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The −174GG Interleukin-6 Genotype Is Protective From Retinopathy and Nephropathy in Juvenile Onset Type 1 Diabetes Mellitus

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ABSTRACT: The aim of our study was to determine an association between the −174G>C IL-6 polymorphism (rs1800795) and occurrence of retinopathy and nephropathy in type 1 diabetes mellitus (T1DM) patients. Two hundred ten children/adolescents with long-standing T1DM (16.5 ± 3.8 y; with diabetes duration of 8.4 ± 3.0 y) were enrolled into the study. A group of 170 healthy young (16.9 ± 5.2 y) sex-matched volunteers was qualified as the control. The IL-6 polymorphism was genotyped with the PCR-RFLP method. Serum and urine IL-6 concentrations were measured by the ultra-sensitive ELISA tests. The −174GG genotype was under represented in the diabetic patients compared with the controls. Patients with this genotype were free from nephropathy and retinopathy. The group of −174GG carriers was characterized by the highest urine IL-6 concentrations in relation to other genotypes. In the multivariate logistic regression analysis adjusted for age, duration of the disease, age of disease onset, HbA1c, and albumin excretion rate, the −174GG genotype was the only independent variable that significantly decreased the risk of jointly analyzed retinopathy and nephropathy [OR = 0.65; 95% CI = 0.52–0.82; $p = 0.0003$]. We propose that the −174GG patients are protected from late diabetic complications by different IL-6 dependent mechanisms. **(*Pediatr Res* 66: 341–345, 2009)**

Low-grade inflammation underlies the course of long-term type 1 diabetes mellitus (T1DM). Diabetic retinopathy and nephropathy entail an exacerbation of inflammatory conditions with the serum IL-6 level correlating with the occurrence of microvascular complications (1). The amount of IL-6 production is dependent on transcriptional regulation of this cytokine. The polymorphic region −174G>C of IL-6 encoding gene is implicated in transcription of this cytokine. The G>C nucleotide substitution creates a potential binding site for the transcription factor NF-1 and the −174G allele, in a reporter gene assay *in vitro* was found to have twice as much promoter strength as the C allele (2). In line with the molecular results, the clinical data place the −174GG genotype as the most proinflammatory one within the rs1800795 polymorphism. High IL-6 *ex vivo* production was documented in the −174GG healthy individuals compared with other genotype carriers (3). The healthy −174G allele carriers turned out to be high IL-6 producers after vaccination with *Salmonella typhii* vaccine (4). It was documented that patients with the −174GG genotype run a risk of an earlier onset and more serious outcome of cardiovascular and cerebrovascular diseases (5–9). Also, the IL-6 promoter polymorphism seems to have influence on various postoperative short-term (10–12) and long-term complications (13,14) as well as on the frequency and severity of different cardiovascular events (15,16).

Although the presented evidence favors the proinflammatory character of the −174GG genotype, some other data may raise doubts about universality of this phenomenon. For example, in the studies of Humphries *et al.* (17), it was the −174C allele carriers that had the increased risk of higher systolic blood pressure and coronary heart disease. In addition, it was the −174C allele carriers that produced *in vivo* the highest amount of IL-6 and had less favorable outcome of coronary revascularization surgery than the GG homozygotes (18).

The data concerning an association between T1DM and the −174G>C IL-6 polymorphism are inconsistent and mostly limited to the assessment of the disease risk (19–25). In a study on 206 Dutch families with juvenile onset of T1DM, no association between the single rs1800795 polymorphism and T1DM has been found (20). In a genome-wide association study comprising 7758 cases and 8852 controls, a borderline significance has been reported for the −174CC genotype indicating an increased susceptibility to T1DM (21). The three other articles dealing with juvenile onset of T1DM imply that the IL-6 genotypic variants may influence the time of disease onset or modulate the risk of diabetes development (22–24). Moreover, a recently published article has observed a trend toward higher frequency of retinopathy within the −174CC genotype in adult diabetes type 1 and type 2 patients (25).

The aim of our study was to determine an association between the −174G>C IL-6 polymorphism (rs1800795) and the occurrence of retinopathy and nephropathy in juvenile onset T1DM patients.

METHODS

Participants. The study was conducted on a group of 210 children/adolescents 16.5 ± 3.8 y with long-standing T1DM (100 boys, 110 girls), from the Outpatient's Diabetology Clinic at the Medical University of Gdańsk, Poland. The diagnosis of T1DM was carried out in accordance with the American Diabetes Association Criteria (26). Patients with coexisting

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Abbreviations: T1DM, type 1 diabetes mellitus

Table 1. *Clinical characteristics of the diabetic patients and healthy group*

	Patients without complications (n = 172)	Patients with complications (n = 38)	Healthy group (n = 170)	Statistical significance (p)
Gender (f/m)	86/86	20/18	80/90	
Age (y)	16.1 ± 1.7	18.8 ± 2.1	16.9 ± 5.2	0.63*
				0.008†
Disease onset	8.1 ± 4.5	8.5 ± 3.5	—	0.03†
Duration of the disease (y)	8.0 ± 3.0	10.3 ± 2.8	—	0.0001†
HbA1c %	8.0 ± 1.4	9.18 ± 2.1	—	0.0001†
Albumin excretion rate (mg/24 h)	16.9 ± 11.1	40.3 ± 5.8	3.5 ± 2.1	0.0001*
				0.0001†
Creatinine in serum (μmol/L)	0.71 ± 0.07	0.79 ± 0.12	0.50 ± 0.01	0.0001*
				0.02†
Systolic blood pressure (mm Hg)	111 ± 11	118 ± 12	107 ± 8	0.0001*
				0.0005†
Diastolic blood pressure (mm Hg)	71 ± 8	75 ± 11	62 ± 6	0.0001*
				0.007†

* Statistical significance between the whole group of diabetic patients.

† Statistical significance between the patients without and with diabetic complications.

autoimmune diseases as well as with additional chronic and acute diseases were excluded from the study. All the patients were treated with humanized insulin. Retinopathy was defined according to the ETDRS Research Group Ophthalmology criteria (27). In all the examined patients, the C-peptide level was found to be <0.5 ng/mL. The urinary albumin excretion was expressed as the average of three 24-h collections obtained during 6 mo before enrolment into the study. The cases that, in at least two out of three urine samples, had albumin excretion within the range of 30–299 mg/24 h were classified as microalbuminuria. The urinary albumin excretion was measured by immunoturbidometric assay using Tina-quant (Boehringer Mannheim GmbH, Germany). Urinary creatinine and IL-6 were measured from the same sample of 24 h urine collection. The glomerular filtration rate (GFR) was estimated by calculation of the creatinine clearance (Cl_{Cr}) from serum creatinine concentration using the Cockroft-Gault formula, which is suitable for children: Cl_{Cr} (mL/min) = [(140 − age) × weight]/(72X_{SCr} [mg/dL]) × 0.85 (if girl); SCr = serum creatinine (28). A group of 170 healthy young age (16.9 ± 5.2 y) and sex-matched volunteers was qualified as the control. Informed consent was obtained from parents of all children enrolled in the study. Children older than 16 y also provided consent. The Ethics Committee of the Medical University of Gdańsk approved this study.

Serum IL-6 and urine IL-6 concentrations. These were measured by the ultra-sensitive ELISA test (Quantikine HS IL-6 kit; R&D Systems, Minneapolis, MN) according to the manufacturer protocol. Minimum detectable concentrations for IL-6 were determined by the manufacturer as 0.03 pg/mL in serum and 0.1 pg/mL in urine.

Genotyping. IL-6 polymorphism was analyzed with the PCR-RFLP according to Fishman *et al.* (2) using the following primers: 5′ AGAAGAACTCAGATGACTGG 3′ and 5′ GCTGGGCTCCTGGAGGGG 3′. The PCR products were cut by the restriction enzyme *Sfa*NI (New England Biolabs) and electrophoresed on 2% agarose gel, stained with ethidium bromide. DNA samples were first sequenced to establish three IL6 polymorphic variants, as a quality control. Next, DNA samples of the −174GG, GC, and CC individuals were routinely added to the examined ones to ensure genotype accuracy.

Statistical analysis. The results were analyzed using the Statistica, Version 7 program (StatSoft, Pl). The Shapiro-Wilk's test was used to evaluate normality of variables. Differences between the groups of normally distributed variables were calculated with the ANOVA or t tests. For comparison of the skew-distributed variables, the nonparametric Kruskal-Wallis ANOVA or Mann Whitney U tests were applied. Nominal variables were analyzed by the χ^2 Pearson's χ^2 test. The two-tailed probability values were calculated. A multivariate logistic regression analyses was performed to assess the association between nephropathy and retinopathy incidence and all the variables significant for late diabetic complications. Finally, a statistical power analysis was carried out to establish whether the number of patients GG was sufficient to conclude a hypothesis about protective effect of this genotype against diabetic complications. The α level was set at 0.05.

RESULTS

Clinical characteristics of the patients and the healthy group are presented in the Table 1. The genotype distribution was significantly different between the patients and healthy group (p = 0.01). The −174GG genotype was under represented in the group of diabetic patients, with only 15% having this genotype, in relation to the control group with the −174GG participants amounting to 30% (Table 2).

Table 2. *The −174G>C genotype distribution in diabetic patients and healthy group*

−174G>C genotypes	Healthy group (n = 170)		Patients (n = 210)	
	n	%	n	%
CC	51	30	69	33
GC	68	40	110	52
GG	51	30	31	15
χ^2 Pearson	0.01*			

* Statistical significance between the patients and healthy group.

Among the 210 diabetic children/adolescents, 38 patients had one or two complications. Twenty-one patients had two complications. Nephropathy was diagnosed in 23 and retinopathy in 36 of them. Two patients with nephropathy were free from retinopathy and 15 patients with retinopathy were free from nephropathy. The patients with nephropathy and retinopathy were within the −174CC and the GC genotypes, whereas no complications were found within the −174GG group. The −174G>C IL-6 genotype distributions within the patients with nephropathy and retinopathy *versus* those free of both late diabetic complications were significantly different (p = 0.005 and p = 0.01, respectively). There were no differences in onset of the disease and its duration between patients with different −174G>C variants (Table 3).

The serum IL-6 concentrations were about four times higher in the patients than in the healthy group. The −174GG patients were characterized by similar IL-6 serum level as the other genotype's groups (p = 0.75). Similarly, no genotype-dependent differences in serum IL-6 level were found among the healthy group (p = 0.9).

The diabetic patients excreted more urine IL-6 than the healthy group. The urinary IL-6 level in the −174GG patients was significantly higher (p = 0.0007) in relation to other genotypes. The urine IL-6 concentration was of similar values in the genotype groups among the healthy children (p = 0.36).

Table 3. *The −174G>C genotype distribution in relation to diabetic complications*

−174G>C genotypes	Diabetic patients (n = 210)	Disease onset	Duration of the disease	Nephropathy n (%) of patients*		Retinopathy n (%) of patients†	
				Free of both complications	With nephropathy	Free of both complications	With retinopathy
CC	69	8.5 ± 5.6	8.5 ± 2.7	59 (85%)	10 (15%)	57 (83%)	12 (17%)
GC	110	8.1 ± 3.9	8.4 ± 3.2	88 (88%)	13 (13%)	86 (78%)	24 (22%)
GG	31	8.2 ± 3.9	8.8 ± 3.2	31 (100%)	0 (0%)	31 (100%)	0 (0%)
χ^2 Pearson		p = 0.774	p = 0.754	p = 0.005		p = 0.01	

* Percent of patients with nephropathy within a given genotype group in relation to the entire genotype group.
† Percent of patients with retinopathy within a given genotype group in relation to the entire genotype group.

Table 4. *Serum and urinary IL-6 in carriers of the polymorphic −174G>C IL-6 variants*

	IL-6 genotype	IL-6 serum (pg/mL)	IL-6 urine (pg/mL)	GFR (CL_{Cr}) (mL/min)	uIL-6/urine creatinine (pg/mg)
Diabetic patients	CC	1.26 ± 1.09	1.74 ± 0.56	102 ± 31	2.4 ± 1.2
	GC	1.34 ± 1.20	1.71 ± 0.81	101 ± 38	4.3 ± 2.5
	GG	1.32 ± 1.01	6.47 ± 2.26	100 ± 29	7.3 ± 4.8
	p	0.75*	0.0007*	0.98†	0.04†
Healthy group	CC	0.39 ± 0.32	0.54 ± 0.32	—	—
	GC	0.35 ± 0.23	0.32 ± 0.07	—	—
	GG	0.35 ± 0.38	0.45 ± 0.20	—	—
Statistical significance	p	0.9*	0.36†	—	—

uIL-6, IL-6 in urine.
* Kruskal Wallis ANOVA.
† ANOVA.

Table 5. *Patients with and without complications representing polymorphic variants of IL-6*

IL-6 genotype	Age (y)	Disease duration (y)	Disease onset (y)	Albumin excretion (mg/24 h)	HbA1c (%)	IL-6 serum (pg/mL)	IL-6 urine (pg/mL)
With complications GC + CC (n = 38)	18.8 ± 2.1	10.3 ± 2.8	8.5 ± 3.5	40.3 ± 5.8	9.18 ± 2.1	1.34 ± 1.40	2.1 ± 2.2
Without complications GC + CC (n = 141)	16.0 ± 1.3	7.9 ± 3.0	8.1 ± 4.7	17.3 ± 11.2	8.0 ± 1.4	1.44 ± 1.52	1.5 ± 1.8
Without complications GG (n = 31)	17.0 ± 3.5	8.8 ± 3.2	8.2 ± 3.9	15.6 ± 11	7.9 ± 1.6	1.56 ± 1.47	6.5 ± 2.2
p	0.003*	0.000000	0.008	0.0005	0.000000	0.6	0.2
	0.07†	0.000000	0.01	0.004	0.00001	0.44	0.04
	0.79‡	0.85	0.56	0.83	0.71	0.59	0.000000

* Differences between values of the GC + CC group with complications *vs* the group GC + CC without complications.
† Differences between values of the GC + CC group with complications *vs* the GG group without complications.
‡ Differences between values of the groups without complications, i.e. the GG *vs* the GC + CC group.

The values of GFR were similar for all −174G>C genotypes (p = 0.98). The urine IL-6 concentration standardized to the urinary creatinine level was highest in the −174GG and lowest in −174CC diabetic patients (p = 0.04) (Table 4).

Next, the presence of nephropathy and retinopathy was analyzed with respect to IL-6 polymorphism. It appeared that the −174CC and GC patients without complications were younger (p = 0.003), had shorter disease duration (p = 0.000000), later disease onset (p = 0.008), lower albumin excretion (p = 0.0005), and lower HbA1c (p = 0.000000) level in relation to the −174CC and GC patients with complications. However, there were no differences between the concentrations of IL-6 in serum (p = 0.6) and urine (p = 0.2). In addition, the −174GG complications free group was younger (p = 0.07) had shorter disease duration (p = 0.000000), later disease onset (p = 0.01), lower albumin excretion (p = 0.004), and lower HbA1c (p = 0.000000) values in relation to the group with complications. The −174GG patients did not differ in a significant way from the complications free 174CC and −174GC genotype carriers with respect to majority of clinical parameters. The only difference between the groups was found in the urine IL-6 concentration that was significantly higher in the −174GG patients (p = 0.000000) (Table 5).

In the multivariate logistic regression analysis adjusted for age, duration of the disease, age of disease onset, HbA1c, albumin excretion rate the presence of −174G allele significantly decreased the risk of the jointly analyzed retinopathy and nephropathy [OR = 0.65; 95% CI = 0.52–0.82; p = 0.0003]. The remaining parameters did not affect the risk of diabetic complications. The significance of the 174G allele was lost when retinopathy and nephropathy were analyzed separately. Moreover, the *post hoc* power analysis revealed that to guarantee a high statistical power of 0.900 with the α value set at 0.05 the number of −174GG complications free patients should be 86 while the patients evaluated in our study amounted only to 31 individuals. The current power of the logistic regression calculations was 0.634.

DISCUSSION

Reduced representation of the IL-6 −174GG genotype found among our patients in comparison to the healthy group points to an association between the −174C allele and juvenile diabetes risk.

Such a genotype distribution profile is in agreement with the data of Gillespie *et al.* (22) who found that the −174G allele decreases the risk of diabetes type 1 onset before the age of 10 y in girls. An analogous conclusion is derived from the article of Hermann *et al.* (23) who found that the −174G allele carrier status is associated with a later onset of diabetes type 1 in children. In a complementary article, Jahromi *et al.* (24) found an enrichment till 69% of the −174G allele in a group of adult T1DM patients compared with normal controls. The results of the cited articles imply that the −174GG genotype is protective against an early T1DM onset but this protection may be lost in the later life.

Although we did not replicate an association between the −174GG genotype and a later onset of the disease, which was done by Gillespie *et al.* (22) and Hermann *et al.* (23), we suggest a protective effect of the −174GG genotype against late diabetic complications in juvenile diabetes. However, one has to treat our results with reservation because they lack the sufficient statistical power (value of power 0.634 with $\alpha = 0.05$), and to make them reliable, the number of −174GG patients should be increased about three times. Our results complement two other articles. A recently published the first large cross-sectional study has found a trend toward a higher frequency of diabetic retinopathy in type 1 and type 2 diabetic patients with the −174CC genotype (25). Moreover, in a genome-wide association study a borderline significance was reported for the −174CC genotype to indicate an increased susceptibility to T1DM (21).

How are we to reconcile the most inflammatory character of the −174GG genotype with the protection from diabetes and its complications? It follows from the literature (2,4,10,16) that an enhanced IL-6 response in the −174GG genotype patients may have beneficial effects.

For example, elevated IL-6 serum level has been found to be associated with higher fasting and postload insulin level in healthy human population, implying that IL-6 may encourage insulin secretion (29,30). In line with this suggestion, it was revealed that IL-6 might directly stimulate insulin secretion from rat pancreatic islets (31) as well as from cultures of these cells *in vitro* (32). Thus, if such conditions really exist in −174GG homozygotes at prediabetes stage, insulin secretion in them may be relatively high until clinical manifestation of the disease.

IL-6 may also delay pancreatic beta cell damage by its anti-inflammatory effect, which contributes to amelioration of the insulitis (33–35). Induction of the circulating IL-1 receptor antagonist and the soluble TNFα p55 receptor demonstrate the well-known anti-inflammatory effects of IL-6 (36). The prophylactic and therapeutic effect of the soluble TNF-α receptor type I, as TNF-α antagonist, against diabetic insulitis has already been documented in female nonobese diabetic (NOD) mice (37). Moreover, IL-6 is responsible for amelioration of local and systemic inflammation by stimulation of IL-10 production (38).

Previous experiments in mice overexpressing IL-6 gene in pancreatic beta cells add a confirmation for a possible protective role of IL-6 in the prediabetes stage. The transgenic nonobese diabetic (NOD) mice, which overexpressed human IL-6 in pancreatic beta cells, had lower average fasting glucose levels, a delayed onset of diabetes, and a longer survival compared with age and sex matched littermates without IL-6 gene overexpression (39). In another study, the high IL-6 production in IL-6 gene overexpressing mice seemed to be specifically protective in the prediabetes phase by stimulating islet cell hypertrophy with beta cell neogenesis suggestive of reparatory processes (40). Thus, it follows from our discussion that in the population of −174GG young diabetic patients, the prediabetic insulitis may last longer due to IL-6-dependent amelioration of insulin deficiency.

It remains in the sphere of speculation as to how the −174GG genotype may shield against diabetic complications. Both retinopathy and nephropathy are associated with inflammatory conditions (41). Recently published articles have shed more light on this problem. Experiments have documented the neuroprotective role of IL-6, which may merit in the protection against diabetic retinopathy. IL-6 produced by glial cell cultures *in vitro* was able to save the retinal ganglion cells from apoptosis both in conditions of elevated pressure, which usually reduce retinal cell survival, and in the ischemic-reperfusion injury (42,43).

The literature that we came across disallows any conclusion about the protective mechanism of IL-6 against nephropathy. IL-6 blood level in our study was not elevated in the −174GG genotype patients who in other pathologic circumstances were characterized as high IL-6 producers (10–12,15,16). The above-mentioned circumstances were, however, associated with an acute inflammatory response while formation of the microvascular complications is due to chronic inflammation. Instead, we showed high IL-6 concentration in the urine of the −1744GG patients, which was much probably produced locally (44,45). It can be easily deduced that the high IL-6 concentration in urine was not a result of both filtration, and a subsequent renal reabsorption may easily be deduced. If IL-6 had been filtered, its urine levels would strongly decrease and that of the blood samples significantly increase (46). Meanwhile, we did not find an elevation of the blood IL-6 in the −174GG group and just opposite the high IL-6 level was visible in urine of these patients. One of the IL-6 protective mechanisms may rely on stimulation of high IL-10 production, which we found in earlier clinical studies (47).

Summarizing, we found that the IL6 −174GG homozygotes were under represented in the T1DM young patients, and they were free from retinopathy and nephropathy. We propose that the −174GG patients are protected from late diabetic complications by different IL-6 dependent mechanisms.

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