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Original Article

Significant Associations of the rs3104413 Single-nucleotide Polymorphism in the HLA Region with Type 1 Diabetes

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Background and Aims: In this study, the effect of rs310441 polymorphism in the human leukocyte antigen (HLA) region on the development of susceptibility or resistance to Type 1 diabetes (T1D) among the people with T1D compared to healthy subjects has been investigated.

Materials and Methods: This research, which is based on the examination of 130 cases with T1D and 98 controls, has been carried out in the city of Hamedan after clinical examination. In order to determine the HLA gene polymorphism, the allele-specific-refractory mutation system-polymerase chain reaction (ARMS-PCR) method was utilized.

Results: This study indicated that there is a significant relationship between the frequency of alleles and genotypes in the patients compared to healthy subjects. The C/C and C/G genotypes were more frequent in patients than controls and G/G genotype was shown to be protective for T1D (p=0.01). Significant difference was found for the G allelic frequency in patients with T1D and in the control group. The allelic frequency was significantly different between the two groups (p=0.0001). Our findings indicate that HLA polymorphism(C/G) and (C/C) genotypes could be considered as genetic risk factors associated with susceptibility and (G/G) genotypes associated with protection for T1D.

Conclusions: This study identified that there is a significant relationship between the frequency of alleles and genotypes in the patients compared to healthy subjects.

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Introduction

Type 1 diabetes (T1D) is a multifactorial disease in which immune system cells destroy pancreatic β-cells responsible for producing insulin in body [1]. T1D is a disease of major public health concern [2]. It is estimated that 366 million people are suffering from T1D and it is expected that, until 2030, this number will reach 552 million in the world [3]. Similar to other autoimmune diseases, the etiology of type 1 diabetes is still unknown. T1D is a chronic autoimmune disease that develops by environmental factors such as infection virus, bacteria and some foods in the people who are genetically susceptible to the disease. It has been known that more than 60 different genes bear a crucial impact in susceptibility of T1D [4]. Almost 30% to 50% of TID susceptibility is due to major histocompatibility complex gene and DQ & DR genes have the most impact [5]. The human leukocyte antigen (HLA) which is a genetic region on chromosome 6p21.31, is responsible for 40% to 50% of the familial aggregation of T1D. By attaching to the peptide antigens and displaying them on the cell surface, this gene facilitates the recognition process for the T-cells [6]. Numerous studies reveal that the existence of HLADRB1 and DQA1*0301, DQB1*0302 and DQA1*0501, DQB1*0201 alleles increases the susceptibility of T1D. If the aforementioned alleles are in linkage disequilibrium with the HLA-DRB1*03(DR3) or HLA-DRB1*04 (DR4), the susceptibility of T1D increases significantly [7-9]. A considerable amount of loci have been identified through genome-wide association studies (GWASs) including infinite number of single nucleotide polymorphisms (SNPs) that are located throughout the genome [10]. It is known that several polymorphism in HLA genes are related to diabetes. In this study, the frequency of rs3104413 polymorphism, which is located in the intergenic region between HLA-DRB1 and HLA-DQA1 in the HLA region in the people with diabetes compared to healthy subjects, has been evaluated. Although the effect of polymorphism has been proven in several populations, no similar study has been carried out in Iran. Due to the considerable effect of different races on the polymorphisms in the genome, studying the relationship between polymorphism and diabetes is highly critical.

Materials and Methods

This study was performed on a group of 69 male and 61 female patients between May 2008 and September 2012, affected with T1D according to the diagnostic criteria established by National Diabetes Data Group (NDDG). The average age of the onset of the disease in this group was between 6 to 12 years. For people without diabetes, the normal range for the hemoglobin A1c level is between 4% and 5.6%. Hemoglobin A1C levels between 5.7% and 6.4% means one has a higher chance of getting diabetes. Levels of 6.5% or higher means having diabetes [11].

The control group consisted of 62 males and 36 females with the same ethnicity and the average

age of 8.3 to 25.4 years with no clinical evidence or T1D history in their family. Informed consents were obtained from all the subjects according to the protocol approved by the Ethical Committee of Hamedan university of medical sciences in Iran and written informed consents were obtained from all the participants. The DNA was extracted from peripheral blood samples of the patients and controls by utilizing a commercially available kit (ArchivePure DNA Kits catalog numbers 2300700, 5Prime, Germany).

Genotyping

We designed an amplification refractory mutation system polymerase chain reaction (ARMS-PCR) for detection of rs3104413 (C/G) (Table 1). These methods are simple, rapid and sensitive to detect the most common mutations [12]. The HLA gene sequences were obtained from the National Center Amplification for Biotechnology Information (NCBI). Polymerase chain reaction (PCR) was performed by using commercially available PCR premix (AccuPower PCR Premix; BIONEER, Daejeon, Korea) according to the manufacturer's instructions. Briefly, 1 µl template DNA (~100 ng/µL), 1 µl of each primer (10 pmol/µl), and 15 µL DNase-free water were added to AccuPower PCR Premix. It was done in 20 µl reaction volume containing 100 ng of genomic DNA. The following thermal profiles were run; 3 min. at 95ºC for initial denaturation, followed by 30 cycles of 95ºC for 20s, 60ºC for 30s and 72ºC for 40 s and final extension at 72°C for 5 min. for position rs3104413 (C/G). The amplified PCR products were analyzed by 2% agarose gel electrophoresis and ultraviolet visualization. The length of the expected PCR products were 372 bp for rs3104413 (C/G) polymorphisms (Fig. 1).

Statistical analysis

The statistical analysis of the data was performed through Chi-square test in SPSS ver. 23 software.

Evaluation of the genotype and allele frequencies in all cases and controls was carried out by calculating the Odds Ratios (OR) with 95% of Confidence Intervals (CI). Significance was assigned when p values less than 0.05 were obtained.

Results

The statistical analysis indicated a significant difference between case and control groups. In other words, a significant association was identified between frequency of genotype GG, CC and CG in rs3104413 polymorphism in 130 patients; they turned out to be 29.23% and 34.62% and 36.15% respectively. On the other hand, the same analysis parameter values of 98 healthy subjects were 75.51%, 7.14%, 17.35% respectively. According to the tables 2 and 3, the comparison between these genotypes (CG, CC, GG) in the patient and healthy groups revealed a statistically significant association. The genotypes CG, CC and GG of the patient groups compared to the healthy subjects showed a statistically significant association as the following: for genotype GG [OR= 0.14 (0.07-0.26) p=0.001], for genotype CC [OR=6.72 (2.73-17.32) p=0.001], and for genotype CG it was [OR=2.72 (1.39-5.39) p=0.00001]. Also, the frequency percentage of

allele C, G in patient and control groups turned out to be 52.27%, 15.82% and 47.72%, 84.18% respectively. Prevalence of genotypic and allelic polymorphisms is shown in tables 2 and 3. By comparing allele frequency between healthy subjects and patients it can be concluded that there is a significant difference between the two groups in terms of diabetes risk [OR=5.83 (3.62-9.42) p=0.00001].

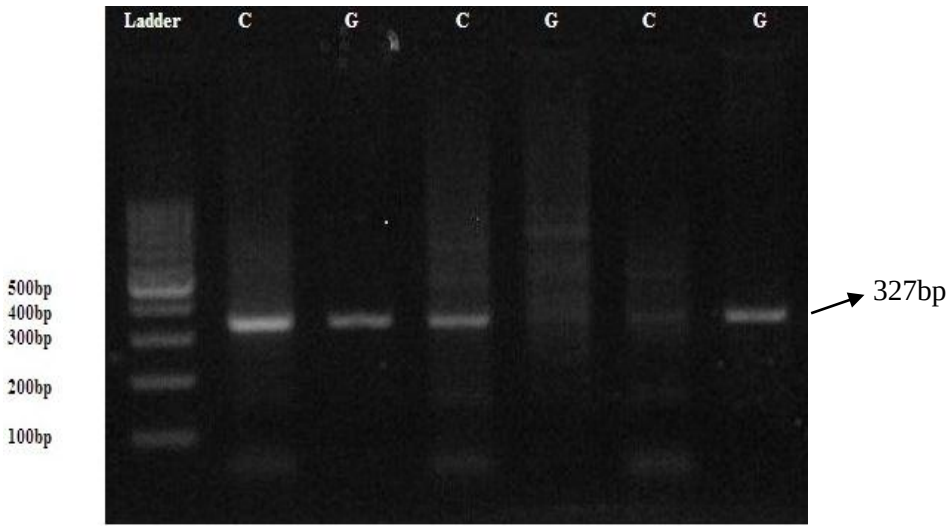

Fig. 1. PCR assay for single nucleotide polymorphism rs3104413 in HLA agarose gel electrophoresis showing the 327 bp

Table 1. The sequences of primers used in the study for rs3104413 single-nucleotide polymorphism

Gene polymorphism	Primers	Sequence (5′ to 3′)	T_m (°C)	Product size
HLA rs1304413	Reverse (C allele)	GGAGAAGCACGACAATAGGAC	59	C and G allele: 327 bp
	Reverse (G allele)	GGAGAAGCAAGCCAATAGGAG	59	
	Forward (common)	CTGCTTTTCACACCAACCTCT	60	

Table 2. Genotypes frequencies of rs1304413 for the case and control groups

Polymorphism HLA	Genotype	Group		OR (95% CI)	P-value
		Patient (n=130)	Healthy (n=98)		
rs1304413	GG	38 (29.23 %)	74 (75.51%)	0.13 (0.07-0.24)	0.001
	CC	45 (34.62 %)	7 (7.14%)	6.88 (2.94 -16.09)	0.001
	CG	47 (36.15%)	17 (17.35%)	2.69 (1.43 -5.08)	0.00001

Table3. Allelic frequencies of rs1304413 for the case and control groups

Polymorphism HLA	Allele	Group		OR (95% CI)	P-value
		Patient (n=130)	Healthy (n=98)		
rs1304413	G	123 (47.72%)	165 (84.18%)	5.83 (3.62-9.42)	0.00001
	C	137 (52.27%)	31 (15.82%)		

Discussion

More than 50 genes have been identified to influence the risk of T1D, with HLA class II genes having the greatest impact on the people's susceptibility [13]. Other loci bear minor impact on the risk for T1D; however, the combination of HLA genotypes and non-HLA single nucleotide polymorphisms has been shown to aid disease prediction [14, 15]. Several studies have demonstrated a fundamental role for the HLA in the susceptibility of, or protection to, T1D [16-18]. So far it has been understood that some polymorphism in HLA genes are related to diabetes. In this study, the effect of rs310441 polymorphism in the HLA region on the development of susceptibility or resistance to T1D among the people with T1D has been studied. The rs3104413 polymorphism is located in the intergenic region between HLA-DRB1 and HLA-DQA1. Several genetic studies have been published related to large-scale polymorphisms and autoimmune diseases one of which is the polymorphisms examined in this probe. By studying 263 patients with rheumatoid arthritis and 374 control cases in 2014, it was found that the rs3104413 in the region between HLA-DRB1 HLA-DQA1 has strong links with rheumatoid arthritis [19]. Cao Nguyen et al. also examined the frequency of high-risk HLA haplotypes in case and control groups. For this, three polymorphisms of the HLA class II loci (rs3104413, rs2854275 and rs9273363) were genotyped in all the samples using custom TaqMan genotyping assay 20x. A study showed that these polymorphisms can predict HLA-DR/DQ haplotypes relevant to T1D with an accuracy (99%) [13, 20]. Three single-nucleotide polymorphisms in the major histocompatibility complex region (rs3104413, rs2854275, rs9273363) were combined to identify carriers of the high- and low-risk HLADR And DQ genotypes known to be associated with autoimmune diabetes (DR3/4, DR3/3, DR4/4, DR3/X, DR4/X, DR4-DQ7, DR4/3-DQ8, DR4-DQ8, DRX/X), where the greatest risk of T1D was found in subjects heterozygous for these types [13]. Since the evaluation of diabetes risk development depends on the type of peoples' HLA and also because the HLA typing method is highly expensive and time consuming, experimenting a limited number of SNPs and designing an algorithm the type of HLA can easily be determined. Furthermore, it has been found that the rs3104413 can be employed in this process. Due to the fact that this polymorphism has a significant association in Iranian population, the findings of Cao Nguyen et al. [13] can be utilized in other forthcoming studies.

Conclusions

This study revealed that there is a significant association between the frequency of alleles and genotypes in the patients compared to healthy subjects. The C/C and C/G genotypes were more frequent in patients than controls and G/G genotype was shown to be protective for T1D. A significant difference was found

for the G allelic frequency in patients with T1D and in the control group. The allelic frequency was significantly different between the two groups. Our findings indicate that HLA polymorphism (C/G) and (C/C) genotypes can be considered as genetic risk factors associated with susceptibility and (G/G) genotypes associated with protection against T1D.

Conflict of Interest

There is no conflict of interest to declare.

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