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Identification of Genotype and Allelic Frequencies of Vitamin D Receptor Gene (Taq1) Polymorphisms in T1DM Patients from Turkey

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Abstract

Changes in the Deoksiribonucleic acid (DNA), or polymorphisms, of the Vitamin D Receptor (VDR) cause the protein to bind more or less tightly to 1.25 Hidroksil (OH). The tighter that vitamin D binds, the stronger and longer lasting the metabolic changes are. Some of the different polymorphisms of the VDR have been associated with an increased risk for Diabetes Mellitus. This study suggests that while Taq1 polymorphisms may be functionally different it may also play a role in serum levels. Therefore, in this study, we selected TT allele of VDR gene has been associated with higher Diabetes Mellitus risk for study and investigated young adults or 100 patients with T1DM (50 women, 50 men) and 120 healthy subjects. The Polymerase chain reaction (PCR) was used for amplification of a 200 bp fragment of the VDR gene. One study found that TT genotype are over-represented in T1DM patients and those with the TT allele had a 3 fold increase in T1DM risk. In addition, the aim of the present study was to adapt PCR amplification, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and the most effective DNA isolation method. The results indicate that the Taq 1 polymorphism in the VDR gene plays a significant role in protection against T1DM. Consequently, We found an association between VDR polymorphism (Taq1) and T1DM in Turk population.

Abbrevations

Diabetes Mellitus (DM); Single Nucleotid Polymorphism (SNP); Polymerase Chain Reaction (PCR); Vitamin D Receptor (VDR), Restriction Fragment Lenght Polymorphism (RFLP).

Keywords: Diabetes mellitus, gene polymorphisms, VDR gene

INTRODUCTION

Diabetes is a disease in which levels of blood glucose, also called blood sugar. People with diabetes have problems converting food to energy. Normally, after a meal, the body breaks food down into glucose, which the blood carries to cells throughout the body. Cells use insulin, a hormone made in the pancreas, to help them convert blood glucose into energy. T1DM, formerly called juvenile diabetes, is usually first diagnosed in children, teenagers, and young adults. T1DM is a complex disease characterized by the autoimmune and progressive destruction of insulin-secreting pancreatic beta-cells. Both genetic and environmental factors are generally accepted as main participants in this autoimmune process that leads to the onset of the disorder. During the last years, diverse studies have emerged to analyze a possible relationship between T1DM and polymorphisms in VDR gene region on chromosome 12 [12, 8]. The action of vitamin D is mediated through binding to its nuclear receptor (VDR). In response to hormone binding, the VDR regulates the transcriptional activity of 1. 25 $(OH)_2$ Vit D_3-responsive genes by complexing with a vitamin D response element located in the promoter region of target genes. The VDR is a candidate locus for different disease such as Prostate cancer, Diabetes mellitus,Breast cancer etc. Due to allelic variation, which affects the activity of the receptor and subsequent downstream vitamin D mediated effects, such as calcium absorption, excretion and modulation of cellular proliferation and differentiation [12]. Achievements of molecular biology provide new prospectives in research on origins of many diseases. Genetic determinations of diabetes are unquestionable. Many studies on variability of candidate genes were performed. The subjects of these studies include genes which code proteins of metabolism. Almost all the studies have used only the Taq1 'A' site, a single nucleotide polymorphism (SNP) in a Taq1 restriction site [2, 3, 7]. All the markers have been described previously Taq1 'A' by [3], Taq1 'B' by [5], Taq1 'D' by [6, 10]. VDR gene polymorphisms are believed to be one of the genetic factors responsible for ethnic difference. At least 22 unique loss of function mutations have been reported 4 in the VDR gene, which shows several common polymorphisms like Bsm1, Apa1, Taq1 variations [4, 5]. The large amount of positive genetic association data in a number of diseases suggests functional consequences of VDR gene polymorphism. We previously reported a positive association of polymorphisms within the VDR gene Taq1. In the present article we extended our previous reports to polymorphisms. Vitamin D and its receptor have been suggested to play a role in the pathogenesis of T1DM. We have therefore studied the influence of VDR gene polymorphisms on susceptibility to T1DM in Turk population. Assessment of VDR gene polymorphisms was performed by the PCR-RFLP method. The genotype-phenotype association for these polymorphisms was analyzed. This study suggests a probable association of the Taq1 polymorphism in VDR receptor gene in Turk population. All samples were analyzed and validity of genotyping ensured.

The aim of this work was to evaluate the contribution of these VDR polymorphisms to the susceptibility to T1DM in the Turk population and it discuss about this methodology. This study suggests that while Taq1 polymorphisms may be functionally different it may play a role in serum levels. Therefore, in this study, we selected TT allele of VDR gene has been associated with higher Diabetes Mellitus risk for study and investigated young adults 100 patients with T1DM (50 women, 50 men) and 120 healthy subjects. In addition, the aim of the present study was to adapt PCR amplification, the PCR-RFLP and the most effective DNA isolation method. We suggest that Taq1 polymorphism indicates susceptibility to T1DM in the Turkey population.

MATERIAL AND METHODS

Patients And Healthy Samples

Blood samples were collected from young adults 100 patients with T1DM (50 women, 50 men) and 120 healthy subjects from the Turkey population with informed consent. We studied on patents with T1DM exposed to fasting plasma glucose (FPG) test which measures blood glucose in a person who has not eaten anything for at least 8 hours. This test is used to detect diabetes and pre-diabetes. Test results indicating that a person has diabetes confirmed with a second test on a different day. Blood specimens were collected in tubes containing Ethylenediaminetetraacetic acid (EDTA). Fifteen milliliters of fresh peripheral blood (5% EDTA) were collected per patient. Nondiabetic subjects were also recruited. The diagnosis of T1DM was based on clinical characteristics, magnitude of residual insulin. The healthy nondiabetic individuals were recruited by selecting those who did not have a family history of diabetes and who demonstrated normal blood glucose, and insulin levels during meal test. Blood samples from fresh material were obtained from the Hematology Departments of Hospitals in Konya in Turkey. We compared the VDR Taq1 and other restriction enzymes RFLP distribution in the female and male population, and no sex-related differences were found in VDR genotype.

Fasting Plasma Glucose (FPG) Test

The FPG test is the preferred test for diagnosing diabetes because of its convenience and low cost. The FPG test is most reliable when done in the morning. People with a fasting glucose level of 100 to 125 milligrams per deciliter (mg/dL) have a form of pre-diabetes called impaired fasting glucose (IFG). A level of 126 mg/dL or above, confirmed by repeating the test on another day, means a person has diabetes.

PCR Assay For Vitamin D Receptor Gene Taq1 Genotyping

The genotypes for restriction fragment lenght polymorphism of the VDR gene were determined by PCR amplification and enzymatic digestion of the product with Taq1. For this purpose, DNA samples were amplified with PCR. Genomic DNA (500 ng) previously was added to a 25 µl or 50 µl reaction mixture containing: 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 200 µM dNTP, 1 unit of Taq polymerase (Fermantase); and 50 pmoles of each primer.

The primers for the amplification of TaqI were:

Taq1 site forward: 5'-GTGGGATTGAGCAGTGAG-3'

Taq1 site reverse: 5'-TGGATCATCTTGGCATAGAG-3'

DNA Isolation

Genomic DNA extraction from blood of patients with T1DM and healthy samples for control groups were carried out by using different procedure including manual and automatically isolation. The genomic DNA was isolated from peripheral blood by using standard phenol chloroform method, EZNA DNA blood kit and EZ1 Nucleic acid isolation analyser techniques. Isolated DNA samples were subsequently stored at -20°C for use in further analyses.

Amplification Of VDR Gene Or Taq1 By Convencional PCR

The Taq1 VDR RFLP was determined by a PCR-based method. Reaction mixtures of 50 µl were used in PCR for the VDR gene start codon polymorphism. DNA samples were amplified in Thermal Cycler (BioRad) with cycling parameters as follows: Amplification was accomplished by 30 cycles of incubation at 94°C for 5 min, at 60°C for 1 min and at 72°C for 2 min. After amplification, a 20 µl aliquot of PCR reaction mixture was digested by the addition of endonuclease, HindIII and Taq1. Reaction mix was incubated at 65°C for 2 h. The electrophoresis was performed on a 2% agarose gel at 100 V. The restriction fragments were visualized by UV.

For the detection of Taq1 polymorphism located in the nineth exon of VDR gene, PCR product was cut with HindIII. An uncut end-products (100-180 bp) showed the presence of Tt allele, while an end-product of three fragments (100 bp) revealed the presence of tt allele. In addition to the homozigot alles, an end-product of two fragments (100 and 180 bp) displayed the presence of the Taq1 site. Codon 352 in exon 9 is polymorphic, existing as either ATC or ATT both of which code for isoleucine, and the C>T change is associated with the loss of a Taq1 restriction site. The resulting alleles are designated t (Taq1 site present) or T (TaqI site absent), and three possible genotypes result: TT, Tt, and tt. Three banding patterns are observed after digestion of the 200 bp amplification fragment, depending upon genotype (Figure 1): (a) homozygous absence of the Taq1 polymorphism (Tt) results in two fragments of 180 bp and 100 bp; (b) homozygous presence (tt) of the Taq1 polymorphism displays in 100 bp fragment. (c) homozygous presence (TT) of the Taq1 polymorphism displays in 180 bp fragment. The 100 bp fragment is constant among all genotypes, having been created by a nonpolymorphic Taq1 site within the amplification fragment, and acts as an internal control for digestion. One study found that TT genotype are overrepresented in T1DM patients and those with the TT allele had a 3 fold increase in T1DM risk. That is to say, there is homozygous presence (TT) of the Taq1 polymorphism displays in 180 bp fragment.

Analysis Of VDR-TaqI Polymorphism

The successful amplification was confirmed using gel electrophoresis on a 2% agarose gel stained with ethidium bromide (0.5 mg/ml). After amplification the three TaqI RFLPs were digested with Taq1 restriction enzymes and HindIII as per the manufacturer's recommended conditions. Subsequently, the digested fragments were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide for visualization. The 100 bp DNA marker ladder was supplied from Fermentase.

Genotyping

Genomic DNA was extracted from peripheral blood

leukocytes collected from the subjects in Turk population. The Taq1 polymorphism in exon 9 of the VDR gene was detected by the PCR-RFLP method previously described by Gross et al. [4] with the primers shown in material (Figure 2). 10µl of the digested reaction mixture was then loaded into 9% Polyacrylamide gel electrophoresis (PAGE) also visualized under white light and photographed with a Alpha Imager 1220 V5. Camera software. The sizes were determined using 100 bp ladder (Roche, Ankara).

Statistical Analysis

Genotype frequencies of the VDR gene polymorphism in normal healthy controls and T1DM patients in Turkey were determined according to Hardy-Weinberg equilibrium and by using the computer software SPSS version 5.1 including the Chi Square (X^2) test.

RESULT AND DISCUSSION

PCR is the most common technique used for genotyping, which has promoted studies on the associations of genotypes with disease risk and prognosis. Type of polymorphism, accuracy of genotyping, number of samples and available PCR equipment are factors to be taken into account when a PCR method is chosen for a given study. PCR-RFLP requires another step for incubation with a restriction enzyme digests DNA products with a particular sequence from the alleles without it. It has been estimated that half of SNPs occur in natural restriction sites. SNPs are scattered throughout the genome and high degree of variability makes these informative genetic markers useful for disease susceptibility. VDR is known to regulate cell proliferation, calcium absorption from the gut, and cell differentiation and may also influence androgen and estrogen activation. PCR-SSCP (single-strand conformation polymorphism) is a genotyping technique to detect differences in the conformation of single strand DNA, which requires electrophoresis equipment to keep a constant temperature [11]. Allele spesific PCR is an alternative for genotyping, whose PCR condition is set to avoid unspesific DNA products. In this work, we have focused on the study of part of the Vitamin D Receptor gene for discrimination between T1DM patients and healthy subjects from the Turkey population. The action of VDR is not only up regulated by vitamin D but also by protein kinase A, parathyroid hormone and growth factors. Any defect in the VDR gene could modulate the metabolism of calcium thereby increasing the risk of developing different diseases e.g. osteoporosis and calcium stones. The VDR gene Fok-1 polymorphism has been widely used as a genetic marker for diseases related to calcium metabolism. In the long run, these studies may help in determining disease susceptibility and clinical management of patients [1]. The haplotype of Taq1 polymorphisms was examined using the PCR-RFLP method. Briefly, a 200-bp fragment from the VDR gene flanking the Taq1 polymorphism sites was amplified by PCR using a forward primer (nt121–144) in intron 8 and a reverse primer (nt337–316) in exon 9 under standard conditions. The PCR products were digested for 1 hour with Taq1 at 65°C. The genotypes were determined by ethidium bromide and UVB illumination of the fragments separated on gels of 3.5% NuSieve GTG agarose (Fermentase). Absence and presence of the Taq1 sites are denoted and T and t, respectively. The subjects were selected at random among unrelated individuals attended at public and private medical health institutions. The number of subjects enlisted at each institution was established based on these socio-economic categories. Knowing the frequency of diabetes mellitus (8/100) we examined a representative sample of DNA (100 individuals). In conclusion, our data indicates an association between the VDR gene and T1DM among the Turkey population. We suggest that the TT genotype may predispose Turkey individuals to T1DM and that this genotype appears to be a marker for T1DM. The role of the VDR gene polymorphism should be studied further in other populations, Taq1 polymorphisms should be analyzed for association with T1DM susceptibility. The genotypes were analyzed with PCR/RFLPs to define three polymorphic sites: Bsm1, Taq1 and Fok1 [9]. We designated with lower case letters the presence of the restriction site for the enzyme and capital letters for its absence. All samples were analyzed and validity of genotyping ensured. Furthermore, we extracted DNA samples from 120 individuals to construct the DNA banks in this study. To conclude, the current study found that Taq1 polymorphism at the vitamin D receptor gene may contribute and polymorphisms were related at T1DM. VDR polymorphisms were determined in representative samples of T1DM among the Turkey population and the effect of the VDR genotype on T1DM is not significant in patients.

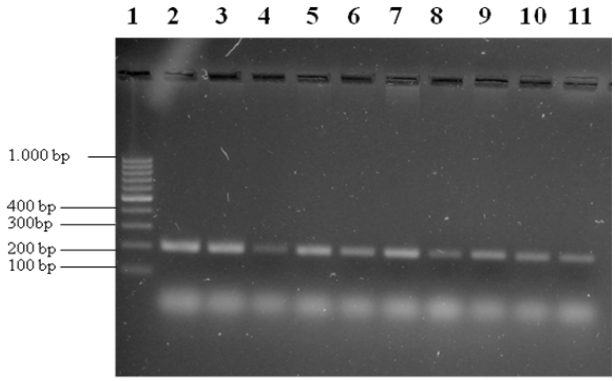

Fig.1. Agarose gel electrophoresis of VDR gene Taq Polymorphism with Diabetes Mellitus patients. Lane 1, 100 bp ladder size standart. Lane 2-11, VDR gene PCR products with Diabetes Mellitus patients.

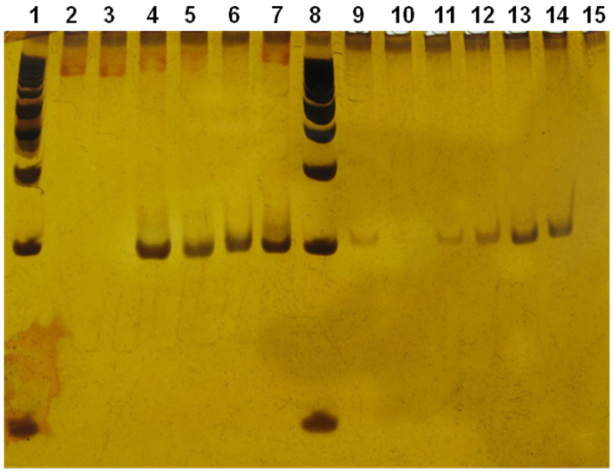

Fig.2. Polymerase chain reaction and restrictrion enzyme single strand conformation polymorphism(SSCP) analysis of Taq gene. Lane 1, 100 bp ladder size standard. Lane 2-7, blood sample genomic DNA PCR products with Diabetes mellitus. Lane 8, 100 bp ladder size standard. Lane 9-14, Restriction enzyme with diabetes mellitus Lane 15, Negative control or none DNA.

VDR polymorphisms have not influence on sex-dependent growth. However, the significance of this result and findings are hard to predict, since T1DM affects male and female equally. In conclusion, our data indicates an association between the VDR gene and T1DM among the Turk population. We suggest that the TT genotype may predispose Turk individuals to T1DM and that this genotype appears to be a marker for T1DM. The role of the VDR gene polymorphism should be studied further in other populations, and other polymorphisms, such as the Bsm1, Fok1 and Taq1 polymorphisms, should be analyzed for association with T1DM susceptibility. We suggest that Taq1 polymorphism indicates susceptibility to T1DM in the Turkish population. It is to be noted that the sample size, especially for the Turk population, was small. With further study, it may be found that there is indeed a difference in the proportion TT allele of Taq1 in Turk population.

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