NeuroD1 Gene and Interleukin-18 Gene Polymorphisms in Type 1 Diabetes in Dalmatian Population of Southern Croatia

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Aim To evaluate the frequency of known polymorphisms in the exon 2 of the NeuroD1 gene and in the interleukin (IL)-18 promoter region in patients with type 1 diabetes mellitus (T1DM) and in healthy control subjects in Dalmatia, Southern Croatia.

Methods A total of 134 unrelated patients (73 men and 61 women) and 132 consecutive unrelated healthy controls (61 men and 71 women) from the Dalmatian region of southern Croatia were recruited for the study. NeuroD1 genotypes (GG, GA, AA) were identified by means of polymerase chain reaction followed by restriction fragment length polymorphism (PCR/RFLP). IL-18 polymorphism in the position –137 of the promoter region was detected by using PCR sequence-specific primers.

Results Genotype distributions of both genes did not show significant difference between patients and controls.

Conclusion Our results suggest that NeuroD1 exon 2 and IL-18 promoter gene polymorphisms are not associated with development of T1DM susceptibility in the population of South Croatia. In addition to previously published positive correlations of these polymorphisms with development of T1DM among different world populations, our findings indicate the existence of ethnic variations in the association of these genes with disease development.

Type 1 or insulin dependent diabetes mellitus (T1DM) is a multifactorial autoimmune disease characterized by destruction of pancreatic β cells (1-3). An autoimmune attack to pancreatic β cells is mediated by autoreactive T cells and apoptosis-inducing cytokines and results in termination of insulin production (4-6). Complex diseases, like T1DM, have a strong genetic component, comprised of multiple gene variants (7). Although major histocompatibility complex (MHC) genotype is an important genetic determinant, several other contributing genes have also been identified (8). Case-control association studies, are a powerful strategy for identifying genes of modest effect in complex diseases (7,9). In this study, we analyzed two candidate genes that could be related with T1DM development: neurogenic differentiation 1 (NeuroD1) gene, which is involved in early differentiation of pancreatic endocrine cells and is also critical for establishing beta-cell-specific and efficient expression of the insulin gene (10-13), and interleukin (IL)-18 gene which is a strong candidate for association with T1DM because of the central role of this inflammatory cytokine in the autoimmunity (14). We performed the study in the population of Dalmatian region in Southern Croatia. Dalmatia is a region situated on the eastern coast of the Adriatic Sea, in Croatia, spreading between the island of Rab in the northwest and the Gulf of Kotor in the southeast. The population of southern Croatia, especially island population, has its own regional genetic differences and therefore provides a unique base for genetic investigations (15,16).

NeuroD1 gene (known as BETA 2 or NeuroD/BETA 2) has been mapped to the long arm of the human chromosome 2 (2q32), where the diabetes susceptibility IDDM7 gene had previously been mapped (2q31-33), implying its possible involvement in diabetes development (17,18). NeuroD1 is a class B basic helix-loop-helix (bHLH) transcription factor and it is expressed in pancreatic endocrine, intestine, and brain cells (10-12). The heterodimer of the NeuroD1 and ubiquitous bHLH (E12/E47) binds insulin E-box complex with a high affinity and also activates the transcription of the insulin gene in pancreatic β cells (12). Naya et al (13) reported that NeuroD1-deficient mice developed severe diabetes and died perinatally because of the striking reduction in the number of insulin-producing β cells. Their results indicated that NeuroD1 gene is required for glucose homeostasis and has an essential role in the morphogenesis or differentiation of pancreatic β cells.

Human NeuroD1 gene comprises two exons. Exon 1 is untranslated, and the protein coding region is located only in exon 2 (19). It is known that there is G→A transition polymorphism in codon 45 of the NeuroD1 gene (rs1801262), leading to Ala/Thr substitution in the NH2-terminus of the protein, although the functional difference between G and A allele is unknown.

There is a possibility that mutation of the human NeuroD1 gene is implicated in the loss of pancreatic β cells, thus allowing T1DM to develop as a consequence of an extensive destruction of pancreatic β cells. In this study, we screened Dalmatian Caucasian patients with T1DM for the presence of the NeuroD1 gene polymorphism and compared them with control subjects.

IL-18 is type 1 cytokine primarily produced by macrophages and closely related to the IL-1 family of cytokines (14). It was shown that IL-18 played a key role in the generation of type 1 cytokine responses by up-regulating the interferon (IFN)-γ production from T cells and natural killer cells through a concerted action with IL-12. An up-regulated production of IL-18 could therefore be an important pathogenic event in the dysregulated production of IFN-γ and other type 1 cytokines thought to predispose immunoinflammatory diseases such as T1DM (14). It was recently reported that IL-18 serum levels are increased in the subclinical stage of T1DM in first-degree relatives of T1DM patients (20).

IL-18 gene locus is located on the chromosome 11q22.2-q23.3 and several polymorphisms in its promoter region have been identified (21). We studied two single-nucleotide polymorphisms of the promoter region of IL-18 gene at the position –137 (rs187238) and –607 (rs1946518). A change at position –137 from G to C changes the H4TF-1 nuclear factor binding site and a change from C to A at position –607 disrupts a potential cAMP-responsive element-binding protein binding site (21). These changes might have an impact on IL-18 gene activity leading to T1DM susceptibility.

Participants and methods

Participants

A total of 134 unrelated Caucasian patients (73 men and 61 women) from Dalmatian region of southern Croatia were recruited for the study. The study included all diabetic patients treated in the Clinical Hospital Split in 2000 and 2001. Type 1 diabetes mellitus was diagnosed according to the World Health Organization criteria (22). The median onset age of T1DM was 98 months (range: 13-201 months) and all subjects were insulin-dependent at the time of the study. Informed consent was obtained from parents of the patients prior to blood sampling. The control group consisted of individuals who came to the Clinical Hospital Split for general health check-ups and had no diabetic data in their personal or family anamnesis. A total of 132 (61 men and 71 women) unrelated control subjects were consecutively selected during the same period and were matched by age and gender to case subjects. The age median was 90 months (range: 12-197 months). All case and control individuals were from the Dalmatian region of southern Croatia and 4% of both groups were inhabitants of Dalmatian islands.

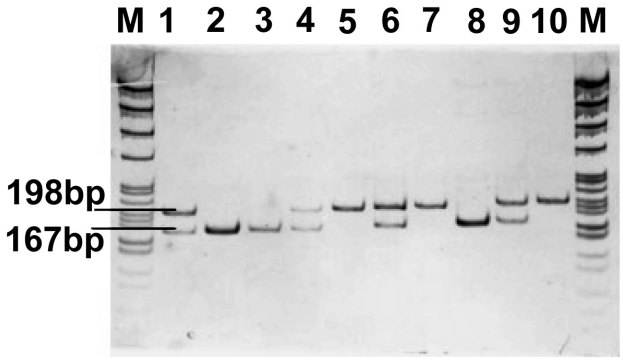

Figure 1. Polyacrylamide gel electrophoresis of PCR fragments after MwoI digestion. Line 2, 3, 8: GG genotype produced two fragments of 167 and 31 bp (the 31-bp band could not be visualized clearly); line 5, 7, 10: AA genotype failed to be cleaved by MwoI; line 1, 4, 6, 9: GA genotype produced three fragments (198 and 167 bp could be visualized); M – size marker.

Gene polimorphism

Genomic DNA was extracted from peripheral blood leucocytes using the Perfect gDNA kit (Eppendorf, Hamburg, Germany).

NeuroD1 genotypes were identified by means of polymerase chain reaction, followed by restriction fragment length polymorphism (PCR/RFLP), according to previous reports (23,24). The PCR product was 198 bp long. G→A polymorphism resulted in a loss of MwoI endonuclease restriction site. Digestion with MwoI (New England BioLabs, Beverly, USA) revealed two fragments, one of 167 and one of 31 bp (the 31-bp band could not be visualized clearly). Samples heterozygous for the respective restriction site were noted as GA (Thr/Ala). Homozygotes GG (Ala/Ala) produced two fragments, while the homozygote AA (Thr/Thr) produced one 198-bp fragment. The digested fragments were separated on 10% polyacrylamide gels and visualized by silver staining (Figure 1). For determination of fragment length DNA marker, pBR322 DNA-MspI Digest (New England BioLabs) was used.

IL-18 polymorphisms were detected by using PCR sequence-specific primers, according to the previous reports (25,26). For the position –137, a common reverse primer (-137 R; 5′ AGGAGGGCAAAATGCACTGG- 3′) and two sequence-specific forward primers (-137 FG; 5′

–CCCCAACTTTTACGGAAGAAAAG-3′ and -137 FC; 5′ –CCCCAACTTTTAC-GGAAGAAAAC- 3′) were used to amplify a 261-bp product. A control forward primer (-137 CTRL; 5′ – CCAATAGGACTGAT-TATTCCGCA- 3′) was used to amplify a 446-bp fragment covering the polymorphic site as an internal positive amplification control. For the position –607, we used another set of sequence specific primers based on literature reports (25,26). Products were visualized by 2% agarose gel electrophoresis and by ethidium bromide staining (Figure 2).

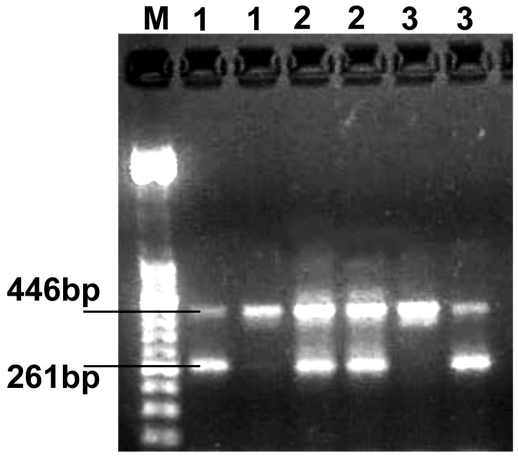

Figure 2. Agarose gel electrophoresis of PCR fragments of IL-18 gene at the position –137. Lines 1, 1: GG genotype; lines 2, 2: GC genotype; lines 3, 3: CC genotype; M – size marker.

Statistical analysis

A comparison between groups was made by χ^2 test, using the Statistica 6.0 (StatSoft. Inc., Tulsa, OK, USA) software package for statistical analysis. A *P*-value of less than 0.05 was considered statistically significant.

Results

The frequencies of the NeuroD1 genotypes, phenotypes, and alleles in the T1DM patients and controls are shown in Table 1. The differences in the genotype frequencies of the G→A polymorphism between cases and controls were not significant (Table 1). The same was true for the subpopulations of men ($\chi^2_1 = 1.64$, $P = 0.440$) and women ($\chi^2_1 = 2.05$, $P = 0.358$), as well as between both genders within the case group ($\chi^2_1 = 0.45$, $P = 0.799$) (Table 1).

Genotype frequencies of IL-18 gene at the position –137 in T1DM patients were similar to those observed in healthy control subjects (Table 2). Genotype frequencies among men ($\chi^2_1 = 1.75$, $P = 0.418$) and women ($\chi^2_1 = 0.87$, $P = 0.648$) of the two examined groups and between genders in the case group ($\chi^2_1 = 2.79$, $P = 0.247$) were also similar (Table 2).

Table 1. NeuroD genotype, allele, and phenotype frequencies in Dalmatian population of southern Croatia in T1DM patients and control subjects

	No. (%) of			
Frequencies	T1DM	controls	χ^2	*P*
Genotype:				
GG (Ala/Ala)	50 (37)	39 (30)		
GA (Ala/Thr)	59 (44)	73 (55)		
AA (Thr/Thr)	25 (19)	20 (15)	3.39	0.184
Phenotype:				
Ala-positive	109	112		
Thr-positive	84	93	0.07	0.788
Minor allele:				
A	0.407	0.428	0.17	0.681

Table 2. IL-18 genotype and minor allele frequencies in Dalmatian population of southern Croatia in T1DM patients and control subjects

	No. (%) of			
Frequencies	T1DM	controls	χ^2	*P*
Genotype:				
GG	67 (50)	67 (51)		
GC	57 (43)	54 (41)		
CC	10 (7)	11 (8)	0.11	0.945
Minor allele:				
C	0.287	0.288	0.0002	0.989

Genotype frequencies for NeuroD1 gene polymorphism and IL-18 gene –137 promoter polymorphism fit the Hardy-Weinberg equilibrium (HWE). However, the genotype frequencies for IL-18 gene –607 promoter polymorphism deviated from the Hardy-Weinberg equilibrium. We performed extensive re-genotyping and obtained the same results.

Discussion

Our study demonstrated that T1DM susceptibility was not associated with NeuroD1 gene and IL-18 gene single nucleotide polymorphisms (SNP) in Dalmatian population of southern Croatia. We found similar distributions of NeuroD1 gene G→A polymorphism and IL-18 gene promoter polymorphism at the position –137 in both T1DM patients and control subjects. Genotype frequencies for SNP at position –607 at IL-18 gene promoter in controls slightly deviated from HWE. Szeszko et al (25,27) reported similar deviations from HWE testing Polish control subjects. They speculated that these deviations occurred because control subjects were sampled from different ethnic groups (27). However, our control group was ethnically homogenous and only 4% of control subjects did not come from the mainland (ie, came from different Dalmatian islands) that would not be sufficient to make significant deviations from HWE. Since we also performed extensive re-genotyping with the same outcome we explain this HWE deviation by the existence of genetic substructures in the population known in literature as "population stratification" (9,28). In order not to have spurious associations, we excluded IL-18 –607 polymorphism from the study. NeuroD1 encodes a transcription factor for the insulin gene and neurogenic differentiation factor (23,24). A recent study on the target disruption of the NeuroD1 gene showed that homozygous NeuroD1-null mice developed severe diabetes and died 3-5 days *postpartum* (13). In the histological examination of these mice, pancreatic islets failed to develop morphologically and the number of specific islet populations, especially β cells, was markedly decreased at the embryonic stage, suggesting NeuroD1 to be essential for the morphogenesis or differentiation of insulin-producing pancreatic β cells. In addition, this study also indicated that apoptosis of the pancreatic β cells was very likely to occur in the NeuroD1 knockout mice. These data raise the possibility that NeuroD1 variant plays a role in the development of apoptosis of pancreatic β cells (13). There is also a possibility that an altered form of NeuroD1 leads to the production of fewer islets in development, or that NeuroD1 may affect the regeneration or differentiation of pancreatic β-cells, thereby leading to a difference in the onset pattern and clinical course of T1DM (13). Some authors reported that deficient binding of NeuroD1 or binding of a transcriptionally inactive NeuroD1 polypeptide to target promoters in pancreatic islets leads to a development of type 2 diabetes and maturity-onset diabetes of the young (MODY) (29,30).

The result of NeuroD1 gene polymorphism and its susceptibility to T1DM in different world populations are controversial. Studies of American, French, or UK population showed no association between NeuroD1 gene polymorphism and T1DM (31-33). Nevertheless, Cinek et al (34) confirmed that the NeuroD1 Ala45Thr polymorphism is associated with childhood-onset T1DM in Czech children. Several other studies of Japanese population found significant association between NeuroD1 gene and T1DM (23,24,35). Malecki et al (36) in their case-control study also suggested that the Ala45Thr polymorphism of BETA2/NeuroD1 may have played a minor role in the risk of T1DM in residents of Massachusetts, USA. Kavvoura and Ioannidis (37) performed meta-analysis of 8 different populations of European and Asian descent and showed that Ala45Thr polymorphism of the NeuroD1 gene had no effect on susceptibility to T1DM. It may, however, be a risk factor for susceptibility to T1DM, in particular for subjects of Asian descent, although bias cannot be totally excluded. Data for NeuroD1 gene in different populations with the susceptibility to T1DM are inconsistent and probably reflect the overall differences in genetic background. According to Mori at al (38), populations of African descent, Caucasians, and Japanese have different population histories of mutation, migration, isolation,

and genetic drift that can result in substantial differences in allele frequencies among these ethnic groups. This may explain data that showed that NeuroD1 gene polymorphism was associated with development of T1DM in Japanese population, but not in other world populations (23,24,31-33,35,36).

IL-18 is a pleiotropic proinflammatory cytokine that plays a pivotal part in the generation of Th1 cytokine responses through its ability to up-regulate IFN-γ production and IL-12-driven Th1 phenotype polarization (14,39). It has been reported that the serum IL-18 level was elevated in subclinical stage of T1DM in anti-islet autoantibody-positive relatives of patients with T1DM, indicating that IL-18 could play a part in the pathogenesis of T1DM (20). The involvement of IL-18 gene in the pathogenesis of T1DM is also supported by in vivo studies in the NOD mouse model of T1DM, showing that intrainsular expression of IL-18 mRNA coincides with, and perhaps promotes, the onset of destructive insulitis (14).

Our results demonstrated that the development of T1DM was not associated with SNP polymorphisms at position –137 in IL-18 gene promoter in the Dalmatian population of southern Croatia. Kretowski et al (25) recently reported that SNP polymorphisms at position –607 and –137 are associated with child T1DM in Polish population. The frequency of –137C allele was increased when compared to controls and the distribution of the –607 and –137 genotypes was different from controls. Frequencies of haplotypes were also different in patients and controls. Similar study of IL-18 gene promoter SNP polymorphisms was performed in Japanese population by Ide et al (26). It showed that distribution of –607 genotypes in T1DM patients differed from controls. However, Novota et al (40) found no association of the polymorphisms at position –607 and –137 in IL-18 gene promoter with disease between adult patients with T1DM and latent autoimmune diabetes in adults. Recently, Szeszko et al (27) concluded that common allelic variation in IL-18 is unlikely to contribute substantially to T1DM susceptibility in a UK population. According to all this data, it is obvious that the distribution of IL-18 gene alleles might vary among different ethnic groups.

It is reported that allele frequencies of autoimmune disease-associated SNPs varied substantially in different ethnic groups and the information on these differences is important for a better understanding of the pathologic mechanisms of polymorphisms (38,41). The European Caucasians are generally regarded as having a relatively homogeneous gene pool, but in fact there is a tremendous variation in genes across Europe and fairly close populations have been shown to exhibit substantial allele frequency variations (28). As we mentioned before, population of southern Croatia and especially island population, has its own genetic specificities. Different distribution of some chromosomal allele frequencies on Eastern Adriatic islands represent reproductive isolates of relatively small size, where genetic drift and founder effect have particularly significant role in shaping the genetic diversity (15,16). These are strong reasons for performing association studies for candidate genes in the population of southern Croatia. Our results of NeuroD and IL-18 genes in Dalmatian population contribute to general understanding of ethnic variations of common disease-susceptible variants and also provide additional knowledge of the investigated genes associated with T1DM.

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