Single-Nucleotide Polymorphism of rs11061971 (+219 A>T) in Adiponectin Receptor 2 (AdipoR2) Gene and Its Association with Risk of Type 2 Diabetes Among an Iranian Population

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Abstract

Genetic modifications in the adiponectin receptor 2 (AdipoR2) gene can affect phenotypes associated with insulin resistance and diabetes. The purpose of this study was to evaluate the possible role of genetic modifications in the AdipoR2 gene, to determine the frequency of genotypes and polymorphism alleles of this gene at rs11061971 (+219 A>T), and to investigate its correlation with type 2 diabetes (T2D) and its related metabolic profile.

In this case-control study, the single-nucleotide polymorphism (SNP) of interest in 116 T2D patients and 102 controls was evaluated using RFLP PCR and FOK I enzyme. Fasting blood sugar, cholesterol, triglyceride, insulin, HDL-C, LDL-C and HbA1c were also measured and their correlation with the studied genetic modifications was assessed. The collected data were analyzed using Chi-square test and Hardy-Weinberg equation.

There was a significant association in AT and TT genotypes in rs11061971 (+219 A>T) with T2D. However, no significant difference was observed in the frequency of alleles between the case and control groups. In addition, in LDL-C and total cholesterol in the control group, there was a significant difference between AA and TT genotypes as well as with AA and AT genotypes. However, no correlation was found between the other serum studied parameters and the genotype of individuals in the rs1106197171 polymorphism.

The role of rs11061971 (+219 A>T) polymorphism in T2D incidence seems to be strong. This study showed that AT and TT genotypes versus AA genotype increase the risk of diabetes.

Introduction

Diabetes, as the most common metabolic disorder, is characterized by chronic hyperglycemia due to impaired insulin secretion by β-cells in the islets of Langerhans or in by insulin resistance [1,2]. Of the four major groups of diabetes, type 2 diabetes (T2D) accounts for 90 to 95% of patients, and is of particular importance [1,3]. Diabetes mellitus is a multifactorial condition influenced by environmental and genetic factors [1]. According to a report, the prevalence of T2D in Iran is the highest among developing countries [4].

The adipose tissue not only acts as the fat-storing parenchymal cells, but also as an endocrine organ and the immune system. One of the most abundant adipocytokines secreted exclusively by adipose tissue is adiponectin, which is a protein hormone that regulates several metabolic processes, including glucose homeostasis and fatty acid catabolism [5]. Adiponectin is the most abundant adipose tissue-derived cytokine, which has anti-inflammatory, anti-diabetic, and anti-atherogenic properties [6,7], with low circulating levels associated with central obesity, insulin resistance (IR), metabolic syndrome (MetS) and T2D [8–10]. Dysregulation of adiponectin and its receptors, which reduces its levels, is involved in the development of various diseases, including obesity, IR, chronic kidney disease (CKD), type 1 diabetes (T1D) and T2D [11,12]. Through its insulin-sensitizing properties, adiponectin is a major regulator of
glucose and lipid homeostasis, and appears to be associated with the development and progression of T2D at lower levels [13,14]. Adiponectin has a direct role in insulin sensitivity. It can activate AMPK and PPAR in skeletal muscle and liver, thereby increasing insulin sensitivity [15,16].

Adiponectin plays an important role in insulin sensitivity and T2D risk through adiponectin receptors 1 and 2 (AdipoR1/AdipoR2) signaling pathways [8,17]. The adiponectin receptors are found at all sites essential for glucose metabolism [17,18]. The AdipoR2 gene is located on chromosome p13.3112, which consists of eight exons [19]. The concomitant impairment of AdipoR1 and AdipoR2 in rats prevents adiponectin binding, leading to processes such as increased tissue triglyceride content, inflammation, and oxidative stress, resulting in IR and glucose intolerance. The alterations in adiponectin mediated pathways have been shown to be associated with glucose intolerance, IR, obesity, and T2D. Genetic modifications in the AdipoR2 gene can affect IR and T2D-related phenotypes [20, 21].

According to the mentioned matters, the adiponectin receptor genes that mediate the anti-diabetic metabolic actions of adiponectin are of the influential factors in the pathogenesis of diabetes. Genetic studies have yielded different results regarding the role of AdipoR1/AdipoR2 genes and the context of T2D risk [22]. Laura G. Rasmussen et al. concluded that AdipoR1/AdipoR2 genes are influential in insulin sensitivity [23], but Kim et al. (2009) found that none of the SNPs in AdipoR1 or AdipoR2 genes has been accompanied with the T2D risk in Korean patients [21]. To the best of our knowledge, the SNPs of AdipoR2 gene in T2D has not yet been fully studied and the effect of its genetic variants has not been extensively studied in Iran. Therefore, the purpose of this study was to evaluate the frequency of single-nucleotide polymorphism of rs11061971 (+ 219 A > T) in AdipoR2 gene and its association with diabetes risk in an Iranian population with type 2 diabetes. Moreover, the possible role of this genetic modification in related metabolic properties was examined.

Materials And Methods

Subjects

This case-control study was conducted on a statistical population including T2D patients and a healthy group. The diabetic subjects included 116 T2D patients aged 40 to 70 years, who had glucose levels above 126 mg/dl and HbA1C above 6.5% (case group). The control group consisted of 102 healthy individuals without underlying disease and with normal glucose levels. One of the exclusion criteria was the subject’s history of other systemic diseases. In both groups, half were men and the other half were women, who were selected by convenience sampling from the people referring to the diabetes control centers in Tehran-Iran.

Sampling

After obtaining the consent of the subjects, 10 ml of venous blood was taken after 12-hour fasting. About 3 ml was poured into a Falcon tube containing EDTA to measure HbA1c, and the rest was poured into an
anticoagulant-free test tube to separate serum, and centrifuged for 10 minutes at 3000 ×g. The serum samples were separated and stored at -20°C.

**Measurement of lipid, glucose, HbA1c and insulin profiles**

Part of the separated serum was analyzed to measure fasting blood sugar, triglycerides, cholesterol, HDL-C and LDL-C levels using a BT 35i autoanalyzer and Biorexfars kits. The HbA1C level was measured by ion exchange chromatography (Audicom). The insulin levels were also measured by ELISA using the Diagnostic Automation/Cortez Diagnostics Inc. (DACDI) Kit (USA). The ELISA insulin kit was based on the sandwich ELISA method.

**DNA extraction**

DNA extraction from white blood cells was performed using a DNA extraction kit (SinaClon Co., Iran) in accordance with the manufacturer's instructions. The quantity of extracted DNA was determined using NanoDrop and the optical density (OD) ratio was measured at 260 to 280 nm.

**PCR process**

The PCR process was performed using PCR 2x Master Mix in which all the necessary materials except DNA template and primers were present. Primers suitable for the desired polymorphism site were designed using Oligo software (Table 1). The final mastermix was prepared using 1 μl of each primer, 6.5 μl of injected water and 10 μl of pre-prepared mastermix. Then, each of the microtubes was added by 1.5 μl of the template DNA corresponding to each patient or control sample and placed in a thermocycler. After PCR, the products were randomly confirmed by 1.5% agarose gel electrophoresis.

**PCR-RFLP method protocol**

The restriction enzyme of FOK 1 was selected in accordance with the designed primers and amplified fragments. Thus, 4 μl of PCR product, 1 μl of enzyme, 1 μl of enzyme buffer and 4 μl of sterile deionized water were mixed and the enzymatic digestion was performed according to the kit protocol. Finally, the restricted product was electrophoresed on 3% agarose gel. The resulting bands were analyzed based on Table 2 and the genotype of individuals was determined.

**Statistical analysis**

Data analysis and charting were performed using SPSS v.18 and Microsoft Office Excel2013 software, respectively. One-way ANOVA was used to statistical analysis. P-value less than 0.05 was considered as a significance level. Hardy-Weinberg equilibrium test was also performed. Statistically mean comparison was carried out by Mann-Whitney test for non-parametric data. Statistically comparison of mean and age was analyzed by independent t-test and comparison of gender ratio by Chi-square test. Ods ratio (95% confidence level, 95%CI) was applied to investigate the role of rs11061971 polymorphism in T2D risk.
Pearson correlation test and correlation coefficient (r) were used to evaluate the correlation between blood parameters studied in diabetics.

**Results**

**Demographic characteristics of study participants**

A total of 218 subjects participated in the present study, of which 103 were male (47%) and 115 were female (53%). This study was performed on two groups of individuals including the control group (n=102, 47%) and the case group (n=116, 53%), whose demographic profiles are listed in Table 3. According to the results, 82 patients (38%) had a family history of diabetes, and the rest had no family history. There was no significant difference in the mean age and sex between case and control groups.

**Evaluation of Hardy-Weinberg equilibrium**

Expected genotype frequency was calculated by Hardy-Weinberg equation. According to statistical analysis, all genotype frequencies followed this equilibrium (Table 4).

**Rs11061971 alleles and genotypes in AdipoR2 gene and their correlation with diabetes risk**

The images obtained by agarose gel electrophoresis were evaluated to determine the genotype of individuals in the rs11061971 polymorphism in the AdipoR2 gene (Figure 1).

According to the obtained results, the frequency and percentage of genotypes in all diabetic and control subjects was determined. Allele A was the wild allele and allele T was the mutant allele. The genotype AT (46%) had the highest frequency in participants, followed by TT (42%) and AA (12%). The Ods Ratio (95%CI) was applied to investigate the role of rs11061971 polymorphism in T2D risk. In individuals with genotype AA, OR=1 (95%CI) was considered for wild alleles. Individuals with genotypes AT and TT in rs11061971 had higher odds ratio of T2D risk than those with genotype AA as wild genotype. The frequency of genotype in rs11061971 region was studied by gender among the subjects (Table 5), and the T2D risk rate (95%CI) in men and women was examined separately.

Regression analysis was performed; according to the results, the risk of T2D in men and women with AT and TT genotype compared to AA wild genotype shows a significant increase.

The frequencies of wild A and mutant T alleles in this SNP in both patient and control populations are shown in Table 6. The frequency of A and T alleles in both diabetic and control groups revealed no significant difference, or in other words, neither was associated with a significant change in T2D risk.

**Serum glucose and lipid profile in different genotypes of AdipoR2 gene**

Mean values of biochemical parameters in terms of genotype were examined in both control and case groups. There was no significant correlation between different genotypes and the mean fasting blood sugar level in both studied groups. In addition, there was no statistically significant correlation between
the mean level of HbA1c and different genotypes in the two groups. The statistical analysis of TG, HDL and fasting insulin showed similar results. However, there was a significant difference in serum LDL-C and cholesterol level between AA and TT genotypes as well as between AA and AT genotypes in control group (P<0.05).

Discussion

Diabetes, with high prevalence and mortality, is associated with other diseases. The disease is associated with biochemical and functional abnormalities in liver, including changes in the metabolism of carbohydrates, lipids and proteins. These changes are especially important because of their effect on liver function in blood glucose homeostasis.

A direct correlation between diabetes and Adipor2 gene polymorphism has been shown in literature. The important role of AdipoR2 in T2D, as observed in the present study, may be due to the unique function of this receptor in the effects of adiponectin in the liver. This organ has been suggested as the main site of adiponectin bioactivity. Adiponectin affects lipid metabolism and increases the fatty acid oxidation by activating AMP-activated protein kinase (AMPK) system. Adiponectin also lowers hepatic glucose levels by reducing expression of the enzymes involved in hepatic gluconeogenesis. Thus, elevated serum triglycerides may reflect low levels of lipid oxidation in patients with metabolic syndrome, leading to the accumulation of toxic intracellular lipid metabolites in the liver and other peripheral tissues and thus inhibiting the insulin signaling [20]. Studies show that AdipoR2 downregulation or dysfunction may be responsible for the IR appearance in peripheral tissues [24]. Therefore, the present study hypothesized that genetic diversity in AdipoR2 may support increase susceptibility to T2D. We investigated this hypothesis by examining the AdipoR2 gene polymorphism in the rs11061971 region and the correlation between the observed sequence change and T2D in a small population of Tehran (Iran).

The present study was performed on blood samples of 102 controls and 116 T2D patients using PCR-RFLP. The results showed that the frequency of AT genotype in patients was 0.763 times higher than in healthy individuals (P < 0.05). The frequency of TT genotype in patients was 0.873 times higher than in controls (P < 0.05). Thus, these two genotypes were significantly associated with T2D risk, and had a higher T2D risk than the AA genotype. Next, we examined the frequency of alleles and statistical analysis showed no significant difference between the two groups.

Putapov et al. in Russia reported that individuals with the TT genotype at rs11061971 had a higher risk of diabetes (OR = 4.45) [20]. They also stated that the T allele of rs11061971 shows a greater correlation with diabetes risk (OR = 2.05). On the other hand, the A allele of rs11061971 was associated with a reduction in T2D risk (OR = 0.49). They concluded that the diversity and types of AdipoR2 increase the T2D risk and correlate with some IR-related phenotypes in the Russian study population [20]; while, the allele frequency revealed no significant difference in our study.

In a study by Ismail et al. in 2016, the AdipoR2 gene SNP was involved in the pathogenesis of T2D with CVD in Al-Najaf Governorate of Iraq [25]. In their study, the homozygous TT and heterozygous AT
genotypes of rs11061971 had a strong association and increased risk of T2D with CVD [25]. Moreover, the T allele frequency of rs11061971 was correlated with increased risk of T2D with CVD. Adiponectin receptors also play an important role in the metabolism of VLDL cholesterol and triglycerides [25]. Similarly, Damcott et al. reported that the T allele of rs11061971 was significantly associated with a higher risk of T2D in the population of Old order Amish [24].

Richardson et al. found a strong correlation between AdipoR2 polymorphism and plasma triglycerides levels, which may have important implications for atherogenesis or dyspepsia due to the potential effect of AdipoR2 genetic modifications on the metabolism of triglyceride-rich lipoproteins in Mexican Americans [26]. Collins et al., meanwhile, suggested that AdipoR2 may not be the most important risk factors for T2D and IR in the UK population; although, more detailed analysis of the gene variants may be needed to determine its potential role in IR and glucose homeostasis [27].

In a study (2018) on the Japanese elderly, it was found that there was a significant correlation between AdipoR2 SNP rs12230440 and renal function and the effects of this polymorphism on the adiponectin receptor may affect renal function in the elderly Japanese [28].

Nikitin et al. (2015) in Russia indicated that the AdipoR2 gene polymorphism was correlated with T2D risk in the Russian population, but there was no correlation between T2D and the AdipoQ or AdipoR1 gene polymorphism [29]. Moreover, recently we studied two adiponectin gene polymorphisms (ADIPOQ rs266729 and rs1501299) in an Iranian T2D population and reported that ADIPOQ rs266729 but no rs1501299 is associated with higher risk of T2D [6].

As can be seen, different results have been obtained in various studies. This discrepancy in findings may be related to racial differences or environmental factors faced by members of the studied community. On the other hand, in biochemical pathways, a protein similar in function to a defective protein may compensate for its defective activity, or at least the function of a defective protein may be sufficient to progress the pathway.

**Conclusion**

In conclusion, the results from the present study, which was conducted for the first time in Iran, revealed a direct correlation between type 2 diabetes (T2D) and adiponectin receptor 2 (AdipoR2) gene polymorphism. The genetic linkages observed between certain types of AdipoR2 and T2D in several studies, including this one, introduce AdipoR2 as a promising target for the treatment of T2D patients, especially those with obesity, insulin resistance and dyslipidemia.

**Declarations**

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Authors' contributions: The author's responsibilities were as follows: Study concept and design: MTG, AAA, MHH and MT; MHH recruited the patients and control subjects and did physical examination; Laboratory tests and analysis: MT, AF, and AMS; Manuscript preparation: MT, AF and AMS; critical revision of manuscript: MTG and AAA; Statistical analysis: MT and MTG. All authors gave their final approval to the submitted manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no any conflict of interest regarding to publication of this article. The authors gave their consent for publication in this journal.

Ethical approval The protocol of this research was reviewed and approved by the ethic committee of Faculty of Medical Sciences at the Islamic Azad University of Shahrood, Iran (IR.IAU.SHAHROOD.REC.1399.048).

Informed consent All patient information remained private and confidential. Informed written consent was obtained from the patients, without any external compulsion or pressure. No additional costs were incurred by patients.

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Tables

Table 1. Sequence of the used primers

| Primers       | Sequence (5'->3') | Length (bp) | Tm (°C) | Amplicon length (bp) |
|---------------|-------------------|-------------|---------|----------------------|
| Forward primer| GGTGATAATGACAGCCACCAAG | 22          | 59.32   | 182                  |
| Reverse primer| CCATCCTCCCTCCAGCAATG | 20          | 60.18   |                      |

Table 2. Number and length of fragments resulting from enzymatic digestion and corresponding genotype

| Number/length of fragments from enzymatic digestion and related genotype | Enzyme | Polymorphism |
|------------------------------------------------------------------------|--------|--------------|
| TT: 49/133bp                                                           | FOK 1  | +219A>T      |
| AT: 49/133/182bp                                                       |        |              |
| AA: 182bp                                                              |        |              |
Table 3
Comparison of demographic characteristics of all subjects

| Variables     | Gender | Case group | Control group | P-value |
|---------------|--------|------------|---------------|---------|
| Age (year)    | Female | 63.10 ± 8.44 | 60.56 ± 8.57  | 0.091   |
|               | Male   | 59.58 ± 8.35 | 61.29 ± 8.31  | 0.301   |
| Gender        | Female (%) | 61 (28.0%) | 54 (24.8%)   | 0.185   |
|               | Male (%)  | 55 (25.2%) | 48 (22.0%)   |

Table 4
Evaluation of Hardy-Weinberg equilibrium in all studied samples

| Parameters                             | Observed frequency | Observed percentage | Expected frequency | Expected percentage |
|----------------------------------------|--------------------|---------------------|--------------------|--------------------|
| Wild homozygous genotype (AA)          | 26                 | 11.93               | 26.50              | 12.15              |
| Heterozygous genotype (AT)             | 100                | 45.87               | 99.01              | 45.42              |
| Homozygous mutant genotype (TT)        | 92                 | 42.20               | 92.50              | 42.43              |
| Wild allele A                          | 152                | 34.86               | --                 | --                 |
| Mutant allele T                        | 284                | 65.14               | --                 | --                 |
| **Chi-square test results**            | 0.021832332        | **P-value** 0.882534 |
| **Outcome**                            | The population is in Hardy-Weinberg equilibrium. |
Table 5
Genotype frequency in rs11061971 region of AdipoR2 gene in subjects by gender

| Gender | Genotypes | Case group frequency/percentage | Control group frequency/percentage | OR (CI = 95%) | P-value |
|--------|-----------|---------------------------------|-----------------------------------|---------------|---------|
| Female | AA        | 8 (6.9%)                        | 5 (4.9%)                          | -             | -       |
|        | TT        | 25 (21.6%)                      | 23 (22.5%)                        | 0.679         | 0.019   |
|        | AT        | 28 (24.1%)                      | 26 (25.5%)                        | 0.673         | 0.012   |
| Male   | AA        | 7 (6.0%)                        | 6 (5.9%)                          | -             | -       |
|        | TT        | 25 (21.6%)                      | 19 (18.6%)                        | 1.128         | 0.016   |
|        | AT        | 23 (19.8%)                      | 23 (22.5%)                        | 0.857         | 0.019   |

Table 6
Allele frequency in the rs11061971 region of AdipoR2 gene in both groups

| Alleles | Case group, frequency(percentage) | Control group, frequency(percentage) | OR (CI = 95%) | P-value |
|---------|----------------------------------|--------------------------------------|---------------|---------|
| Wild A allele | 81 (34.9%) | 71 (34.8%) | 0.995 | 0.208 |
| Mutant T allele | 151 (65.1%) | 133 (65.2%) |     |       |

Figures
Figure 1

Analysis of the bands appeared on agarose gel electrophoresis for genotyping adiponectin receptor gene (M: 50-bp marker, 11: negative control (NTC), 1, 2, 4, 5 and 8: heterozygous AT, 10: undigested PCR product (UnDigest), 3 and 9: homozygous AA, 6 and 7: homozygous TT)