prediabetes and diabetes using random blood glucose. Further research is needed to demonstrate the feasibility, effectiveness, and cost-effectiveness of screening in this setting.

PAX3

Maternal diabetes significantly increases the risk of congenital malformations in humans and in animal models. These malformations arise at the beginning of organogenesis, during the first 8 weeks of gestation in human embryos, and during the first 7–10 days in mouse and rat embryos. Diabetic embryopathy can affect any developing organ system, although defects of the neural tube and heart are among the most common. In studying neural tube defects (NTDs) in a mouse model of diabetic embryopathy, we showed that expression of Pax3, a gene that regulates neural tube closure, is significantly reduced in embryos of diabetic mice before the manifestation of morphological defects. Because loss-of-function Pax3 mutation causes the same kind of NTDs as those caused by maternal diabetes (open neural tube defects, particularly exencephaly and spina bifida) in 100% of mutant embryos, this suggests that impaired expression of Pax3 is sufficient to prevent normal formation of the neural tube. We also showed that the excess glucose surrounding the embryo, which is a consequence of maternal diabetes, is responsible for the adverse effects of diabetic pregnancy. Therefore, a unifying mechanism possibly explaining the spectrum of malformations associated with diabetic embryopathy is that exposing the embryo to excess glucose at critical times during induction of developmental control genes can compromise subsequent morphogenetic processes (41).

PVT1

Plasmacytoma variant translocation 1 gene (PVT1) is proved to have an influence on uncontrolled cell growth especially in the mesangial cell expansion which is identified to be an important hallmark in diabetic-related kidney diseases. It is located at the chromosomal location 8q24. PVT1 gene is recognized to interfere at both the developing as well as the progression stage of DKD. A prominent point is that PVT1 is a substantial noncoding RNA which have an association with renal diseases. A genome-wide association study carried out among the Pima Indians has shown a positive association of the genetic variant rs2648875 of PVT1 in end-stage renal disease. An upregulation of PVT1 was seen which was instigated by the transforming growth factor-beta 1 (TGFβ1) as well as increased hyperglycaemic levels. There are very few studies that are not enough to come to a final conclusion. Hence, further investigations in diverse ethnic populations are essential to discover relevant proof showing the link between genetic polymorphisms of PVT1 in the progression of DN (42).

MATERIALS AND METHODS

sampling

The criteria for entering the study were patients with type 2 diabetes, and the criteria for leaving the patients were those who did not fill out the questionnaire and did not want to cooperate on the part of the patient. The inclusion criteria in the control group are healthy people and the exclusion criteria are people who do not fill out the questionnaire and do not want to cooperate on behalf of

the patient. 60 blood samples from patients with type 2 diabetes in Milad Hospital in Tehran were included in the study. These samples were collected in advance and stored at minus 80 degrees Celsius. Type 2 diabetes patients were confirmed using diagnostic tests.

DNA extraction

1. In order to purify DNA from blood samples from patients with type 2 diabetes, a blood DNA extraction kit was used in this project.

2. The name of the sample was written on three microtubes.

3. The frozen blood samples were placed at room temperature to melt, then they were vortexed for about 5 seconds to loosen the clots.

4. 500 microliters of blood sample were taken and poured into the first microtube. 1000 microliters of buffer 1 was added to the sample and pipetted several times to mix well.

5. The resulting solution was centrifuged at 8500 rpm for 4 minutes and the supernatant was discarded.

6. Again, 1500 microliters of buffer 1 was added to the microtube (first, 1000 microliters of buffer were well pipetted to completely dissolve the sediment with the buffer, and then another 500 microliters of buffer 1 was added to the sample).

7. The resulting solution was centrifuged at 8,500 rpm for 4 minutes (white sediment without red blood cells was obtained), then the supernatant was discarded.

8. 350 microliters of buffer 2 was added to the sediment and pipetted until the sediment was dissolved with the buffer.

9. microliters of SDS along with 15 to 20 microliters of proteinase k were added to the sample and placed in a heater at 50°C for 30 minutes until it dissolved.

10. 100 microliters of NaCl were added along with 600 microliters of chloroform and vortexed for 15 seconds (milky liquid) and centrifuged for 4 minutes at 8500 rpm.

11. 3 phases were formed in the microtube. The sample was taken from the upper phase carefully, so as not to mix with the lower phases, with a sampler and transferred to the second microtube. The sample was centrifuged at 13,000 rpm for 3 minutes.

12. The supernatant was transferred to the third microtube and 700 microliters of absolute ethanol was added (at this stage the DNA coil was visible). The sample was centrifuged at 13,000 rpm for 2 minutes.

13. The supernatant was discarded and 600 to 700 microliters of 70% ethanol was added to the sample and centrifuged for 2 minutes at 13,000 rpm. Finally, the alcohol was slowly discarded and the microtube was placed in a heater to dry.

14. 20 microliters of distilled water were added to the sample to wash the wall of the microtube. To confirm the extracted DNA concentration, all samples were measured by nanodrop system in the ratio of A260 to A280.

TETRA-PRIMER ARMS PCR

The T-ARMS PCR technique is a simple and low-cost method to determine the genotype of single nucleotide polymorphisms (SNP). Using this method, natural and mutant alleles are amplified together with the control fragment during a PCR reaction in a tube. The region near the mutation site is amplified by two external primers and produces an allele non-specific control amplicon.

Two internal primers are designed in opposite directions and in combination with normal primers can simultaneously amplify both natural and mutant amplicons. Two allele-specific amplicons have different lengths, which are easily separated by standard gel electrophoresis method. Considering that the control amplicon and at least one of the two allele-specific amplicons are always present, the T-ARMS PCR method creates an internal control against the negative error caused by PCR failure. Genomic DNA reservoirs were used as templates for PCR amplification and product sequencing. Based on the nucleotide sequence of PAX3 and PVT1 genes, the primer pairs in Table 1 were used to determine the sequence and the genotype.

Tab 1. Primers used in T-ARMS PCR for amplification rs2305619 (PAX3) and rs2648875 (PVT1)

Target gene	Primer sequence
PAX3-FO-439 Outer primer product = 439 bp	5'- ACTCAGCTCACTTGAGAGTCTCCTCCCG -3'
PAX3-RO	5'- CGCCGGTTATGTAGCTCAAAACAGTCAA -3'
PAX3-FI-Aallele A/inner product = 255 bp	5'- CCATCCCACTGAGGACCGTAAGTGCA-3'
PAX3-RI-Gallele G/inner product = 230 bp	5'- AGTCAGGGTTAGCAGAGAAACAGTTAACGC-3'
PVT1- FO Outer primer product = 390 bp	5'- GGTTACATGTGTAAGCTCATCCAATCCC -3'
PVT1- RO	5'- TAGTAGAGACGGGGTTTCACCATGTTAGC -3'
PVT1- FI G/inner product = 239 bp	5'- GTAAGTCATTGGTCTTTGCAGGTGATGA -3'
PVT1 - R A/inner product= 207 bp	5'- TGACTTTCTTTTTTCCTGCCTCACACTC -3'

Electrophoresis

After amplification, the samples were loaded in 2% agarose gel wells stained with DNA Safe Stain, and using an electric power source, an electric current of 90 volts was established in the

electrophoresis tank filled with TBE1X buffer, and electrophoresis was performed for 1 hour. The results of band formation were checked using gel dock.

Statistical analysis

SPSS software version 23 was used for genotypic and allelic distribution. To check the quantitative data in these 2 groups, after checking the normality of the data, t-test or Mann-Whitney test was used.

RESULTS

This study included 60 patients with type 2 diabetes and 60 controls (Table 2). The average age of healthy people was 55.58±11.96 and the average age of sick people was 57.57±11.86. Statistical analysis with the Mann-Whitney test showed that the two studied groups did not differ significantly

in terms of age (p=0.361). Therefore, the two groups selected for the study did not differ in terms of age. Gender distribution was investigated in two healthy and diabetic groups. The results showed that in the control group, the number of male and female samples was equal, so that there were 13 samples in both groups. In the diabetic group, 36 of the samples were male (60.0%) and 24 were female (40.0%). Statistical analysis with chi-square test also showed that there is no significant difference between the two groups in terms of gender distribution (p=0.271).

The average BMI of healthy people was 27.63±2.63 and the average BMI of sick people was 27.95±2.89. Statistical analysis with the Mann-Whitney test showed that the two studied groups did not differ significantly from each other in terms of BMI (p=0.726). Therefore, the two groups selected for the study did not differ in terms of BMI.

The average FBS of healthy people was 82.20±9.27 and the average FBS of sick people was 164.42±20.02. Statistical analysis with the Mann-Whitney test showed that the level of FBS in the group of diabetic patients increased significantly compared to the control group (p<0.001). HbA1C level of healthy people was 4.94±0.49 and HbA1C level of sick people was 7.03±0.49. Statistical analysis with the Mann-Whitney test showed that the level of HbA1C in the group of diabetic patients increased significantly compared to the control group (p<0.001). Cholesterol level was 176.92±14.05 in healthy people and 192.13±37.31 in sick people. Statistical analysis with Mann-Whitney test showed that the cholesterol level in the group of diabetic patients increased significantly compared to the control group (p=0.029). Triglyceride level was 174.67±21.44 in healthy subjects and 195.77±45.09 in diabetic subjects. Statistical analysis with Mann-Whitney test showed that the level of triglycerides in the group of diabetic patients increased significantly compared to the control group (p=0.005).

Tab 2. Demographic parameters between two control groups and diabetic patients

p-value	**Patient group)n=60(**	**Control group)n=60(**	**Specifications**
0.361	57.57±11.86	55.58±11.96	**age (years)**

0.271	36 (60.0%) 24 (40.0%)	30 (50.0%) 30 (50.0%)	**Man** **Woman**	**gender**
0.726	27.95±2.89	27.63±2.63	**BMI**	
<0.001	164.42±20.02	82.20±9.27	**FBS**	
<0.001	7.03±0.49	4.94±0.49	**HbA1C**	
0.029	192.13±37.31	176.92±14.05	**Cholesterol**	
0.005	195.77±45.09	174.67±21.44	**Triglyceride**	

Investigation of polymorphism in PVT1 gene between the group of healthy people and patients with type 2 diabetes

The type of polymorphism of this gene can be understood by examining the amplified fragments using specific primers of PVT1 gene on agarose gel. Based on this, the 390bp band that includes

both sides of the polymorphism region are a complete fragment, which indicates the correctness of the PCR reaction. Using the internal primers designed specifically for each SNP, the 239bp and 207bp band is amplified, which indicates the presence of G and A alleles in different samples, respectively. Therefore, based on the position of the band, the type of polymorphism and allele can be determined. Figures 1 show the gel image of samples 1 to 12 of patients and control (representing all samples) following their PCR amplification of PVT1 gene polymorphism. Based on the pattern of the formed bands, different genotypes were identified and used to perform statistical tests. 20 people in the control group and 10 people in the patient group have GG genotype. AG heterozygous genotype was observed in the control group in the number of 39 people and in the patient group in the number of 49 people. AA homozygous genotype was observed in 1 person in the control group and 2 people in the patient group.

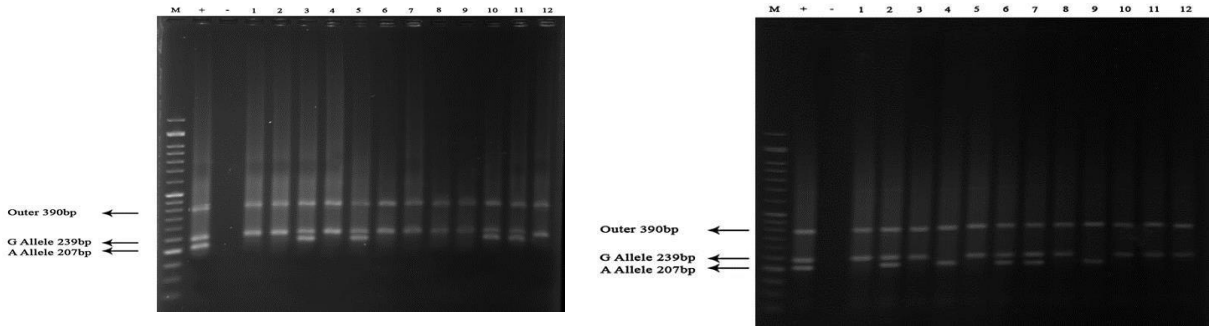

Fig 1. Left: Agarose gel image of Tetra-Arms PCR reaction products of PVT1 polymorphism (rs2648875) in healthy samples (Lane M: marker (100 to 2000bp), lane +: positive control, lane -: negative control. Wells 1 to 12: healthy samples. Samples 1, 2, 4, 6, 7, 8, 9 and 12 have GG genotype and samples 3, 5, 10 and 11 have AG genotype). Right: Agarose gel image of Tetra-

Arms PCR reaction products of PVT1 polymorphism (rs2648875) in patient samples 1 to 12 (Lane M: marker (100 to 2000bp), lane +: positive control, lane -: negative control. Wells 1 to 12: patient samples. Samples 1, 3, 5, 8, 10, 11 and 12 have GG genotype, samples 2, 7 and 9 have AG genotype and samples 4 and 9 have AA genotype).

Investigation of Hardy-Weinberg equilibrium in PVT1 gene

To check the Hardy-Weinberg equilibrium, the number of mutant heterozygous, mutant homozygous and normal homozygous genotypes in the statistical population was compared with the Hardy-Weinberg equilibrium population. As shown in Table 3, the value is p-Value>0.05 (more than 5 percent). Therefore, the analysis of the collected data showed that the PVT1 gene polymorphism in the control and patient groups is in Hardy-Weinberg equilibrium.

Tab 3. Investigation of Hardy-Weinberg equilibrium in the study population of PVT1 polymorphism

	Number of observed	The observed percentage	Expected number	Expected percentage	Results
GG	73	60.83	68	56.87	
AG	35	29.17	45	37.08	
AA	12	10.00	7	6.05	
wild allele	181	75.42			
Mutant allele	59	24.58			

Allelic and genotypic frequencies for PVT1 polymorphism in healthy groups and diabetic patients are presented in Table 4. The genotypic frequency of each of the GG, AG and AA genotypes in the healthy group was 63.3%, 31.7% and 0.5%, respectively. The genotypic frequency of each of GG, AG and AA genotypes in the group of diabetic patients was equal to 58.3%, 26.7% and 15.0%, respectively. The frequency of G and A alleles in the healthy group was 79.17% and 20.83%, respectively, while these ratios were 71.7% and 28.3% in the group of diabetic patients, respectively. Although the genotypic distribution showed a difference between the healthy group and diabetic patients, the data analysis with chi-square test showed that the genotypic distribution was not significantly different between the two groups of healthy and diabetic samples (p=0.184). In addition, allelic distribution was not significantly different between two groups of healthy and diabetic samples (p=0.177). The results showed that the A allele was significantly higher in patients with diabetes than in the control group (28.3% vs. 20.83%). However, no statistically significant changes in genotype were observed in the studied groups.

Tab 4. Genotypic and allelic frequency of PVT1 gene in control and patient groups

P-value	Number and percentage		
	(n=60) patients	(n=60) Control	**Genotype**
	35 (58.3%)	38 (63.3%)	GG
0.184	16 (26.7%)	19 (31.7%)	AG
	9 (15.0%)	3 (5.0%)	AA
			Allel
0.177	86 (71.7%)	95 (79.17%)	G
	34 (28.3%)	25 (20.83%)	A

DISCUSSION AND CONCLUSION

This study was conducted with the aim of investigating the role of polymorphism of PAX3 and PVT1 genes as effective factors in type 2 diabetes in Iran and the effect of different demographic factors on gene polymorphism. Unfortunately, there have been very limited studies on the relationship between polymorphism of PAX3 and PVT1 genes with diabetes, which makes it difficult to discuss the results of this study and other similar works. In the rest of this chapter, we will refer to similar works as well as investigate the mechanism of connection between the serum levels of these expressed proteins and type 2 diabetes. In addition, it should be noted that these two genetic polymorphisms show a positive link in the progression to diabetes types in other populations. PVT1 affects aberrant cell growth, especially the expansion of mesangial cells, which is a hallmark of diabetic kidney disease. This gene has been shown to affect uncontrolled cell growth, particularly the proliferation of mesangial cells, which is an important feature of diabetes-related kidney disease. This gene is located at chromosomal location 8q24. PVT1 gene is known to interfere in the development stage as well as the progression of diabetic kidney disease DKD. Notably, PVT1 is a significant non-coding RNA associated with diseases such as diabetic nephropathy. A genome-wide association study conducted among Pima Indians showed a positive association of the rs2648875 PVT1 genetic variant with end-stage renal disease. An upregulation of PVT1 was observed, stimulated by transforming growth factor beta 1 (TGFβ1) as well as increased levels of hyperglycemia. There are very few studies on the polymorphism of this gene, which is not enough to reach the final result. Hence, further research in different ethnic populations is necessary to uncover the relevant evidence that shows the association between PVT1 genetic polymorphisms in the development of diabetes (43).

The PAX3 gene belongs to a family of PAX genes that plays an important role in the formation of tissues and organs during embryonic development. The PAX gene family is also important for maintaining the normal function of certain cells after birth. PAX3 is produced by several cell types, most prominently by endothelial cells and monocytes in response to inflammation. PAX3 is a sensitive biomarker of local inflammatory reactions and is associated with a variety of clinical diseases, including coronary artery disease, rheumatoid arthritis, sepsis, and chronic kidney disease (CKD). Previous studies have shown that diabetes is a low-grade chronic inflammatory disease

and changes in PAX3 plasma levels are associated with the development of diabetes. As plasma PAX3 level is inversely correlated with eGFR, but positively correlated with albuminuria/proteinuria levels in patients with diabetes, therefore, PAX3 may be involved in the development of diabetes. Single nucleotide polymorphisms (SNPs) in PAX3 affect circulating PAX3 levels and are associated with the risk of certain diseases including early graft dysfunction after lung transplantation, pulmonary tuberculosis and the development of hepatocellular carcinoma in patients with chronic hepatitis C (CHC) are related. However, no data are available on the association between PAX3 polymorphisms and the development of diabetes. A separate study, conducted in a Chinese population, showed an association between PAX3 genetic polymorphisms with diabetes susceptibility in patients with diabetic nephropathy. PAX3 is synthesized in extrahepatic tissues and cells including atherosclerotic lesions, adipose tissue, vascular endothelial cells and macrophages. A previous study in a mouse model showed that PAX3 is expressed in renal peritubular endothelial cells. In humans, PAX3 expression is observed in renal proximal tubular epithelial cells (PTECs), primary mesangial cells, and renal fibroblasts in kidney tissues. In addition, increased plasma levels of PAX3 were observed in patients with CKD and diabetic nephropathy. Furthermore, plasma PAX3 levels and urinary protein excretion can be normalized by treatment of the renin-angiotensin system, and/or with calcium channel blockers, in patients with type 2 diabetes presenting with proteinuria. All of the aforementioned evidence suggests that abnormal expression of PAX3 may contribute to renal injury (44).

This study was conducted with the aim of investigating the role of polymorphism of PAX3 and PVT1 genes as effective factors in type 2 diabetes in Iran and the effect of different demographic factors on gene polymorphism. Analysis of the amplified fragments in the present study on agarose gel showed that there are polymorphisms in (rs2305619) PAX3 and (rs2648875) PVT1, and the presence of formed bands indicated the presence of A and G alleles in both genes, respectively. Statistical calculations showed that there was no significant relationship between polymorphism of PAX3 and PVT1 genes and type 2 diabetes in the studied population. The highest relative genotypic frequencies in AA and GG genotypes were seen in PAX3 and PVT1, respectively, which had the greatest role in type 2 diabetes. The obtained results ruled out the existence of any significant relationship between PAX3 (rs2305619) and PVT1 (rs2648875) polymorphisms, as well as age, gender, and BMI factors, while cholesterol levels, fasting blood sugar, triglycerides, and HbA1C have no effect on type 2 diabetes. had a lot and was reported in a meaningful way.

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