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Original Article

Polymorphisms associated with type 1 diabetes mellitus

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Abstract

Background Type 1 diabetes mellitus (T1DM) is an organ-specific autoimmune disease characterized by T cell-mediated destruction of pancreatic islets. The genetic factors involved consist of at least five vulnerability genes: HLA, INS, CTLA-4, PTPN22, and IL2RA/CD25.

Objective To investigate for associations of PTPN22-1123 G>C SNP and CTLA-4 +49A/G polymorphisms with T1DM.

Methods Case and control groups underwent CTLA-4 +49A/G gene examination from June to December 2017, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.

Results The study population consisted of 30 T1DM patients and 30 healthy subjects with no family history of diabetes or autoimmune diseases. With regards to the PTPN22-1123 G>C SNP, significantly more subjects with T1DM had the GC genotype than the GG genotype (OR 7.64; 95%CI 1.48 to 39.29; P=0.007). For the CTLA-4 +49A/G polymorphism, although the total number of G alleles in the case group was more than that of the control group (OR 2.286; 95%CI 0.804 to 6.945; P=0.118), there were no significant relationships between the frequency of G alleles (P=0.248) and genotypes GG or AG (P=0.293) with the incidence of T1DM. However, the PTPN22-1123 G>C SNP had a significantly positive association with T1DM, and may be considered as a risk factor for T1DM. In contrast, the CTLA-4 +49A/G polymorphism was not recognized as a risk susceptibility factor for T1DM.

Conclusion These study confirms an association between PTPN22-1123 G>C SNP and T1DM, but no significant association between CTLA-4 +49A/G polymorphism and T1DM. **[Paediatr Indones. 2018;58:274-9; doi: http://dx.doi.org/10.14238/pi58.6.2018.274-9]**.

Keywords*: T1DM; PTPN22-1123 G>C; CTLA-4+49A/G; PCR-RFLP; polymorphism*

Type 1 diabetes mellitus is an autoimmune disease characterized by the infiltration of pancreatic beta cells by immune cells.[1] Beta cells act as regulators of blood sugar levels in the body by producing and secreting insulin when blood sugar levels rise, and maintaining blood sugar levels within physiological limits.[1] Damage may be caused by autoimmune or idiopathic processes. In T1DM patients, insulin secretion is reduced or stalled.[2] In a state without insulin, blood glucose levels rise to higher than normal. The genetic factors involved consist of several vulnerability genes, at least five of which are as follows: HLA, INS, CTLA-4, PTPN22, and IL2RA / CD25.[3]

Bottini *et al.* discovered that tyrosine phosphatase was associated with susceptibility to T1DM, as it is a lymphoid protein encoded by the PTPN22 gene on chromosome 1p13. The PTPN22 gene contributes to susceptibility to T1DM by increasing T-cell regulatory activation.[3] In 2008, Ikegami *et al.*

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sequenced the PTPN22 gene from Japanese and Korean subjects and found five new SNPs in the sample. One of these, the -1123 G>C SNP in the promoter region, was associated with type 1 diabetes in both Japanese and Korean populations.[4] Since genetic markers in disequilibrium are closely related to etiology of disease, polymorphisms can be used to detect relationships. Conventionally, previous studies had shown that certain gene candidates were considered to cause the disease.[4] This approach was used to evaluate the relationship between T1DM and lymphoid-specific phosphatase (LYP) encoded by PTPN22. The LYP belongs to the family of tyrosine phosphatase proteins (PTP) that are involved in preventing spontaneous T-cell activation by dephosphorylating and deactivating T-cells of related receptor kinases and their substrates. The PTPN22 is specifically expressed in lymphocytes and associated with the SH3 domain of the CSK kinase, which suppresses the mediator signaling of T-cell receptors, i.e., Src family kinases (Lck and Fyn). The LYP is one of the most powerful inhibitors of T-cell activation. Recently, missense mutations in the PTPN22 gene were found to be associated with several autoimmune diseases including T1DM, rheumatoid arthritis, and systemic lupus erythematosus. Mutations in this gene were shown to reduce the binding affinity of LYP to CSK, indicating functional relevance for T-cell activation.[3]

There are four polymorphisms in the CTLA-4 gene, one of which is a single nucleotide polymorphism (SNP) in exon 1 (+49 A/G) encoding the co-stimulatory molecules expressed on the surface of activated T-cells.[5,6] The CTLA-4 and CD28 (also located at 2q33) are part of the immunoglobulin superfamily and bind to the B7 molecule on antigen-presenting cells.[6,7] The CTLA-4 has a greater affinity for B7 molecules than CD28, and it downregulates T-cell function. Therefore, CLTA-4 is highly likely to play an important role in T-cell-mediated autoimmunity and susceptibility to autoimmune disease, including T1DM. Several studies confirmed the association between CTLA-4 + 49 A/G and T1DM, in populations in Spain, France, Korea, Italy, America-Mexico, Belgium, Japan, and Iran.[7,8] However, there was a negative correlation in the population of Turkey, Chile, China, England, Egypt, Portugal, and Brazil.[7,8] We aimed to investigate for associations of the PTPN22-1123 G>C SNP and CTLA-4 +49A/G polymorphisms with T1DM.

Methods

This case-control study was done in a South Sumatera population. The case group had 29 children and 1 adult (16 males, 14 females) with T1DM and the control group had 30 healthy subjects (16 males, 14 females) with no family history of T1DM or other autoimmune diseases. Patients were followed up at the Pediatric Endocrinology Outpatient Unit, at Sriwijaya University School of Medicine, Mohammad Hoesin Hospital. All T1DM patients were diagnosed before the age of 15 years and were insulin dependent. Parameters included in the statistical analysis were gender, age at the time they entered the study, severity at onset (presence or absence of ketoacidosis), and family history of T1DM in at least one first-degree relative. The control group consisted of children aged 10-11 years and in the 5th grade of elementary school in South Sumatera. Informed consent was obtained from adult participants and parents or guardians of subjects under 18 years. The study protocol was approved by the Ethics Committee of the Universitas Sriwijaya Medical School.

The PCR-RFLP method involved four stages: (a) genomic isolation of deoxyribonucleic acid (DNA), (b) polymerase chain reaction (PCR), (c) incubation of PCR products with restriction enzymes, and (d) electrophoresis. Three-mL blood specimens were collected from all subjects in EDTA-anticoagulant tubes. PCR was used to identify PTPN22 and CTLA-4 genes inT1DM. PCR reaction specimens contained the following: 0.25 μg genomic DNA specimen in a 25μl PCR reaction containing 50 μM of each dNTP (Boehringer, Germany), 2U of Taq DNA polymerase, 2.5 μL of 10×PCR buffer, and 0.8 μM of each primer. The reaction mixture was first heated at 94°C for 4 min, then amplification was done for 33 cycles in a PCR thermocycler by denaturation at 94°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 45 sec per cycle. All PCR products were screened and the PTPN22 gene was found in all samples. After PCR, restriction fragment length polymorphism (RFLP) with the Rsa1 enzyme was done. This enzyme serves as an analyzer tool with direct sequencing of

PCR-RFLP for PTPN22. Amplification of the 162 bp genomic region of PTPN-22 genes was performed with forward (5'-CTGATGGTTCCCCACTGTCT-3') and reverse (5'-CTCCACCCCTAAGCACAAAG-3'). Amplification of the CTLA-4 genes was performed with forward (5'-GCTCTACCTCTTGAAGACCT-3'), reverse (5'AGTCTCACTCACCTTTGCAG- 3') primers. The RFLP analysis of CTLA-4 was done using FastDigest BbvI (Fermentas, Germany) in 30 μL total volume by mixing 10 μL of PCR products, 1.0 μL of BbvI restriction enzyme, 2.0 μL 10 X FastDigest green buffer, and 17 μL nuclease-free water. The mixture was incubated at 37 °C for 10 min followed by heating at 65°C for 10 min. DNA fragments were resolved in 2.0% agarose gels. The A allele does not create a restriction site (162 bp), while the G allele creates A restriction site producing two fragments, 88 bp and 74 bp.

The PCR-RFLP analysis of the PTPN22 gene was done using genomic DNA obtained from peripheral blood leukocytes and sequence-specific primers, followed by PCR product digestion with the Rsa1 restriction enzyme. The PCR amplicon was 323 bp. After digestion by Rsa1, we would expect that the wild type allele G (homozygous) would remain undigested (323bp). However, the 323 bp PCR products of heterozygous G and C alleles would be digested by RsaI, yielding fragments of 287 bp and 36 bp (**Figure 1**). In addition, the homozygous C alleles would also yield fragments of the same size as the heterozygous G and C alleles.

The PCR-RFLP analysis of the CTLA-4 gene was done using genomic DNA extracted from peripheral blood of individuals using an MBI Fermentas DNA isolation kit. After PCR amplification, the products (162bp) were digested with the BbvI restriction enzyme. After digestion by BbvI, we would expect that the wild type allele A (homozygous) would remain undigested (162bp). However, the 162bp PCR products of the heterozygous A and G alleles would be digested by BbvI, yielding fragments of 88bp and 74 bp (**Figure 2**). In addition, the homozygous G allele would also yield fragments of the same size as the heterozygous A and G alleles.

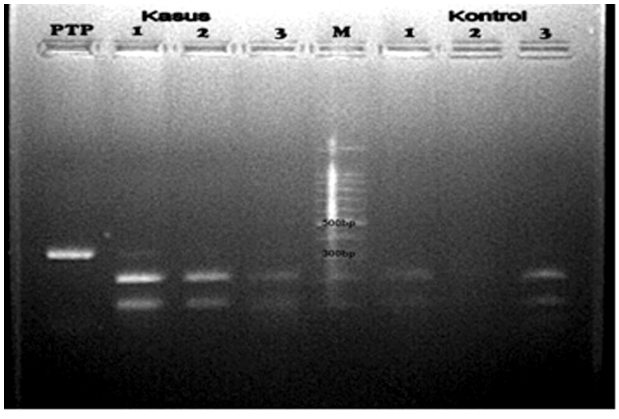

Figure 1. Agarose gel electropherogram of PCR amplicons of the PTPN22 G>C genotype (323bp)

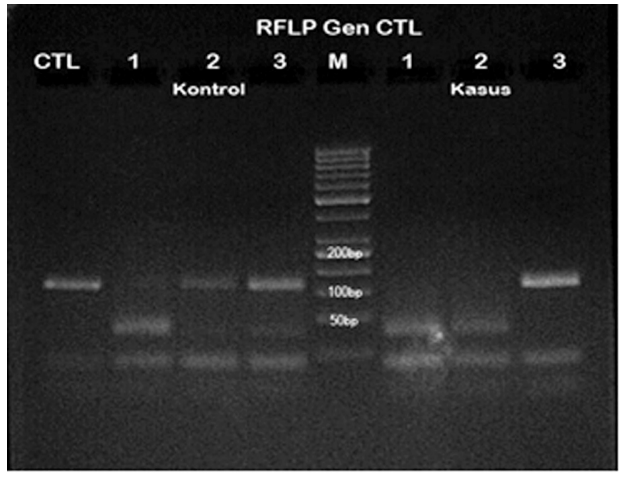

Figure 2. Agarose gel electropherogram of CTLA-4-+49A/G gene PCR amplicon

Data were analyzed using *IBM SPSS Statistics 18* software. The differences in frequencies of genotypes and alleles between case and control groups and among patient subgroups based on qualitative variables, were analyzed by Chi-square or Fisher's exact tests. Normal distribution of quantitative variables was examined using the Kolmogorov-Smirnov test. The level of statistical significance was defined to be $P < 0.05$.

Results

The characteristics of subjects are shown in **Table 1**. Most subjects were male in both groups (16 subjects;). The median age of subjects was 10.5 years, ranging from 3to 29 years. T1DM subjects' nutritional status was mostly well nourished (23 subjects, 77%), while 7/30 subjects were malnourished.

The most common age grouping of T1DM subjects was 6-11 years (15 subjects). All control group

Table 1. Characteristics of subjects

Characteristics	Case (n=30)
Age by group, n	
0-5 years	3
6-11 years	14
12-16 years	8
17-25 years	4
26-35 years	1
Sex, n	
Male	16
Female	14
Nutritional state, n	
Well-nourished	23
Malnourished	7
Race, n	
Austronesia	30
Tribe, n	
Palembang	14
Jawa	1
Minang	1
Komring	1
OKI/OKU/Ogan	4
OI/Kayu Agung	2
Lahat/Gumai	1
Muaraenim/Semendo	2
Banyuasin	2
Sekayu	1
Rawas	1

subjects were aged 6-11 years. All subjects were of the Austronesian race.

One specimen was undetermined, so the total number of specimens was only 59 samples, 30 from the case group and 29 from the control group. With regards to the PTPN22 gene, 43 subjects had a GC genotype. T1DM subjects had significantly more GC genotype and fewer GG genotype (OR 7.647; 95%CI 1.488 to 39.290) than the control group (**Table 2**). Also, the CC genotype was not statistically significant as a risk factor for T1DM (P=0.245). The G allele was significantly more common in the case group than the control group, but presence of the G allele was not a risk factor for T1DM (OR 0.131; 95%CI 0.025 to 0.672; P=0.007). The C allele did not significantly differ between the case and control groups and was also not a risk factor for T1DM. The GG genotype was a significant protective factor against T1DM (OR=0.136; 95%CI 0.27 to 0.690; P=0.02). However, the GC genotype was a significant (P<0.05) risk factor for T1DM (OR=4.53; 95%CI 1.26 to 16.60; P=0.03). The frequency of the allele PTPN22-1123 G>C SNP

Table 2. Correlations between PTPN22 genotypes and alleles with T1DM

Variables	Control (n=29)	Case (n=30)	OR (95%CI)	P value
Genotype, n			7.647	0.007*
GG	10	2	(1.488 to 39.290)¥	
GC	17	26		
CC	2	2		
Allele, n				
G	74	60	0.131 (0.025 to 0.672)	0.007*
C	42	60	0.654 (0.084 to 5.095)£	0.536

* = significant with P<0.05; ¥=OR between groups of GG and GC genotypes; £=Fisher's exact test

was significantly higher in the case group compared to the control group (OR 7.647; 95%CI 1.488 to 39.290; P=0.003).

In the case group, 3/30 subjects had the AA genotype, 11/30 had the AG genotype, and 16/30 had the GG genotype. In the control group, 4/29 subjects had the AA genotype, 16 /29 children had AG, and 10/29 had GG. There was no significant difference in genotype distribution between the case and control groups (P=0.293) as shown in **Table 3**. The A allele was present in 17/30 cases and 24/29 control subjects. The G allele was present in 43 cases (54.4%) and 36 (45.6%) controls. The distribution of A and G alleles was not significantly different among groups (OR 1.68, 95%CI 3.61 to 3.68; P=0.248). For the CTLA-4 +49A/G polymorphism, although the total number of G alleles in the case group was more than that of the control group (OR 2.286; 95%CI 0.804 to 6.945; P=0.118), there were no significant relationships between the frequency of G alleles (P=0.248) and genotypes GG or AG (P=0.293) with the incidence of T1DM.

Table 3. Correlations between CTLA-4 genotypes and alleles with T1DM

Variables	Case (n=30)	Control (n=30)	P value
Genotype, n			
AA	3	4	0.293*
AG	11	16	
GG	16	10	
Allele, n			
A	17	24	0.248
G	43	36	

* = significant with P<0.05

Discussion

Previous studies reported similar T1DM incidences in boys and girls: 2.1 per 100,000 boys and 1.9 per 100,000 girls.[1,3] In our study, the sex distribution was similar in both groups (P= 0.796).

In our study, all T1DM cases were diagnosed at <11 years of age, but the previous study had a mean age <15 years for age at the time of T1DM diagnosis.[9] A limitation of our study was that we could not be sure that the controls did not have T1DM or other autoimmune diseases.

We found a significant relationship between the PTPN22 -1123 G>C gene polymorphism and T1DM. The TIDM cases had a 7.6 times higher chance of having the PTPN22 -1123 G> C polymorphism (P=0.007). The first report of a relationship between the PTPN22 -1123 G> C SNP and T1DM was by Kawasaki et al. in 2006. No other studies have shown that the PTPN22 -1123 G>C SNP could contribute to the tendency of having T1DM, especially in the Asian population. Nonetheless, the researchers concluded that further study was still needed to support this finding.[4]

With regards to CTLA-4, there was no difference in genotype distribution between the two groups (P=0.293). This result was consistent with an Egyptian study which reported no association between the CTLA-4 +49A/G polymorphism and the incidence of T1DM.[10] Similarly, a cohort study in several families in Egypt also found no association between CTLA-4 +49 A/G polymorphism genes and the incidence of T1DM.[11-13] A previous study concluded that the CTLA-4 +49 A/G gene polymorphism was selective for Caucasian race.[13] Studies in Korea, Turkey, Portugal, Chile, and Azerbaijan also found no evidence of a relationship.[7,12] In addition, we found no association of the G allele and genotypes GG and AG with the incidence of T1DM (P=0.248). In contrast, various Middle East studies (Lebanon, Tunisia and Iran) and in Japan showed the relationship between high frequency G allele or GG genotype with the incidence of T1DM.[14,15] Polymorphisms in the CTLA-4 gene have the potential to generate abnormal signals in T helper cells, which may impact the formation of antibodies (by plasma cells) to host cells, including pancreatic β cells, resulting in T1DM.

In conclusion, we confirm the association of PTPN22-1123 G>C SNP with T1DM, but find no significant association between the CTLA-4 +49A/G polymorphism and T1DM. More studies are needed with a larger study population to further confirm these findings.

Conflict of Interest

None declared.

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