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Relationship between polymorphisms 804C/A and 252A/G of lymphotoxin-α gene and −308G/A of tumor necrosis factor α gene and diabetic retinopathy in Japanese patients with type 2 diabetes mellitus

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Abstract

To clarify whether polymorphisms of the lymphotoxin-α (LTA) gene and tumor necrosis factor α (TNF-α) gene were related to diabetic retinopathy (DR), we performed a case-control study in 251 Japanese patients with type 2 diabetes mellitus participating in a multicenter research protocol. Genetic analyses were performed by using a fluorescent allele-specific DNA primer assay system. Diabetic retinopathy was diagnosed in a masked manner by an independent ophthalmologist using fundus photographs and was classified as nondiabetic retinopathy (NDR), nonproliferative retinopathy (NPDR), and proliferative retinopathy (PDR). The results showed that the genotype frequencies of 804C/A in exon 3 and 252A/G in intron 1 of the LTA gene were not significantly different among patients with NDR, NPDR, and PDR. A allelic frequency of the TNF-α gene (−302A/G in promoter) was also identical among NDR, NPDR, and PDR groups. Multivariate logistic regression analyses showed that significant associations with DR were glycosylated hemoglobin level and diabetes duration, but not polymorphisms of the LTA gene or TNF-α gene. In conclusion, the present study showed no association between polymorphisms 804C/A and 252A/G of the LTA gene and −302A/G of the TNF-α gene and DR in Japanese type 2 diabetic patients.

1. Introduction

Variation of development of diabetic retinopathy (DR) among individuals is dependent on diabetes duration [1], poor metabolic control [2], and hypertension [3], which promote their progression over time. DR is divided into 2 major categories, nonproliferative retinopathy (NPDR) and proliferative retinopathy (PDR). However, why PDR unlike NPDR affects only a subgroup of patients despite the same glycemic control and diabetes duration remains elusive. In addition to conventional risk factors, many genetic studies [4-11] have suggested the contribution of genetic factors to DR.

Insulin resistance is one of the features of type 2 diabetes mellitus (T2DM) and now has clear linkage with cardiovascular disease and mortality in the diabetic population [12,13]. It has been evident that DR, especially PDR, is associated with insulin resistance [14] and with increased cardiovascular morbidity and mortality [15]. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and lymphotoxin-α (LTA), previously known as TNF-β, play putative roles for insulin resistance [16,17] and hence contribute to the inflammatory process in atherosclerosis. Associations between polymorphisms of TNF-α [18,19] and LTA genes [20,21], and insulin resistance and/or T2DM have been reported. Recently, polymorphism (252A/G in intron 1 and 804C/A in exon 3) of the LTA gene has been identified as a major risk factor for myocardial infarction (MI) in Japanese individuals [22]. Although there have been few reports on the association of

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tumor necrosis factor allelic polymorphism with DR in Caucasian [6] and Indian populations [7], whether this is true for Japanese is currently unknown. We therefore conducted this study to clarify the relationship between polymorphisms 804C/A in exon 3 and 252A/G in intron 1 of the LTA gene as well as −308G/A TNF-α promoter variant and DR in Japanese patients with T2DM.

2. Materials and methods

A case-control study was conducted in 251 Japanese patients with T2DM participating in a multicenter research protocol. The inclusion criteria were age 30 years or older at diagnosis of diabetes and known duration of diabetes of 5 years or more. Diabetes was diagnosed according to 1999 World Health Organization criteria [23]. An ophthalmologist who was masked to the clinical and laboratory data of the patients performed the retinal examination independently. Photographs of 7 standard 30° fields of each eye were taken through dilated pupils in stereo pairs with a Canon CF60 UV fundus camera (Tokyo, Japan). Based on the International Clinical Retinopathy Severity Scale [24], DR was classified as no apparent DR (NDR), nonproliferative retinopathy (NPDR), and proliferative retinopathy (PDR). NDR denotes no signs of DR; NPDR denotes signs of microaneurysm, intraretinal hemorrhage, exudates, macular edema, venous dilatation, soft exudates, peripheral ischemia on fluorescein angiography, intraretinal microvascular abnormalities, and diffuse intraretinal hemorrhage; and PDR denotes signs of neovascularization at the optic disc, neovascularization elsewhere, vitreous hemorrhage, fibrovascular proliferation, and rubeosis iridis. The patients were treated with diet alone (125.5 kJ/kg standard body weight per day) or in combination with oral hypoglycemic agents or insulin therapy. The study protocol was approved by the Institutional Ethics Committee, and all patients gave informed consent.

2.1. Genotyping

Genomic DNA was extracted from peripheral blood. The genotypes of 804C/A and 252 A/G of the LTA gene and −308G/A TNF-α promoter variant were determined with a fluorescent allele-specific DNA primer assay system as described elsewhere [25]. Briefly, the polymorphic regions of the LTA gene and TNF-α gene were amplified by polymerase chain reaction with 2 allele-specific sense primers labeled at the 5′ end with either fluorescein isothiocyanate (5′-GTGAGCAGCAGGTTTGAGXGT-3′ for 804C/A; 5′-CACATTCTCTGTTTCTGCCATXGT-3′ for 252A/G of the LTA gene; 5′-ATAGGTTTTGAGGGGCATXGG-3′ for −308G/A TNF-α gene) or Texas red (5′-GTGAGCAGCAGGTTTGAGXTT-3′ for 804C/A; 5′-CACATTCTCTGTTTCTGCCATXAT-3′ for 252A/G of the LTA gene; 5′-AATAGGTTTTGAGGGGCATXAG-3′ for −308G/A TNF-α gene) and with an antisense primer (5′-ACACCTTCA-GCTGCCCAGAC-3′ for 804C/A; 5′-GTCAGAGAAACCCCAAGGTGAG-3′ for 252A/G of the LTA gene; 5′-TAGGACCCTGGAGGCTGAA-3′ for −308G/A TNF-α gene) labeled at the 5′ end with biotin. The reaction mixture (25 μL) contained 20 ng DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, and 1 U of DNA polymerase (rTaq, Toyobo, Osaka, Japan) in either DNA polymerase buffer. For determination of the genotype, amplified DNA was incubated in a solution containing streptavidin-conjugated magnetic beads in the wells of a well plate at room temperature and measured for fluorescence with a microplate reader (Fluoroscan Ascent, Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 and 538 nm, respectively, for fluorescein isothiocyanate and of 584 and 612 nm, respectively, for Texas red.

2.2. Statistical analysis

Statistical analysis was performed by using StatView version 5.0 (Abacus Concept, Berkeley, CA). Analysis of variance was used to compare clinical and laboratory characteristics among the 3 different groups, and χ^2 analysis was performed between NDR and either NPDR or PDR. We compared the frequency between AA and CC + CA genotypes for 804C/A and between GG and AA + AG for 252A/G of the LTA gene, and GA vs GG for the TNF-α gene. Multivariate regression analysis was used to adjust risk factors, with DR as the dependent variable and age, body mass index, glycosylated hemoglobin (HbA_{1c}), diabetes duration, blood pressure, and the genotype of each polymorphism as the independent variables. We conducted analyses that combined both level of affection status (NPDR and PDR) into a single category (DR) and calculated the odds for affection with any level of retinopathy. The odds ratio (OR) and 95% confidence interval (CI) were also calculated. A P value less than 5% was considered significant. Data are shown as mean ± SD.

Table 1
Clinical characteristics of diabetic subjects with NDR, NPDR, and PDR

	NDR group (n = 176)	NPDR group (n = 36)	PDR group (n = 39)
Age (y)	61.9 ± 10.5	59.7 ± 9.2	64.71 ± 8.1
Sex (male/female)	124/65	26/13	15/25
Duration of diabetes (y)	12.3 ± 6.3	12.6 ± 5.0	15.8 ± 7.2*,#
BMI (kg/m^2)	23.4 ± 3.0	23.2 ± 3.1	24.2 ± 3.3
HbA_{1c} (%)	7.2 ± 1.2	7.6 ± 1.4	7.9 ± 1.3*
SBP (mm Hg)	133 ± 16	129 ± 14	136 ± 13#
DBP (mm Hg)	79 ± 10	76 ± 11	77 ± 11
Total cholesterol (mg/dL)	205 ± 33	195 ± 33	210 ± 44
Triglyceride (mg/dL)	124 ± 74	114 ± 54	98 ± 50
HDL-C (mg/dL)	57 ± 16	60 ± 14	59 ± 16

Data are means ± SD. BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

* $P < .05$ vs NDR.

\# $P < .05$ vs NPDR.

3. Results

Clinical and laboratory data are shown in Table 1. The PDR group showed a longer duration of diabetes, higher levels of HbA_{1c}, and higher systolic blood pressure than the NDR group.

The genotypic distribution of the LTA 804C/A polymorphism was in Hardy-Weinberg equilibrium (C/C, 41.8%; C/A, 41.8%; A/A, 16.4%), and the A allele frequency was 0.37. We found that the genotype of the LTA 252A/G polymorphism in each individual completely corresponded with that of the LTA 804C/A polymorphism (804C/C→252A/A, 804C/A→252A/G, 804A/A→252G/G) and that the allele and genotypic frequencies of the 252A/G polymorphism (AA, 41.8%; AG, 41.8%; GG, 16.4%; G allele frequency, 0.37) were the same as those of the LTA 804C/A polymorphism. In the −308G/A polymorphism in the promoter of the TNF-α gene, 5 (2.0%) subjects were AG heterozygotes and 246 (98.0%) were GG homozygotes. The frequency of the A allele was 0.01. Table 2 shows the relationship between the prevalence of DR and genotypes of the LTA gene and the TNF-α gene. The genotype frequencies of the LTA 804C/A and 252A/G polymorphisms in both NPDR and PDR groups were not significantly different from that in the NDR group. The frequency of genotypes of the TNF-α gene was similar among NDR, NPDR, and PDR groups. There were no differences between A allele carriers of the 804C/A and G allele carriers of the 252A/G in the LTA gene regarding age, duration of diabetes, blood pressure, HbA_{1c}, and serum lipid levels (data not shown). Multivariate logistic regression analyses showed that independent risk factors for DR were diabetes duration (OR, 1.037; 95% CI, 1.001-1.075; $P = .0486$) and HbA_{1c} level (OR, 1.251; 95% CI, 1.014-1.543; $P = .0364$). However, we did not find any significance in polymorphism 804C/A and 252A/G of the LTA gene (OR, 0.851; 95% CI, 0.403-2.1.793; $P = .6705$) and −308G/A genotypes of the TNF-α gene (OR, 0.710; 95% CI, 0.108-4.647; $P = .7206$).

Table 2
Relation between prevalence of retinopathy and genotypes of the LTA gene and TNF-α gene

Variant	NDR (n = 176)	NPDR (n = 36)	PDR (n = 39)
LTA exon 3 804C/A			
Genotype			
CC	71 (40)	14 (39)	20 (51)
CA	77 (44)	17 (47)	11 (28)
AA	28 (16)	5 (14)	8 (21)
χ^2 test (AA vs CC + CA)			
P (vs NDR)		.761	.442
OR (95% CI)		0.853 (0.305-2.382)	1.364 (0.568-3.275)
LTA intron 1 252A/G			
Genotype			
AA	71 (40)	14 (39)	20 (51)
AG	77 (44)	17 (47)	11 (28)
GG	28 (16)	5 (14)	8 (21)
χ^2 test (GG vs AA+AG)			
P (vs NDR)		.761	.442
OR (95% CI)		0.853 (0.305-2.382)	1.364 (0.568-3.275)
TNF-α promoter −308G/A			
Genotype			
GG	172 (98)	35 (97)	39 (100)
GA	4 (2)	1 (3)	0 (0)
χ^2 test			
P (vs NDR)		.856	.342
OR (95% CI)		0.988 (0.585-1.668)	0.485 (0.026-9.198)

Data are n (%) unless otherwise indicated.

4. Discussion

Many genetic studies have reported on the association of gene polymorphisms with the development of DR [4-11]. However, the linkage of these gene polymorphisms with DR is still controversial because some specific genotypes of genes are associated with a more rapid course of DR, whereas others do not increase the frequency of DR. In the present study, we examined whether polymorphisms 804C/A and 252A/G of the LTA gene and −308G/A TNF-α promoter variant, which are related to insulin resistance and/or T2DM [18-21], were associated with DR in Japanese patients with T2DM, but did not identify any significant relationship between these variants of the LTA and TNF-α genes and DR.

There has been evidence that DR, especially PDR, was associated with insulin resistance [14] and increased cardiovascular morbidity and mortality [15]. These observations suggest a possible contribution of the inflammatory cytokines and polymorphisms of these genes to the pathogenic process, resulting in diabetic microvascular complications in T2DM. In particular, TNF-α has been known to play a causal role for insulin resistance [16,17] by interfering with the insulin signaling [26]. Several reports have provided the evidence for positive associations between −308G/A TNF-α promoter variant and insulin resistance and/or diabetes [18,19]. In the present study, the frequency of the −308G/A polymorphism of the TNF-α gene was 0.01, which is the same as that (0.01) previously reported in the Japanese [27] but much lower than those (0.11-0.17) in Caucasians [28,29]. Hayakawa et al [27] reported that this polymorphism did not influence the clinical and metabolic characteristics of insulin resistance in Japanese, accounting for one of the reasons for racial difference of insulin resistance.

Lymphotoxin-α is another proinflammatory cytokine that is known to play multiple roles in the regulation of the immune system and inflammatory reactions. The relationship between LTA and insulin resistance has been investigated because the LTA gene and the TNF-α gene are located close to each other in the HLA class III region [30]. The last exon of the LTA gene shares 56% sequence homology with the TNF-α gene. An *Nco*I polymorphism of the first intron of LTA gene influenced the secretory

capacity of TNF-α, and this polymorphism was related to insulin resistance and fasting glycemia in Japanese and Caucasian subjects [27,31]. Evidence implicating the polymorphism in the LTA gene (T60N) with increased susceptibility to T2DM has emerged from Japanese and Danish studies [20,21]. Moreover, a recent Japanese genomewide study [22] showed a high association between variations in the LTA gene and MI. In contrast, another study has provided no evidence that the specific LTA or TNF-α variants influence susceptibility to T2DM in UK populations [32], and no relationship of polymorphisms of the LTA gene with MI was observed in Japanese [20]. Nevertheless, involvement of the LTA gene in susceptibility to T2DM or cardiovascular disease is still controversial and needs large collaborative investigations.

Notably, the present study ascertained for the first time that there was no significant relationship between LTA 804C/A and 252A/G polymorphisms as well as −308G/A TNF-α promoter variant and DR in the Japanese population. In this study, the genotype of the LTA 804C/A polymorphism in each individual completely corresponded with that of the LTA 252A/G polymorphism, which is consistent with other reports showing that 804C/A and 252A/G polymorphisms of the LTA gene exhibited marked linkage disequilibrium in Japanese subjects [20,22]. Multivariate logistic regression analyses showed that significant associations with DR were HbA_{1c} level and diabetes duration, but not LTA 804C/A and 252A/G polymorphisms. There have been a few reports on the association of tumor necrosis factor allelic polymorphism with DR in Caucasian [6] and Indian populations [7], which were inconsistent with our study. Differences of allele frequencies of the genes examined among studies may explain the discrepancies of the results. In diabetes, hyperglycemia itself is the major determinant for development of diabetic microangiopathy via activation of protein kinase C or formation of advance glycated end products and so on [33,34]. Nonetheless, the present study has some limitations and needs cautious interpretation because this is a cross-sectional study investigating only 3 polymorphisms: 804C/A and 252A/G of the LTA gene and −308G/A of the TNF-α gene. Furthermore, we did not perform haplotype evaluation because the genotype of the LTA 804C/A polymorphism in each individual completely corresponded with that of the LTA 252A/G polymorphism, and the A allele frequency of the −308G/A polymorphism of the TNF-α gene was very low (0.01). Therefore, additional untyped variants in the gene and environmental factors may affect the development of DR in T2DM, and a large prospective genomewide study will be needed to elucidate the development of DR.

In conclusion, we showed that polymorphisms 804C/A and 252A/G of the LTA gene as well as −308G/A TNF-α promoter variant were not related to possible genetic modification of susceptibility to DR in Japanese subjects with T2DM.

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