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Polymorphisms rs1800795 of interleukin-6 and rs2228145 of interleukin-6 receptor genes in Euro-Brazilians with adult-onset type 1 diabetes mellitus

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Genet. Mol. Res. 18 (3): gmr18260
Received February 02, 2019
Accepted July 22, 2019
Published July 26, 2019
DOI http://dx.doi.org/10.4238/gmr18260

ABSTRACT. Type 1 *Diabetes mellitus* (T1D) is caused by the immune-mediated destruction of insulin-producing islet β cells, and its pathogenesis involves cytokines. Genetic background may influence cytokine signals, and polymorphisms may determine their impact on T1D autoimmunity. Several polymorphisms in and close to Interleukin-6 (IL-6) and Interleukin-6 Receptor (IL-6R) have been identified as associated with pathological processes. We investigated the IL-6 promoter -174G>C (rs1800795) and IL-6R Asp358Ala (rs2228145) polymorphisms in 141 Euro-Brazilian patients with adult-onset type 1 diabetes (diagnosis > 18 years old) and 150 healthy controls, matched by gender and age. Genotyping for both polymorphisms was performed by PCR-RFLP. PCR fragments for rs1800795 with *Hsp92*II and rs1800795 with *Hind*III were resolved by 15% polyacrylamide gel electrophoresis. The polymorphisms in both groups were in Hardy-Weinberg equilibrium. IL-6 rs1800795 was not different between healthy controls and T1D subjects, showing 27.3% (95% CI, 20 - 35%) and 30.1% (23 - 38%) for the C minor allele (-174C), respectively. For IL-6R rs2228145, the

genotype (P = 0.046) and allele (P = 0.021) were different in the groups. The frequencies for rs2228145 C minor allele (358Ala) were 34.7% (27 - 43%) and 44.0% (36 - 51%) for controls and T1D subjects, respectively. In conclusion, IL-6 rs1800795 was not associated with adult-onset T1D; however, IL-6R rs2228145 was associated with T1D development in adulthood, and carriers of the minor C allele are at increased risk for adult-onset T1D (OR = 1.48; 95% CI = 1.06 - 2.07).

Key words: Promoter polymorphism; -174G>C; Adult-onset T1D; IL6R; Asp358Ala; Genetic susceptibility

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune-mediated disease caused by death of pancreatic β cells (Eisenbarth, 1986; Atkinson, 2014; Roep et al., 2019), and its incidence has increased in recent years (You and Henneberg, 2016). Genome-wide association studies (GWAS) have identified more than 60 susceptibility regions to T1D across the human genome, which are marked by single nucleotide polymorphisms (SNPs) (Ram and Morahan, 2017). Several non-HLA type 1 diabetes susceptibility genes have been demonstrated to influence disease progression, and it has been suggested that their contribution may be partly associated with the β-cell, in which several gene products have demonstrated expression in cytokine-stimulated islets (Eizirik et al., 2012).

Cytokine signals may be influenced by genetic background, and polymorphisms may determine their impact in T1D autoimmunity (Lee et al., 2015). Proinflammatory cytokines, such as TNF-α, IL-1β, IFN-γ, IL-1α, IL-6, and IL-18, have been implicated in the pathogenesis of T1D (Grunnet et al., 2009). Therefore, interleukins and interleukin receptor genes are among the genetic risk factors of T1D (Ram and Morahan, 2017).

Interleukin-6 (IL-6, Chr7, 7p15.3; OMIM 147620) plays a critical role in the immune response (Feigerlova and Battaglia-Hsu, 2017). It binds to an IL-6 membrane receptor (IL-6R, Chr1, 1q21.3; OMIM 147880) or soluble receptor (sIL-6R), and the complex IL-6–IL-6R or IL-6–sIL-6R couples with gp130 protein to trigger intracellular signaling (Mihara et al., 2012). The soluble sIL-6R is generated by shedding the membrane-bound form or by proteolytic cleavage of the ectodomain (Mullberg et al., 1994; Horiuchi et al., 1998; Hurst et al., 2001), which triggers trans-signaling (Rose-John, 2012). There is evidence that IL-6 trans-signaling possesses a prevalent pro-inflammatory role, whereas classic IL-6 signaling via the membrane bound IL-6R is needed for regenerative or anti-inflammatory processes (Rose-John, 2012; Hodes et al., 2016).

Dysregulation of the IL-6/IL-6R system has been associated with the pathogenesis of several autoimmune and inflammatory diseases in humans (Tanaka and Kishimoto, 2012; Tanaka et al., 2014). In T1D, IL-6 signaling is a complex process. No difference between T1D and healthy subjects was identified; however, a putative post-translational regulation was suggested (Feigerlova and Battaglia-Hsu, 2017).

Several polymorphisms in and close to IL6 and to IL6R genes have been identified. There are studies investigating the genetic association between IL6 and IL-6R

polymorphisms and type 1 diabetes, type 2 diabetes, insulin resistance, and other features of the metabolic syndrome (Fishman et al., 1998; Wang et al., 2005).

The IL-6 promoter -174G>C (rs1800795) polymorphism has been suggested to functionally affect IL6 promoter activity (Fishman et al., 1998). The rs1800795 G allele in homozygous (GG genotype) was associated with higher concentrations of IL-6 increasing the immune response (Larcombe et al., 2008; Nadeem et al., 2017).

IL-6R rs2228145 is located in exon 9, a putative extracellular cleavage, domain and the A>C polymorphism promotes a non-synonymous change of aspartic acid to alanine in the position 358 (Asp358Ala; D358A) (Galicia et al., 2004). In vivo studies have shown higher concentrations of the IL-6R soluble form (dIL-6R) in carriers of the C allele (350Ala). However, polymorphism effects in diabetes are controversial (Ferreira et al., 2013).

Type 1 diabetes manifests predominantly at a young age (childhood onset) and less frequently in adults (adult onset). Both forms of T1D have shown differences based on genetic influences (Howson et al., 2012).

We examined the association between adult-onset T1D and the IL-6 promoter rs1800795 and IL-6R rs2228145 in a Euro-Brazilian population.

MATERIAL AND METHODS

Ethical considerations

The study was approved by the Ethics Committee of the Federal University of Paraná (CAAE 01038112.0.0000.0102). All subjects participating in the research provided written consent after being properly informed of the potential benefits and potential harm associated with the study.

Subjects

This cross-sectional study was conducted with Euro-Brazilian adults who attended the Clinical Hospital of the Federal University of Parana, Curitiba, Parana State, in Southern Brazil. Briefly, 291 unrelated adults were classified as healthy (control, n = 150) or as adult-onset T1D diagnosed at age ≥18 years (T1D, n = 141) according to international guidelines (American Diabetes Association, 2019). Clinical and anthropometric data were obtained from all patients. The control group was obtained from blood bank donors. The groups were matched by sex and age.

Genotyping

DNA was extracted from peripheral blood leukocytes (buffy coat) by the “salting out” method of Lahiri and Nurnberger (1991). DNA samples with an A260/A280 absorbance ratio between 1.6 and 1.9 were used, and the concentrations of all samples were normalized to 100 ng/μL for polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

The rs1800795 *IL*-6 and rs2228145 *IL6*-R were genotyped by PCR-RFLP. PCR was performed using the following primers: rs1800795F-5'-TTCCCCCTAGTTGTGTCTTGC-

3', rs1800795 R-5'-TGGGGCTGATTGGAAACCT-3'; and rs2228145 F-5′-CCTCTTCCTCCTCTATCTTCAATTTT-3′, R-5'- AATGTGGGCAGTGGTACTGAA-3' as described by Ruzzo et al. (2014). The amplification protocol for genomic DNA (final volume 20 µL) contained 100 ng genomic DNA, 10 pmol each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.0 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), and 1× reaction buffer (Invitrogen).

The PCR amplification protocol, for both polymorphisms, was started with an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 30°C for 30 s, and extension at 72°C for 30 s, with a final step at 72°C for 5 min. The rs1800795 IL-6 and rs2228145 IL6-R PCR products were digested with *Hsp92* II and *Hind* III (Promega, Madison, WI, USA), respectively. Restriction fragments were resolved by 15% polyacrylamide gel (29 : 1) electrophoresis, stained with ethidium bromide (0.5 µg/mL) and photo-documented with L-PIX (Loccus Biotechnology, São Paulo, Brazil) using UV light (302 nm). The expected sizes of the fragments, as described by Ruzzo et al. (2014), for rs1800795 IL-6 were genotypes GG (75 bp), GC (75 bp + 50 bp + 25 bp), and CC (50 bp + 25 bp), and rs2228145 IL6-R were genotypes AA (73 bp), AC (73 bp + 43 bp + 30 bp), and CC (43 bp + 30 bp). PCR-RFLP showed clear genotype discrimination in all samples.

Biochemical markers

The biochemical parameters were measured using the Architect Ci 8200 automated system (Abbott Diagnostics, Lake Forest, IL, USA) or Labmax 400 (Labtest, Labtest, Inc., Delta, British Columbia, Canada) with reagents, calibrators, and controls from the equipment manufacturer. Glycated hemoglobin (HbA1c) was measured using immunoturbidimetry, and 1,5-anhydroglucitol was measured enzymatically (GlycoMark, Tomen America, New York, NY, USA) in an automated system (Labmax 400).

Statistical analysis

Continuous variables with normal distribution, verified by the Kolmogorov-Smirnov test, were compared by the two-tailed Student's *t* test for independent variables. The Mann-Whitney U test was used to compare groups without normal distribution. The chi-square test was used to compare categorical variables. Hardy-Weinberg equilibrium and allele comparisons were calculated using the DeFinetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Further, 95% confidence intervals (95% CI), odds ratio (OR), and all statistical analysis were calculated with the MedCalc Statistical Software version 18.11.3 (MedCalc Software bvba, Ostend, Belgium). A P-value of less than 5% ($P < 0.05$) was considered significant.

RESULTS

Clinical data of the study group are shown in Table 1. The groups were matched by age and gender. The age at diagnosis in the T1D group was between 18 and 38 years (adult onset T1D). T1D subjects were determined to be leaner than control subjects, as assessed by

body mass index. The average time of diabetes was 14 years, and 69.2% had a family history of diabetes.

As expected, the glycemia concentration in the T1D group was significantly higher ($P < 0.001$) when compared to the healthy controls, which shows consistency in the sample classification. Furthermore, glycemic biomarkers were statistically different between groups (Table 2). From the results obtained, we observed inadequate glycemic control in the T1D group (poor glycemic control criteria: fasting glycemia >7.2 mmol/L, HbA1c >7%, and 1,5-AG <61 µmol/L) (Van Leeuwen and Bladh, 2015; SBD, 2017; ADA, 2019).

Table 1. Anthropometric and clinical parameters for adult-onset Type 1 diabetes (T1D) patients and healthy controls.

Parameters	Control (n = 150)	T1D (n = 141)	P
Age, years	44 (40–49)	45 (34–52)	0.602*
Male/Female, n	53/97	48/93	0.817**
Weight (kg)	74 (64–83)	68 (60–82)	0.016*
Height (cm)	166.2 ± 8.9	163.3 ± 9.8	0.009
BMI (kg/m^2)	27.0 ± 4.2	25.8 ± 4.3	0.018
Duration of diabetes, years	NA	14.8 ± 10.8	-
Family history of diabetes %	NA	69.2%	-

Control, healthy subjects; BMI, body mass index. Values are mean ± SD or median (interquartile range). NA, Not Applicable. P-value, Independent Student *t* test (two-tailed), *Mann-Whitney U test, or ** chi-square test. P values in bold are significant ($P < 0.05$).

Serum concentrations of albumin and total proteins showed no evidence of malnutrition or protein loss. The highly significant concentrations of both markers in the T1D group could be associated with minor dehydration associated with polyuria in T1D subjects.

The concentrations of kidney function biomarkers, creatinine and urea, were in the reference range, suggesting no renal damage in the T1D group (Ceriotti et al., 2008). The highly significant concentration of these biomarkers in T1D compared with healthy subjects could be associated with hydrodynamic changes, as expected in this pathology.

Table 2. Concentrations of biochemical parameters in adult-onset type 1 diabetics (T1D) and healthy controls.

Parameters	Control (n = 150)	T1D (n = 141)	P
Glycemia (mmol/L)	5.3 (4.6 - 5.8)	9.5 (5.8 - 13.8)	< 0.001
HbA1c (%)	5.4 (5.2 - 5.6)	8.8 (7.6 - 9.7)	< 0.001
HbA1c (mmol/mol)	36 (33 - 38)	73 (60 - 83)	
1,5-AG (µmol/L)	108 (102 - 114)	4.1 (2.3 - 5.9)	< 0.001
Total protein (g/L)	69 (66 - 72)	82 (74 - 88)	< 0.001
Albumin (g/L)	39 (38 - 40)	41 (38 - 43)	< 0.001
Creatinine (µmol/L)	49.5 (42.4 - 59.2)	72.4 (70.7 - 88.4)	< 0.001
Urea (mmol/L)	3.8 (3.3 - 4.4)	5.3 (4.1 - 6.4)	< 0.001

Controls, healthy subjects. Values are medians (interquartile range, 25th - 75th). ND, no information available. P-values are Mann-Whitney U test. P-values in bold are significant ($P < 0.05$).

Genotypes and allele frequencies of the polymorphisms were in Hardy-Weinberg equilibrium ($P < 0.05$; Table 3). The genotypic and allelic frequencies of the rs1800795

polymorphism did not differ between the study groups. The rs1800795 polymorphism of the *IL-6R* gene showed an association with T1D, with significant differences between the genotypic and allelic frequencies between the study groups and in the dominant model. The minor frequency C allele was associated with a higher risk for T1D, with an odds ratio of 1.48 (95% CI 1.06 - 2.07) calculated for the allele frequency.

Table 3. Genotypic and allelic frequencies of *IL-6* rs1800795 and *IL-6R* rs8182284 in adult-onset type 1 diabetics (T1D) and healthy controls.

Polymorphism		**Control (n = 150)**	**T1D (n = 141)**	**P**
(rs1800795)	G/G	75 (50.0)	63 (44.7)	0.599
G>C	G/C	68 (45.3)	69 (48.9)	
IL-6	C/C	7 (4.7)	9 (6.4)	
MAF	C	27.3	30.1	0.350
[95% IC]		[20 - 35]	[23 - 38]	
Dominant	GC+CC vs GG	75/75	78/63	0.749
Recessive	CC vs GC+GG	7/142	9/132	0.521
(rs2228145)	A/A	59 (39.3)	39 (27.7)	0.046
A>C	A/C	78 (52.0)	80 (56.7)	
IL-6R	C/C	13 (8.7)	22 (15.6)	
MAF	C	34.7	44.0	0.021
[95% CI]		[27 - 43]	[36 - 51]	
Dominant	AC+CC vs AA	91/59	102/39	0.035
Recessive	CC vs AA+AC	13/137	22/119	0.069

Controls, healthy subjects. Values were n (%); MAF, minor allele frequencies; 95% CI, 95% confidence interval; *P*, probability, chi-square test. Hardy-Weinberg equilibrium (P-value) in healthy controls and T1D for rs1800795 (0.084; 0.081) and rs2228145 (0.070; 0.072). Dominant model (others vs. prevalent homozygous genotype) and recessive model (rare homozygous genotype vs. others). Odds ratios (95% Confidence Interval) for rs2228145 C allele frequencies and dominant model (AA genotype) were 1.48 (1.06 - 2.07) and 0.59 (0.36 - 0.97), respectively.

We associated the polymorphic genotypes with anthropometric data and several common serum biomarkers for glycemic control (fasting plasma glucose, HbA1c, and 1,5-AG), renal function (urea and creatinine), and proteins (total protein and albumin), applying regression analysis. Our observations did not show any association between the study polymorphisms and the factors mentioned above in both studied groups (data not shown).

DISCUSSION

Interleukin 6 (IL-6) is a multifunctional cytokine that is involved in inflammatory and autoimmune diseases (Hundhausen et al., 2016). IL-6 is a pleiotropic cytokine with a number of important biological activities, including regulation of acute-phase reactions and generation of immune responses (Duncan and Berman, 1991). IL-6 has also been shown to have an essential role in the pathogenesis of islet β cell destruction in animal models (Campbell et al., 1991). IL-6 levels and actions are mainly controlled by sIL-6R, and several studies have shown increased sIL6R levels in different autoimmune diseases (Marinou et al., 2010; Eleftherohorinou et al., 2011).

Hundhausen et al. (2016) described IL-6 signaling increases in some individuals with T1D, presumably favored by metabolic, immunological, or perhaps genetic mechanisms.

There is evidence that the activity levels of IL-6 and its receptor are regulated by functional polymorphisms in corresponding genes (Smith and Humphries, 2009). Consequently, these polymorphisms may contribute to the genetic background of T1D by modifying amplification of the immune response (Ferreira et al., 2013; Woo and Humphries, 2013).

Carriers of genetic variants that up-regulate IL-6 and sIL-6R secretion may represent patient sub-groups with host-related features that favor T1D. Notably, we found that the minor C allele of the IL-6R variant (rs2228145) showed an association with T1D. Carriers of the C allele showed 1.48-fold more susceptibility to T1D. This result is contradictory to that obtained by Ferreira et al. (2013), which found an association of the C allele with protection from T1D (OR 0.94; 0.91 - 0.99). However, these authors discuss that the protective effect seems to be a paradox, as the C allele was associated with higher circulating soluble forms of the IL-6 receptor (sIL-6R) (Reich et al., 2007; Ferreira et al., 2013) and IL-6 levels (Reich et al., 2007; IL6R Genetics Consortium Emerging Risk Factors Collaboration et al., 2012; Naitza et al., 2012).

However, the C allele results in nearly a 2-fold increase in sIL-6R levels in homozygous carriers of the SNP rs2228145 (Garbers et al., 2014). These authors demonstrated that these individuals were protected from a number of inflammatory diseases, including coronary heart disease and rheumatoid arthritis (Ferreira et al., 2013). It has been suggested that this finding can be explained by the increased capacity of sIL-6R/gp130 to buffer for IL-6 in the blood of SNP rs2228145 carriers (Scheller and Rose-John, 2012).

The functional C allele impairs classical IL-6 receptor signaling, which is considered to have homeostatic and anti-inflammatory effects, and IL-6 trans-signaling mainly regulates pro-inflammatory reactions (Rose-John, 2017). Similarly, IL-6 trans-signaling via the sIL-6R has the potential to lead to stronger and longer intracellular signaling than classic IL-6 signaling via the membrane-bound IL-6R (Rose-John, 2012).

Notably, T1D patients studied by Ferreira et al. (2013) were under 17 years of age at diagnosis (childhood onset), while our study was composed of subjects with T1D diagnosis of ≥18 years (adult onset). Similar effects have been reported by other cytokines (IL-10); however, polymorphisms modulate the degree of islet destruction and the age-at-onset of type 1 diabetes, suggesting that variability of the age-at-onset of this disease is genetically determined (Ide et al., 2002). Others studies conducted with juvenile onset of T1D imply that IL-6 genotypic variants may influence the time of disease onset or modulate the risk of diabetes development (Jahromi et al., 2000; Gillespie et al., 2005; Hermann et al., 2005).

These results are different from our findings where no association was identified between IL-6 -174G>C (rs1800795) and adult-onset T1D in the present study. Similarly, Tsiavou et al. (2004) did not find an association of IL-6 -174G>C (rs1800795) with LADA (Latent Autoimmune Diabetes of the Adult). Additionally, some studies suggest that the -174GG genotype is protective against early T1D onset; however, this protection may be lost later in life (Jahromi et al., 2000; Gillespie et al., 2005; Hermann et al., 2005; Mysliwska et al., 2009).

Frequencies of the rare C allele of IL-6 -174G>C (rs1800795) found in this study for the control (27.3%, 95% CI 20 - 35%) and T1D groups (30.1, 95% CI 23 - 38%) were lower than that reported for other Caucasian populations, such as British (control 42.6% and

T1D 44.0%) (Cooper et al., 2007), Greeks (control 35.9%) (Tsiavou et al., 2004), Polish (control 50.0% and T1D 58.8%) (Mysliwska et al., 2009), and Slovaks (control 38.6% and T1D 48.7%) (Javor et al., 2010).

The frequency of the minor C allele of IL-6R Asp358Ala (rs2228145) found in this study for the control (34.7%, 95% IC 27 - 43) was similar to Americans (39.6%) (Qi, Rifai and Hu, 2007), Spanish (39.3%) (Cenit et al., 2012), Polish (33.2%) (Kapelski et al., 2015) and lower than in Korean (44.5%) (Kim et al., 2004) populations. We have not found information about this polymorphism in T1D.

There are many examples in the literature, suggesting that relevant analyses in different patient populations with complex diseases result in controversial data due to gene-environment/gene-gene interactions or their different racial/ethnic origins and overall genetic composition (Howson et al., 2012). It is important to highlight this is a prospective study. Therefore, future studies with larger sample sizes will be required to confirm our findings.

In conclusion, IL-6 rs1800795 was not associated with adult-onset T1D. However, IL-6R rs2228145 was associated with T1D development in adulthood, and carriers of the minor C allele are at increased risk for adult-onset T1D (OR = 1.48; 95% CI = 1.06 - 2.07).

ACKNOWLEDGMENTS

This study was funded by the government agencies CNPq (National Council for Scientific and Technological Development), Araucaria Foundation, and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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