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Short communication

TCF7L2 Polymorphisms in Type 2 Diabetes, Insight from HRM and ARMS Techniques

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ABSTRACT

Introduction: Diabetes is a biological problem of life in a new way in human societies, especially in developing countries. Environmental and genetic factors are mutually influential in the incidence and exacerbation of this disease. One of the genes is the transcription factor TCF7L2 that has been proven in many studies in different communities to play a role in diabetes and is located on chromosome 10. Two SNPs for this gene, rs12255372 and rs7903146, have been recorded on the NCBI site and have a direct and significant correlation with type 2 diabetes. In this study, the genotypic frequency of these two SNPs was studied using ARMS and HRM techniques.
Materials: This study was performed on 100 patients with type 2 diabetes and 100 healthy individuals as non-diabetic controls. Diabetics were selected from patients referred to Ali Asghar Diabetes Clinic in Zahedan. The control group consisted of individuals who did not meet the criteria for diabetes or had no family history of diabetes in first- or second-degree relatives. DNA extraction was performed using the phenol-chloroform method and finally, PCR was performed for a specific primer.
Results: ARMS results showed that the number of people with SNP rs7903146 in diabetic patients is significantly higher than those in the control. However, the difference was not significant for rs12255372. HRM results were also highly correlated with ARMS and showed very precise allelic differentiation in the studied population for both positions.
Conclusion: In general, since HRM is a relatively inexpensive technique and a large number of samples can be analyzed in a few hours, the results of this study can be used in the preparation of diagnostic kits based on this method in these two and other sites related to diabetes.
Keywords: Diabetes, Genotype, HRM, ARMS.

1. Introduction

Type 2 diabetes is the most common metabolic disease that is influenced by environmental and genetic factors. The disease is the final stage of a chronic and progressive disorder, which is caused by insulin resistance, decreased function of pancreatic beta cells, and increased production of glucose by the liver. Type 2 diabetes is currently diagnosed when an excessive increase in blood sugar or

clinical manifestations of diabetes is present [1-3].

Unlike other mono-gene diseases, in which the mutation is affected by a mutant allele in one gene locus, in conditions similar to type 2 diabetes, the disease incidence depends on several gene loci that have a small to moderate effect [4, 5]. A study has shown that 26% of patients with type 2 diabetes have genetic mutations in the insulin receptor gene [6].

Over the past two decades, many genes associated with type 2 diabetes have been studied, such as KCNJ11 (Potassium Inwardly Rectifying Channel Subfamily J Member 11) and TCF7L2 (Transcription factor 7-like 2) [7]. Two positions in TCF7L2, rs12255372 and rs7903146, have a positive correlation with the occurrence of T2D (Type 2 Diabetes). The TCF7L2 gene is located on chromosome 3 with 217 kb length and 14 exons. The length of these transcripts varies from 3880 kb in transcript 6 to 4092 kb in variant 7. The mRNAs of this gene are expressed in all body tissues, but the highest level of expression is observed in the heart, skeletal muscle, and muscles. The TCF7L2 protein is responsible for expressing the beta-catenin gene, and its absence prevents cell formation [8].

The TCF7L2 gene encodes a huge transcription factor called the HMG (high mobility group), which plays a vital role in the Wnt signaling pathway. This protein is very important in the balance of blood sugar. The main and proven function of this protein is involved in the Wnt signaling pathway by altering the MYC by binding to the promoter of this gene. In the absence of ctnnB1, it acts as a repressor and in its presence as an activator[8].

There are various genotyping methods in single-nucleotide mutations, the two most important of which are ARMS and HRM. ARMS technique is based on an allele-specific extension system with two internal primers (wish is consistent for wild type and mutant position) and two extend primers with a different distances of targeted positions. Two and one amplification are represented heterozygote and homozygote genotypes, respectively [9].

In this study, using the above two techniques, the number of target mutations in the TCF7L2 gene was evaluated. The accuracy of each was calculated, and the results were compared with each other.

2. Materials and methods

This study was performed on 100 patients with type 2 diabetes and 100 healthy individuals as non-diabetic controls. Diabetic's patients were selected from the patients who referred to Ali Asghar Diabetes Clinic in Zahedan. The control group consisted of individuals who did not meet the criteria for diabetes or had no family history of diabetes in first- or second-degree relatives.

Diabetes mellitus, according to the WHO criteria, was defined in the form of fasting blood sugar (FBS) above 126 mg / dL, or blood sugar two hours after glucose intake above 200, or taking anti-diabetic medication prescribed by a physician. Sampling was performed after receiving written consent from individuals.

The collection of whole blood samples was performed in tubes containing EDTA for further studies, and genomic DNA was extracted from whole blood using the phenol-chloroform method. The primer design for the ARMS technique was designed using the Primer plex option in Allele ID7 software. In this option (Primer plex), the specific primers for each allele are called the Allele-Specific Primer Extension (ASPE). In this case, the target nucleotide site must be determined for each of the two sites of

rs12255372 and rs7903146, and then a primer must be designed separately for each of the two locations (Table 1).

Table 1. Primer design for ARMS technique using Primer plex option in Allele ID7 software for two target positions of rs12255372 and rs7903146.

Primer Forward (external)	Reverse Primer (internal)	Amplitude length (bp)
GAGTTTAGCCCAGGTTCTGTTtCT	GGGTGCCTCATACGGCAATTAAATTATAcAG	292
GAGTTTAGCCCAGGTTCTGTTtCT	GAGGATCATTTGAACCCAAGaGT	898
AACAATTAGAGAGCTAAGCACTTTTTAGAcAT	GAGGATCATTTGAACCCAAGaGT	568
ACTTGCTAATCCAACCTTATACATTcCA	CAGAGGCCTGAGTAATTATCAGAATATGgTA	547
ACTTGCTAATCCAACCTTATACATTcCA	AGCAGTAACAGGTTTGGAGcTG	694
TGCCCAGGAATATCCAGGCAAGAaTG	AGCAGTAACAGGTTTGGAGcTG	203

PCR reaction for ARMS technique in a final volume of 25 µl as 200 ng of target DNA, 5 picomoles of each reverse and forward primer, 2.5 µl of 10X PCR buffer, 1 mM of MgCl2, 1 mM of dNTPs, and one unit of Taq DNA Polymerase were prepared. The PCR reaction conditions were as follows: At the beginning of the PCR reaction, genomic DNA was performed for 5 min at 95 °C, then 35 cycles including 35 seconds at 95 °C, 40 seconds at 64 °C, and 50 seconds at 72 °C.

The specificity of the PCR product on 1.5% agarose gel was observed as a band of 298bp. PCR products were sent to the South Korean Macrogen company for sequencing to ensure that the amplified sequences in PCR are the target region of the TCF7L2 gene (three products per site). After identifying healthy and diseased populations for each gene locus in the ARMS test, and the agarose gel results were confirmed by sequencing, those samples were also used for the HRM method. Samples were selected to have both wild and mutant types for both sites.

HRM analysis was performed by 5x Hot FIRE POL EvaGreen qPCR Mix (Salice Biodyne) with raising the temperature of 0.01 °C from 70 to 85 °C. This technique could discriminate synthesized wild-type and mutant groups.

The primer for HRM was designed using Beacon designer software and the HRMA option. The length of the amplified fragment was 392 at rs7903146. Amplification program in the real-time PCR-cycler Rotor-Gene 6000 including enzyme activation at 95 ° C for 15 minutes and 40 cycles including 95 ° C desorption for 15 seconds and binding of primers at 60 ° C for 30 seconds, combined expansion at 72 ° C For 20 seconds, and the melting curve of temperature increase from 50 to 99 degrees was performed. Melting temperatures for wild and mutant types and heterozygous states were 60, 59.5, and 59, respectively (Table 2).

SNPs were measured in 100 patients and controls, and a t-test in SPSS software was performed between the two groups. Finally, the mean comparison (in dependent-sample test) between controls and patients was performed to check for the presence or absence of differences using SNPs.

Table 2. Primer sequences (5'>3'). Tm (°C) and amplicon size (bp). Primers designed using HRMA module of beacon designer 8.1 software for two target positions of rs12255372 and rs7903146

Primer	Sequence Primer	Tm	Amplicon
rs7903146 F	GGTAATGCAGATGTGATGAGA	56.69	81
rs7903146 R	GAGAAAGCCCATGAATTTGTC	56.66	
rs1225537 F	GAGACAATCTAAAGACCCAACAA	56.94	84
rs1225537 R	TTAATGACCACATAACAGTCCATAA	57.41	

3. Results

One hundred peripheral blood samples of 100 peripheral blood samples of patients and 100 peripheral blood samples of healthy individuals were collected. Patient samples were selected in terms of gender, age, and race. The number of male patients was 40 and the number of female patients was 60. The number of healthy patients was 45 males and 55 females who did not have a history of high blood sugar (Fig. 1).

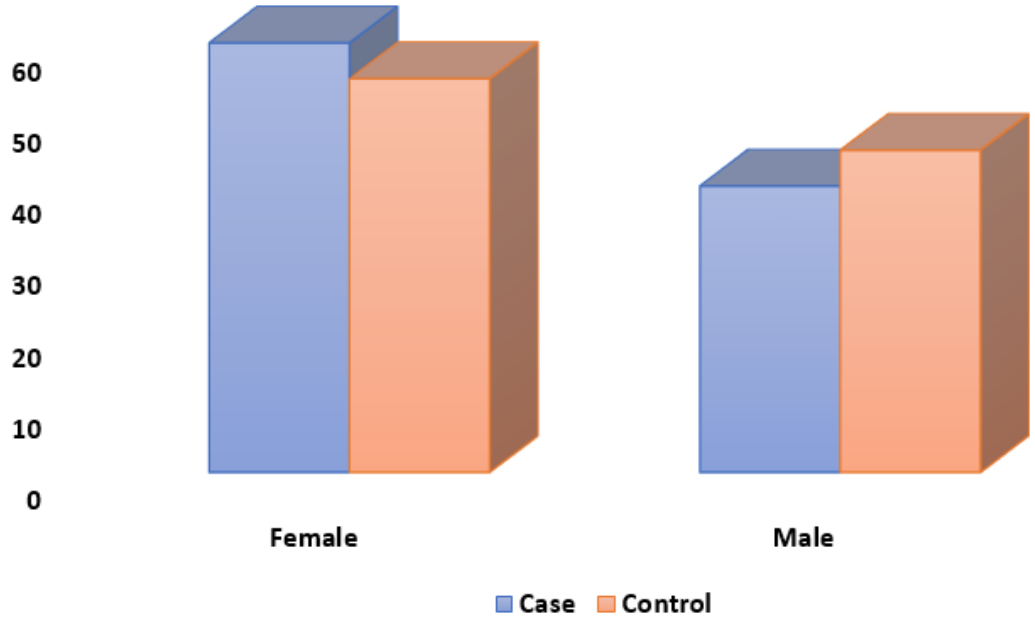

Figure 1. The percentage of men and women participating in the study in both patient and control groups

In this study, 100 DNA samples of diabetic individuals and 100 DNA samples of healthy individuals were evaluated to obtain rs12255373 and rs7903146 SNPs using ARMS and HRM techniques. Despite the precise design of the primers due to the combination of 8 primers, the performance of PCR was drastically reduced. In addition to the target bonds, non-specific bands were also observed. Six amplified fragments were identified as an agarose gel and sent to South Korea Macrogen Company for sequencing to confirm correct amplification (Fig. 2).

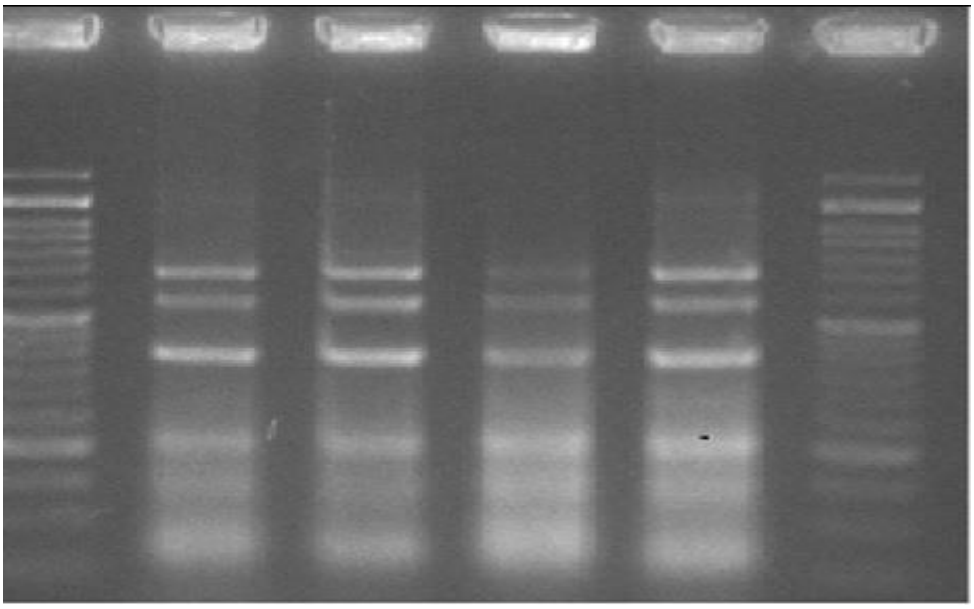

Figure 2. Agarose gel four samples of PCR product, resulting from a combination of eight primers

Simultaneous application of several primers in one reaction (also called Multiplex PCR) reduces the performance of PCR due to the formation of hairpin or heterodimer structures between the primers.

High resolution melting analysis is based on a different melting temperature of a target sequence even for one nucleotide. In this research, we applied both ARMS and HRM systems for allelic discrimination of two SNP of TCF7L2 gene in T2D samples. In the HRM technique with Rotor gene 6000 software, several graphs were drawn in the first stage and before the start of amplification (plot) (Fig. 3).

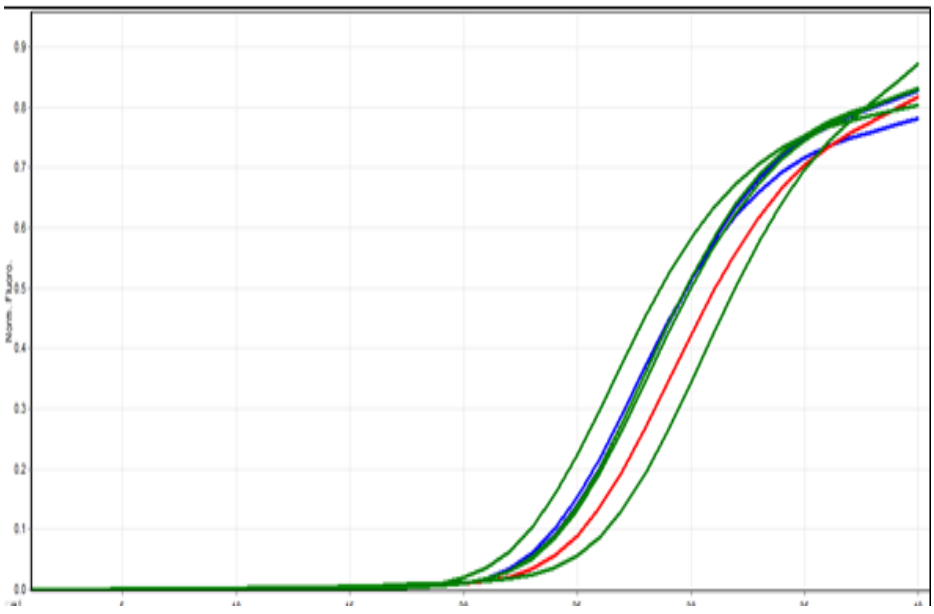

Figure 3. The amplification curve of the TCF7L2 gene around rs7904136 is one of the principles of HR, which is the same as the original pattern, which, despite control, shows a slight difference

The x-axis diagram shows the reaction cycles, and the y-axis shows the amount of fluorescence emitted from each reaction (each tube).

In HRM, the identity of these plots indicates that the original DNA is the same. Figures 4 and 5 show the results of the study.

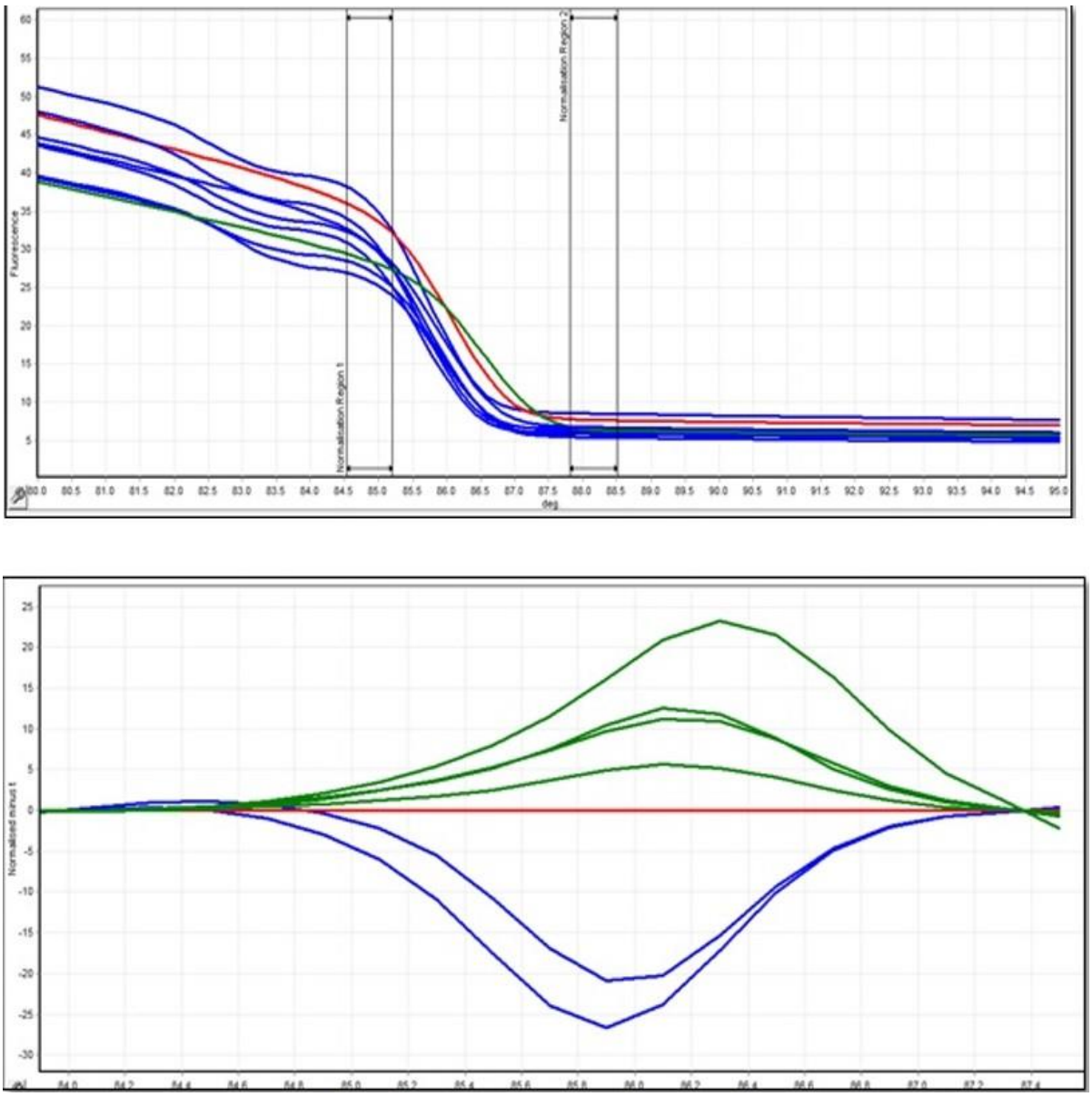

Figure 4. For HRM analysis, we focused on ± 2 ° C of temperature melting of each PCR product. In dissociation cure, red line represents heterozygote samples. Green and blue line represent wild and mutant samples, respectively.

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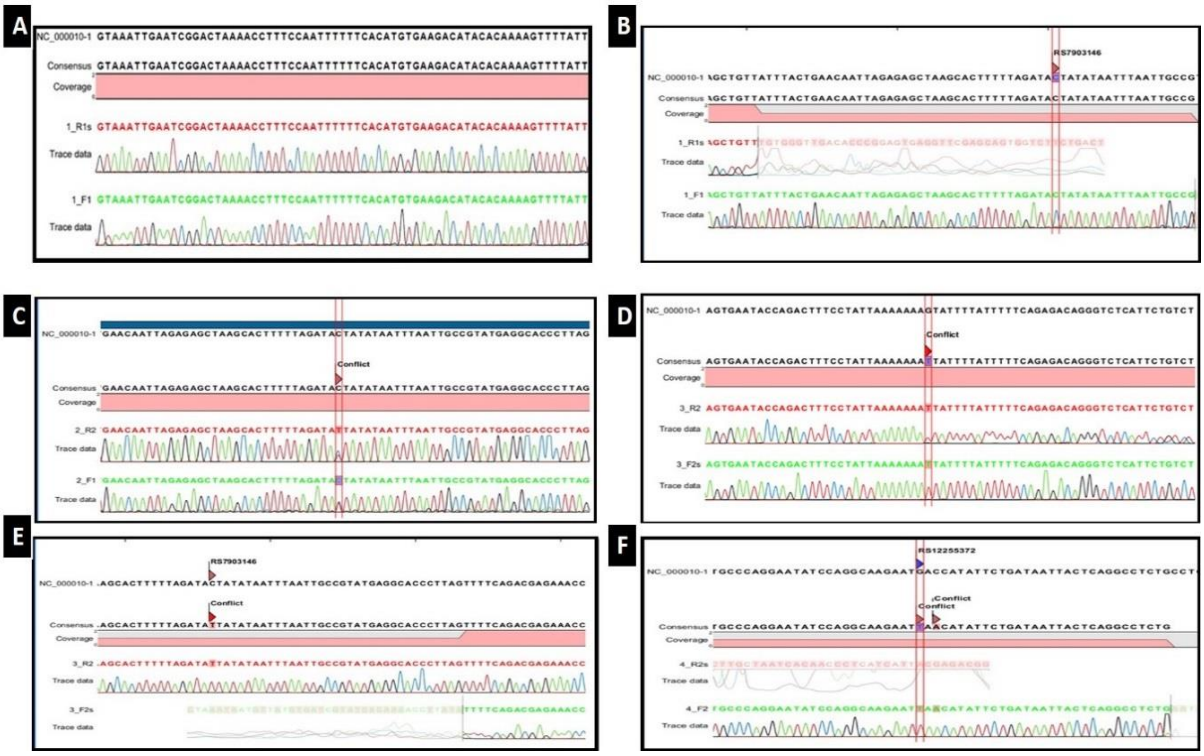

Figure 5. The amplification of the TCF7L2 gene using eight primers in which the obtained fragments were sequenced and the results showed that both mutant and wild types were amplified utilizing this system. Components in order A relate to the basis for comparing Forward Reverse sequences; B indicates cytosine nucleotide (wild type state); C is Observing two red-blue peaks on top of each other indicates the presence of heterozygosity for this nucleotide; D is the reference sequence containing G whereas the same location in query sequences contain T which is known as a conflict; E is the presence of thymine nucleotide at SNP site rs7904136, and F at SNP site rs12255373, in which there is a mutant type in this region.

$$\boldsymbol{p} = \frac{[\mathbf{2} \times (\ \mathbf{AA}\ observed)] + (\ \mathbf{AB}\ observed)}{\mathbf{2} \times [(\mathbf{AA}\ observed) + (\mathbf{AB}\ observed) + (\mathbf{BB}\ observed)]}$$

$$\boldsymbol{p} = \frac{[\mathbf{2} \times (\mathbf{195})] + (\mathbf{5})}{\mathbf{2} \times [(195) + (\mathbf{5}) + (\mathbf{0})]} = \mathbf{0.985}$$

$$\boldsymbol{q} = \mathbf{1} - \boldsymbol{p} = \mathbf{1} - \mathbf{0.985} = \mathbf{0.0125}$$

Based on the obtained genotypes, the allelic frequency of the experiment is obtained, then the expected frequency of each genotype is calculated based on the following formulas.

$$\boldsymbol{AA}\ \text{expected} = \boldsymbol{np^2} = \mathbf{200} \times \mathbf{0.9875} = \mathbf{197.5}$$

Then chi² was calculated.

$$chi^{\,2} = \sum \frac{(O-E)^2}{E}$$

$$chi^{\,2} = \frac{(195-197.5)^2}{197.5} + \frac{(5-4.93)^2}{4.93} + \frac{(0-0.031)^2}{0.031} = 0.06299$$

Chi-square score in the corresponding table with the degree of freedom 1 (difference in the number of genotypes from the allele) is 3.84. Therefore, because the calculated chi-square is smaller than the chi-square in the table, the zero curve based on a significant

difference or deviation from Hardy-Weinberg equilibrium is rejected and the studied population is in equilibrium according to SNP, rs7903146. This indicates that specific selective pressures, such as gender and environment, have not been significant. In this study, allele A is the presence of G in the rs12255373 position and allele B is the presence of adenine in this position (Fig. 6).

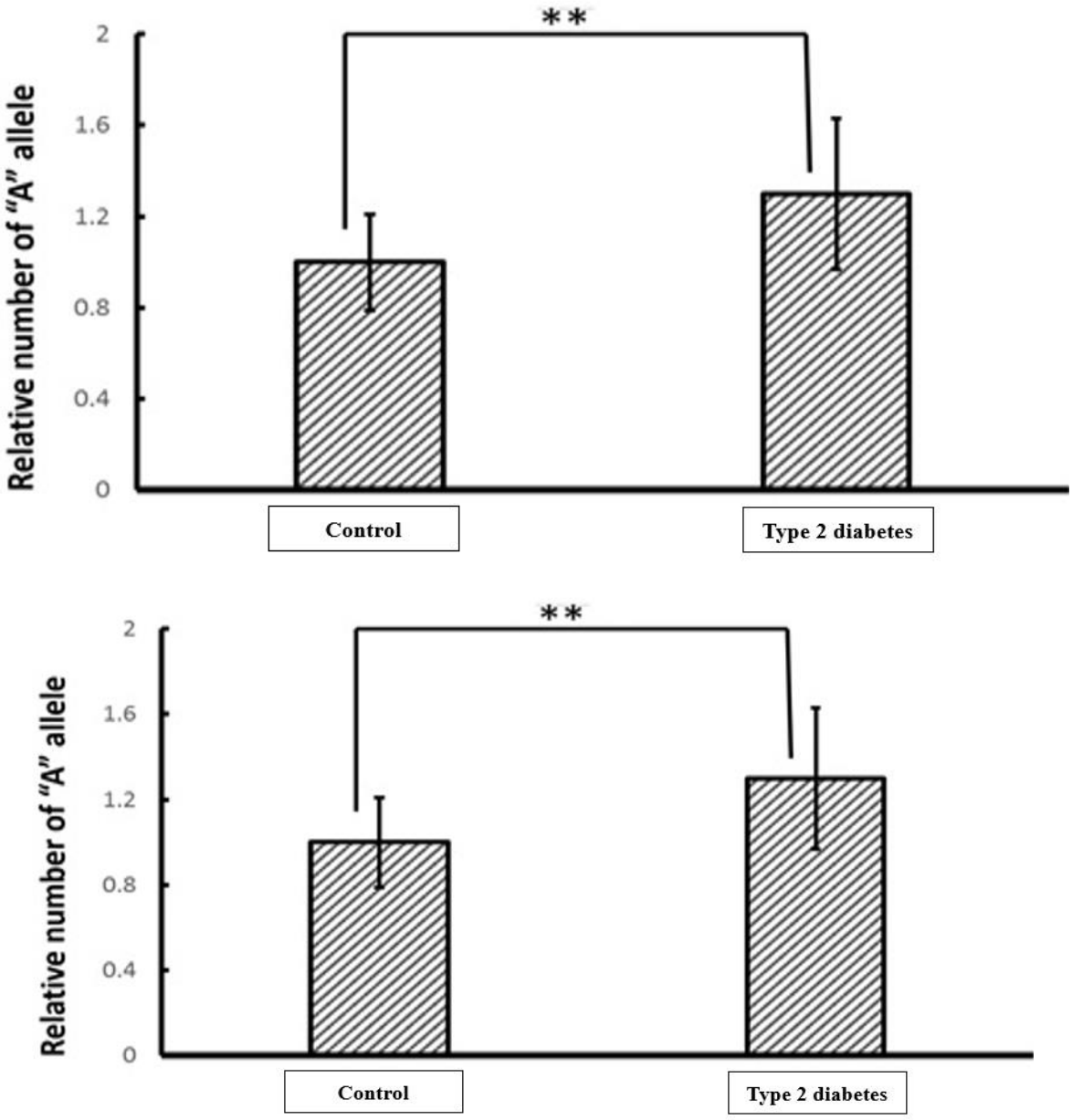

Figure 6. Diagram of the difference between thymine mutant allele between samples of diabetic patients and healthy individuals in SNP rs7904136

In the present study, 100 blood samples from diabetics and 100 samples from healthy individuals with normal blood sugar levels were used. After DNA extraction using ARMS primers, alleles A and G in rs7904136 and G and T for rs12255373 were obtained separately for sick and healthy samples. Figure 6 shows bar charts comparing mutant alleles for these two sites.

The number of mutant A alleles in rs7903146 was about 45% higher among diabetic samples than healthy individuals (Fig. 4).

4. Discussion

ARMS technique has been significantly developed in recent years and also to simultaneously find 12 significant SNPs in breast cancer. The confirmation of the results of this group was similar to the present study with sequencing [10]. Dutra *et al.* (2008), like the present study, used the ARMS technique to determine the relationship between SNP rs7903146 with type 2 diabetes and introduced this technique to determine the susceptibility to type 2 diabetes very quickly and efficiently [11].

In a study examining the expression of the TGF-B gene in diabetic patients using the ARMS technique, it was shown that a mutation in codon number 10 of this protein was directly related to diabetes [12]. A study of 581 diabetic patients using the ARMS-PCR technique showed that G/C polymorphism at position 1059 of CRP protein was significantly higher among patients than the control group [13]. In many studies, the ARMS technique has been used to identify plant varieties (8). Another curve in HRM is the Normalization plot. In this curve, the Tm range of the desired part for the final HRM analysis can be specified by default or manual to correct the variations at the beginning and end of a PCR reaction [14].

5. Conclusion

One of the objectives of this study was to evaluate the feasibility of using eight primers simultaneously in a tube, a PCR reaction, and electrophoresis well to separate a maximum of six bonds. In another study, the same thing was done for four different genes LR4, IL1B, IL-6, and IL12, with the difference that in the present study, the HRM technique was used. Therefore, achieving an integrated and easy genotype evaluation method can be a specific goal in the production of commercial kits.

Abbreviation

ARMS-PCR: amplification refractory mutation system- PCR
FBS: fasting blood sugar
HRM: High Resolution Melting PCR
MYC: The *c-myc* promoter is regulated by scores of signals, transcription factors, and chromatin components.
PCR: Polymerase chain reaction
SNPs: single nucleotide polymorphisms

FootnotesAuthors' Contribution

All authors had equal role in study design, work, statistical analysis, and manuscript writing.

Conflict of Interests

The authors declare no conflict of interest.

Ethics approval and consent to participate

No human or animals were used in the present research. The study protocol was approved by the Ethics Committee of University of Zabol, IR.UOZ.REC.1400.0015

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