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Polymorphisms of interleukin–8 and –18 genes and diabetic retinopathy

Research Article

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Abstract: We evaluated possible roles of interleukin-8 gene polymorphisms (1633T/C-rs2227543, 251A/T-rs4073) and interleukin-18 gene polymorphisms (-607C/A-rs1946518, -137G/C-rs187238) in the development of diabetic retinopathy (DR) in Caucasians with type 2 diabetes. 271 patients with DR and 113 without diabetic retinopathy were enrolled in this cross-sectional study. We did not observe an association between either interleukin-8 gene polymorphisms (1633T/C, 251A/T) or interleukin-18 gene polymorphisms (-607C/A, -137G/C) and diabetic retinopathy in Caucasians with type 2 diabetes. We did not find statistically significant differences in interleukin-8 serum levels between diabetics with the TT and AA genotype and those with other genotypes. The interleukin-18 serum levels between diabetics with the CC genotype of the -607C/A polymorphism and those with other genotypes (AA, AC) were not significantly different. Moreover, we did not observe a statistically significant effect of the tested polymorphisms of either interleukin-8 or interleukin-18 genes on serum levels in diabetics. In conclusion, our study indicates that the examined polymorphisms of interleukin-8 (1633T/C, 251A/T) and interleukin-18 (-607C/A or the -137G/C) genes are not genetic risk factors for diabetic retinopathy. Therefore, they may not be used as genetic markers for diabetic retinopathy in Caucasians with type 2 diabetes.

Keywords: *Interleukin 8 • Interleukin 18 • Gene Polymorphism • Diabetic retinopathy • Genetic markers • Cross sectional study*

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1. Introduction

Diabetic retinopathy (DR) is a progressive disease predominantly affecting the integrity of the microscopic vessels found in the retina [1]. It is one of the primary causes of visual loss worldwide. The major risk factors for DR, reported from several epidemiologic studies, are the duration of diabetes, hyperglycemia/glycated hemoglobin value, hypertension, hyperlipidemia, pregnancy, and nephropathy/renal disease [2]. After 20 years of type 2 diabetes most diabetics have some form of DR [1,3]. Moreover, in earlier studies higher incidence rate of DR was reported in comparison with later reports [1,3].

DR is caused by alterations of the retinal microvasculature leading to the breakdown of the blood – retina barrier and pathological angiogenesis [2,3]. Angiogenesis is a complex multistep process that includes proliferation, migration, and differentiation of endothelial cells, degradation of extracellular matrix, microtubule formation, and sprouting of new capillary branches. Inappropriate angiogenesis – neovascularization – occurs in many pathological situations. As new capillaries are fragile and prone to

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hemorrhage, neovascularization, occurring in the eye, can trigger pathological conditions such as retinopathy of prematurity (ROP), diabetic retinopathy, retinal vein occlusion, and age-related macular degeneration [4,5]. The prevalence of DR increases with the duration of diabetes; up to 21% of patients with type 2 diabetes have retinopathy at the time of their first diagnosis of diabetes, and most develop some degree of retinopathy over time [3].

In DR and other angiogenesis-associated diseases, increased levels of cytokines, inflammatory cells, growth factors, and angiogenic factors are present [6-9]. Interleukin-8 (IL-8) and interleukin-18 (IL-18) belong to the family of inflammatory cytokines. There is a large body of evidence indicating that IL-8 contributes to the pathogenesis of diabetic retinopathy. These small basic heparin-binding proteins are proinflammatory and primarily mediate the activation and migration of neutrophils into the tissue from peripheral blood. It is produced by mononuclear phagocytes, neutrophils and other cells, such as endothelial cells [10,11]. Clinical studies have identified increased levels of IL-8 in the vitreous of diabetic probands compared to non-diabetics [2,8,12]. Higher levels of IL-8 in the vitreous may promote retinal vascular damage in diabetic patients and may lead to diabetic retinopathy [8].

IL-18 is a pleiotropic cytokine produced by activated monocytes, glial cells and dendritic cells, and it plays an important role in many inflammatory diseases [13]. Two polymorphisms in the promoter region of the *IL-18* gene, at positions -607 and -137, appear to have functional associations between genotype and serum concentrations of IL-18 [14]. It has been shown that IL-18 has a role in angiogenesis. Cao *et al.* reported that it suppressed corneal neovascularization (NV) induced by the fibroblast growth factor and tumour angiogenesis, and proposed that IL-18 acted as an angiogenesis and tumour suppressor [5,15]. *IL-18* gene expression has been detected in the normal iris and retina but the function of this protein in the eye remains unclear [2,5]. Interleukin-18 upregulates the synthesis of proinflammatory cytokine IL-8, as well as the expression of adhesion molecules [16]. IL-8 is a potent chemoattractant for neutrophils and triggers firm adhesion of rolling monocytes to the vascular endothelium [17].

In this cross-sectional study, we investigated a possible association between either *IL-8* gene polymorphisms (1633T/C- rs2227543, 251A/T - rs4073) or *IL-18* gene polymorphisms (-607C/A - rs1946518, -137G/C - rs187238) and DR among patients with type 2 diabetes.

2. Experimental Procedures

384 unrelated Caucasians with type 2 diabetes mellitus with a defined ophthalmologic status were enrolled in this cross-sectional study (from Ljubljana, Slovenia and Tuzla, Bosnia and Herzegovina). Patients were classified as having type 2 diabetes according to the current American Diabetes Association criteria [The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997].

Fundus examination was performed by a senior ophthalmologist (M.P.) after pupil dilatation (tropicamide and phenylephrine 2.5%) using slit lamp biomicroscopy with a non-contact lens, and was electronically documented with a 50°-angle fundus camera (Topcon-TRC 40-IX; Tokyo, Japan). The staging of diabetic retinopathy was determined according to the Early Treatment Diabetic Retinopathy Study (ETDRS) retinopathy severity scale [EDTRS 1991].

The study group consisted of 271 patients with diabetic retinopathy: 196 subjects with proliferative diabetic retinopathy (new vessel formation and/or fibrous proliferation with or without vitreous hemorrhage) and 75 subjects with non-proliferative diabetic retinopathy (microaneurysms, retinal hemorrhages, hard exudates) [EDTRS 1991]. The control group consisted of 113 subjects with type 2 diabetes of duration of more than 10 years, who had no clinical signs of diabetic retinopathy.

To avoid the confounding effect of impaired kidney function, the patients with overt nephropathy were not enrolled. The study was approved by the national medical ethics committee. After an informed consent for participation in the study was obtained, a detailed interview was conducted.

The serum IL-8 and IL-18 levels were analyzed in a subpopulation of 70 consecutive diabetics and in a subpopulation of 22 subjects without diabetes. IL-8 and IL-18 in the sera were measured by using ELISA kits (R&D Systems, Minneapolis, MN). The ELISA was performed according to the manufacturer's instructions.

Genotyping of the 2 polymorphisms 251A/T and 1633C/T was performed by PCR-RFLP. The polymorphism 251A/T was typed by using the primer pair 5'-CCA TCA TGA TAG CAT CTG TA-3' and 5'-CCA CAA TTT GGT GAA TTA T*TA A-3' (annealing temperature 55°C; *change to the original sequence to create a restriction site for an endonuclease). After PCR, the product was digested with 1 U of AseI (New England Biolabs, Beverly, Mass) overnight, and the fragments were resolved on a 2% agarose gel. The polymorphism C1633T was typed by using the primer pair

5'-CTG ATG GAA GAG AGC TCT GT-3' and 5'-TGT TAG AAA TGC TCT ATA TTC TC-3' (annealing temperature 56°C). After PCR, the product was digested with 2 U of NlaIII (New England Biolabs) overnight, and the fragments were resolved on a 2% agarose gel.

For the position -607C/A IL-18 specific PCR, a common reverse primer 5'-TAACCTCATTCAGGACT-TCC-3' and two sequence specific forward primers 5' - GTTGCAGAAAGTGTAAAAATTATTAC - 3' and 5'-GTTGCAGAAAGT-GTAAAAATTATTAA-3' were used. An amplification product of 196-bp was detected. A control forward primer 5'-CTTTGCTATCATTCCAG-GAA-3' was used to amplify a 301-bp fragment covering the polymorphic site as an internal positive amplification control.

For the position -137G/C specific PCR, a common reverse primer 5'-AGG-AGGGCAAAATGCACTGG-3' and two sequence specific forward primers 5'-CCC and CAACTTTTACGGAAGAAAAG-3' and 5'-CCCCAACTTTTACGGAAG-AAAAC-3' were used. An amplification product of 261-bp was detected. A control forward primer 5'-CCAATAGGACTGATTATTCCGCA-3' was used to amplify a 446-bp fragment covering the polymorphic site to serve as an internal positive amplification control.

Genotyping was performed by two researchers (D.P., I.C.), blinded to the retinopathy status of the patients. The chi-square test was used to compare discrete variables. Continuous clinical data were compared by unpaired Students t test. In addition, all variables that showed significant differences by univariate methods (chi-square test, unpaired Students t test) were analysed together in a logistic regression analysis. A statistical analysis was performed using the SPSS programme for the Windows version 17 (SPSS Inc. Illinois).

3. Results

The characteristics of the cases and controls subjects are listed in Table 1. Cases had an earlier onset of diabetes and a longer duration of type 2 diabetes compared to the diabetics without diabetic retinopathy. Additionally, they had a higher incidence of insulin therapy than the controls (diabetics without diabetic retinopathy). There were no significant differences in hypertension, smoking, total LDL, HDL cholesterol and triglyceride levels between the cases and control subjects (Table 1).

The *IL-8* genotype distributions in patients (DR group) and controls (subjects without DR) were compatible with Hardy-Weinberg expectations (1633: DR group χ^2 0.31, P=0.58; controls χ^2=0.04, P=0.84; 251: DR group χ^2=0.63, P=0.43; controls χ^2=0.36, P=0.54; Table 2). The *IL-18* genotype distributions in patients (DR group) and controls (subjects without DR) were compatible with Hardy-Weinberg expectations (607: DR group χ^2=0.70, P=0.4; controls χ^2=2.07, P=0.15; 137: DR group χ^2=2.61, P=0.11; controls χ^2=0.81, P=0.37).

We analysed serum IL-8 and IL-18 levels in 70 subjects with type 2 diabetes and 22 subjects without diabetes. Serum IL-8 levels in 70 diabetics were significantly higher than those of 22 controls without diabetes (49.4±48.8 ng/l *vs.* 30.6±12.9 ng/l; P=0.01).

Characteristics	Cases n (%)	Controls n (%)	P
Number	271	113	
Age (years)	65.6 ± 9.5	66.9 ± 11.5	0.3
Male sex (%)	132 (48.7)	42 (37.2)	0.2
Duration of diabetes (years)	19.4 ± 8.6	16.5 ± 6.6	0.001
Patients on insulin therapy (%)	191 (70.4)	46 (40.7)	<0.001
Age of diabetes onset	46.5 ± 11.3	53.3 ± 12.1	<0.001
HbA_{1c} (%)	8.0 ± 1.6	8.2 ± 1.6	0.1
Systolic blood pressure (mm Hg)	144 ± 22	145 ± 20	0.9
Diastolic blood pressure (mm Hg)	84 ± 10	84 ± 9	0.7
BMI (kg/m^2)	27.9 ± 4.6	27.7 ± 4.4	0.9
History of hypertension (%)	205 (75.6)	75 (66.4)	0.6
Smokers (%)	30 (11.1)	12 (10.6)	0.4
Total cholesterol (mmol/l)	5.4 ± 1.2	5.5 ± 1.2	0.1
HDL cholesterol (mmol/l)	1.1 ± 0.4	1.2 ± 0.4	0.1
LDL cholesterol (mmol/l)	3.1 ± 1.0	3.2 ± 0.9	0.2
Triglycerides (mmol/l)	2.2 ± 1.3	2.6 ± 1.9	0.1

Table 1. Characteristics of patients with diabetic retinopathy (cases) and patients without diabetic retinopathy (controls). The values represent mean±S.D.

	Cases (271)	Controls (113)	OR (95 % CI)[1]	P
IL-8 C1633T				
Genotype TT	49 (18.1)	20 (17.7)	1.0 (0.6-1.7) [2]	1[2]
Genotype TC	137 (50.5)	57 (50.4)	1.0 (0.6-1.6) [3]	0.9[3]
Genotype CC	85 (31.4)	36 (31.9)		
	271	113		
IL-8 A251T				
Genotype AA	56 (20.7)	24 (21.2)	0.9 (0.5-1.6) [2]	0.8[2]
Genotype AT	142 (52.4)	59 (52.2)	0.9 (0.5-1.6) [3]	0.9[3]
Genotype TT	73 (26.9)	30 (26.6)		
	271	113		
IL-18 C607A				
Genotype CC	83 (30.6)	44 (38.9)	0.7 (0.4-1.1) [2]	0.1[4]
Genotype AC	140 (51.7)	47 (41.6)	1.1 (0.6-2.0) [3]	0.7[5]
Genotype AC	48 (17.7)	22 (19.5)		
	271	113		
IL-18 C137A				
Genotype CC	17 (20.8)	11 (20.9)	0.7 (0.3-1.5) [2]	0.3[4]
Genotype AC	122 (52.2)	42 (52.7)	1.3 (0.8-2.0) [3]	0.3[5]
Genotype AC	132 (27.0)	60 (26.4)		
	271	113		

Table 2. Distribution of *IL-8* and *IL-18* genotypes in patients with diabetic retinopathy (cases) and in those without diabetic retinopathy (controls).

[1]Odds ratio (95% confidence interval), [2]P-value and OR for recessive model (1633: TT versus TC plus CC; 251: AA versus AT plus TT), [3]P-value and OR for dominant model (1633: TT plus TC versus CC; 251: AA plus AT versus TT),[4]P-value and OR for recessive model (607: CC versus AC plus AA; 137: CC versus AC plus AA), [5]P-value and OR for dominant model (607: CC plus AC versus AA; 137: CC plus AC versus AA)

Serum IL-8 levels in 16 diabetics with DR were not statistically significantly different from those of 54 diabetics without DR (48.6±33.9 ng/l *vs.* 51.3±44.6 ng/l; P=0.9).

Serum IL-18 levels in 70 diabetics were not significantly higher than those of 22 controls without diabetes (310.0±234.1 ng/l *vs.* 250.3±190.4 ng/l; P=0.3). Serum IL-18 levels in 16 diabetics with DR were not statistically significantly different from those of 54 diabetics without DR (315.6±234.9 ng/l *vs.* 250.3±190.4 ng/l; P=0.3).

Serum levels	Genotypes (number)	Genotypes (number)	P
IL-8			
IL-8 C1633T	TT (12)	TC + CC (58)	
IL-8 (ng/l)	47.5 ± 33.9	52.5 ± 54.0	0.8
IL-8 A251T	AA (12)	AT+TT (58)	
	46.8 ± 38.5	50.2 ± 51.1	0.9
IL-18			
IL-18 C607A	CC (13)	AC+AA (57)	
IL-18 (ng/l)	287.3 ± 221.7	323.3 ± 257.3	0.6
IL-18 C137A	CC (13)	AC+AA (57)	
IL-18 (ng/l)	241.5 ± 132.7	340.2 ± 167.4	<0.05

Table 3. The serum IL-8 and IL-18 levels in a subpopulation of 70 consecutive diabetics according to different genotypes of IL-8 and IL-18 polymorphisms.

We did not find any statistically significant difference in IL-8 serum levels in diabetics with the TT genotype (12 subjects) compared to those with other (TC + CC) genotypes (Table 3). Moreover, we did not find any statistically significant difference in IL-8 serum levels in diabetics with the AA genotype (12 subjects) compared to those with other (AT + TT) genotypes (Table 3).

We did not find any statistically significant difference in IL-18 serum levels in diabetics with the CC genotype of the 607C/A polymorphism (13 subjects) compared to those with other (AC + AA) genotypes (53 subjects) (Table 3). However, a statistically significant difference in IL-18 serum levels was found in diabetics with the CC genotype of the 137G/C polymorphism (13 subjects) compared to those with other (AC + AA) genotypes (53 subjects) (Table 3).

However, we failed to demonstrate an association between either *IL-8* gene polymorphisms (1633T/C, 251A/T) or *IL-18* gene polymorphisms (607C/A, 137G/C) and DR in Caucasians with type 2 diabetes (Table 2).

4. Discussion

We did not observe an association between either *IL-8* gene polymorphisms (1633T/C, 251A/T) or

IL-18 gene polymorphisms (607C/A, 137G/C) and DR in Caucasians with type 2 diabetes. To our knowledge, the present study is the first attempt to examine whether *IL-8* and *IL-18* gene polymorphisms are involved in the development and progression of DR. Balasubramanyam and co-workers reported that preretinal proliferative membrane formation, which is regulated by various cytokines, is a very important step in the pathogenesis of proliferative diabetic retinopathy [18]. Moreover, significantly higher vitreous levels of IL-8 were reported in patients with proliferative diabetic retinopathy in comparison with the control subjects [19]. Additionally, increased levels of IL-8 in proliferative diabetic retinopathy were reported to be associated with a higher extent of large-vessel gliotic obliteration [20]. The tested polymorphisms of *IL-8* and *IL-18* (1633T/C, 251A/T, 607C/A, 137G/C) have recently been reported to be associated with various forms of cancer [20-25]. Moreover, the -607C/A and the -137G/C polymorphisms of the *IL-18* gene were reported to be associated with susceptibility to type 1 diabetes; the C allele of-137 polymorphism was found to be a risk allele for DR and the A allele of – 607 was found to be a protective allele against DR [14].

In our study, serum levels of IL-8 and IL-18 in 70 subjects with type 2 diabetes and 22 subjects without diabetes were analysed. Serum IL-8 levels in type 2 diabetics were significantly higher compared to non-diabetic subjects, whereas serum IL-18 levels in type 2 diabetics did not statistically significantly differ from those in non-diabetic subjects. Serum IL-8 and IL-18 levels of patients with DR were not statistically significantly different from those of type 2 diabetics without DR. Kretowski and co-workers reported that polymorphisms in the *IL-18* gene were associated with alterations in serum concentrations of IL-18 [26]. Katakami and co-workers reported that serum IL-18 levels are significantly higher in type 1 diabetic subjects compared with non-diabetic subjects (192±80 *vs.* 122±69 ng/ml, P=0.0005), but they also failed to detect a significant association between serum IL-18 levels and the severity of diabetic microangiopathy [27].

Due to the fact that this was a cross-sectional study, cases with DR and control subjects (diabetics without DR) were not matched in age, sex and other clinical parameters. Cases had an earlier onset of diabetes and a longer duration of type 2 diabetes compared to the diabetics without diabetic retinopathy. We suggest that the lack of relationship between these polymorphisms and diabetic retinopathy in our study may be due to the multifactorial nature of DR. These polymorphisms may make either little or no detectable contribution to DR. We speculate that local factors in the eye have a more important role in the pathogenesis of DR than genetic factors. Another explanation for the negative result of the association study might be a type II statistical error, and this type of error is frequently due to sample sizes being relatively small.

In conclusion, our study indicates that the examined polymorphisms of the *IL-8* gene (1633T/C- rs2227543, 251A/T - rs4073) and the polymorphisms of the *IL-18* gene (-607C/A - rs1946518, -137G/C - rs187238) are not associated with DR. Therefore, they may not be used as genetic markers for DR in Caucasians with type 2 diabetes.

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