Promoter polymorphism at the tumor necrosis factor / lymphotoxin-alpha locus is associated with type of diabetes but not with susceptibility to sight–threatening diabetic retinopathy.

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Abstract

Aims: To investigate, in a large cohort of 2494 individuals with diabetes mellitus (DM) whether functional single nucleotide polymorphisms (SNPs) in the promoter region of *Tumor necrosis factor* (*TNF)* and *lymphotoxin-alpha* (*LTA)* genes are associated with type of DM or presence of diabetic retinopathy (DR).
Methods: 334 type 1 DM (T1DM) and 999 type 2 DM (T2DM) participants with sight threatening DR (STDR), and 260 TIDM and 901 T2DM participants with no DR or minimal NPDR, were genotyped for 2 SNPs (rs1800629 and rs361525).
Results: The A allele of rs1800629 was associated with T1DM ($p<0.001$; OR 0.62). After adjustment for age, sex, DM duration, HbA1c, hypertension and nephropathy, no significant association was found between rs1800629 or rs361525 and STDR.
Conclusions: An association between the A allele of rs1800629 and type of diabetes was found. No association was found between 2 promoter variants of *TNF* and *LTA*, and DR in a large cohort of Caucasian patients with T1DM and T2DM.

Key words

Diabetic retinopathy, genetics, tumor necrosis factor, lymphotoxin-alpha, diabetes

Running heading

TNF / LTA locus and diabetic retinopathy

Acronyms

DM	Diabetes Mellitus
DR	Diabetic Retinopathy
DN	Diabetic Nephropathy
AGEs	Advanced Glycation End Products
TNF	Tumor Necrosis Factor
LT-α	Lymphotoxin-α
SNP	Single nucleotide polymorphism
HLA	Human Leukocyte Antigen
T2DM	Type 2 Diabetes Mellitus
T1DM	Type 1 Diabetes Mellitus
HREC	Human Research Ethics Committees
ETDRS	Early Treatment Diabetic Retinopathy Study
STDR	Sight-threatening DR
NPDR	Non-proliferative DR
PDR	Proliferative DR
DME	Diabetic Macular Edema
SPSS	Statistical Package for Social Sciences

DM is a disease of increasing prevalence, associated with significant mortality and morbidity. Microvascular complications of DM, including DR and DN, are of complex etiology. Pathological changes to retinal microvasculature are caused by a cascade of events involving the formation of AGEs, in the presence of sustained hyperglycemia [1]. The interaction of AGEs with their receptors (RAGEs) on the surface of macrophages and endothelial cells results in an increase in the synthesis and secretion of pro-inflammatory cytokines including TNF (previously known as TNF-α)[1]. TNF is implicated in the pathogenesis of DR, through its contribution to blood-retinal barrier breakdown and neovascularisation[1]. Another pro-inflammatory cytokine LT-α (previously known as TNF-β) binds the same TNF receptors, with similar downstream effects [2].

TNF promoter polymorphisms have been extensively studied across a range of diseases with rs1800629 (TNF -308) and rs361525 (TNF -238) being the most widely investigated SNPs. Individuals homozygous for the less common TNF -308 A allele have been shown to have higher circulating TNF levels than those homozygous for the G allele, and have worse outcomes in response to infectious diseases[3]. The TNF -238A allele (rs361525) has also been implicated in a number of disease states with the A allele conferring protection against autoimmune diseases including rheumatoid arthritis[3].

Both the *TNF* and *LTA* genes are located on chromosome 6 in close proximity to each other, in the HLA class III region. We conducted this study to investigate the regulatory regions of the *TNF* and *LTA* genes, by investigating 2 promoter polymorphisms (rs1800629 and rs361525) known to influence *TNF* and *LTA* expression, and their relationship to DM and DR in a large and well-characterised cohort of 2494 Caucasian patients with T1DM and T2DM.

Methods:

Individuals with DM were recruited from ophthalmology and endocrine clinics in Australia and the United Kingdom, including: Flinders Medical Centre, The Royal Adelaide Hospital, The Queen Elizabeth Hospital, The Royal Melbourne Hospital, Royal Victorian Eye and Ear Hospital, St Vincent's Hospital, Sydney Eye Hospital, The Repatriation General Hospital and Canberra Hospital in Australia; and The National Institute for Health Research Biomedical Research Centre at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology, London, United Kingdom. The study was approved by HREC in Australia (Southern Adelaide Clinical HREC; Royal Adelaide Hospital HREC; TQEH/LMH/MH HREC; Royal Melbourne Hospital HREC; Royal Victorian Eye and Ear Hospital HREC; StVincent's Hospital HREC; South Eastern Sydney Illawarra HREC) and The NHS Health Research Authority in London. Written informed consent was obtained from each participant and the project conformed to the tenets of the Declaration of Helsinki.

A detailed description of recruitment protocols and study participants has been reported previously [4]. In brief, patients were required to meet the following inclusion criteria: (1) at least 18 years of age, and (2) on medical treatment for DM (either oral hypoglycemic agents or insulin therapy). Individuals with T2DM were required to be on medical treatment for DM for at least 5 years prior to inclusion in the study. Social, demographic and medical history was collected via a questionnaire. Retinopathy status was established via ophthalmic examination in accordance with the modified ETDRS criteria. STDR was based on the patient's worst ever DR grading, and was defined as the presence of either severe NPDR, PDR, or DME, in at least one eye. Controls were defined as those whose retinopathy grading had never been worse than minimal NPDR, with no history of DME in either eye. An additional 1445 Individuals with DM included in this analysis were recruited since the previous report using identical protocols.

DNA was extracted from whole blood using QIAamp Blood DNA Maxi Kits (Qiagen). SNPs rs1800629 and rs361525 were genotyped using iPLEX Gold chemistry on an autoflex Mass Spectrometer (Sequenom, San Diego, CA). SNPs were in Hardy-Weinberg equilibrium. Baseline characteristics of cases and controls were

compared using the Mann-Whitney U test for non-parametric, continuous variables, and χ^2 test for dichotomous variables, using SPSS version 20.0 for Mac OS X (IBM SPSS Statistics 20.0, SPSS Inc., Armonk, NY, USA). Testing for association of each SNP with DR was undertaken with the χ^2 test for univariate analysis and binary logistic regression for multivariate analysis in PLINK (version 1.06).

Findings and discussion:

2494 individuals with DM were recruited and genotyped for this study. 1161 of these had No DR or minimal NPDR (260 T1DM and 901 T2DM), 189 had severe NPDR (25 T1DM and 164 T2DM), 734 had PDR (252 T1DM and 482 T2DM) and 909 had diabetic macular edema (DME) (140 T1DM and 769 T2DM). 499 of those with DME also had co-existing PDR or severe NPDR.

Neither rs1800629, nor rs361525 are associated with STDR, PDR or DME:

This study found no association between SNPs rs1800629 and rs361525 and STDR, PDR or DME in either T1DM or T2DM patients, in a multivariate logistic regression analysis controlling for age, sex, duration of DM, HbA1c, hypertension and nephropathy (Table 1).

The rs1800629 promoter SNP has previously been investigated in relation to DR, in a large study including 742 T1DM and 2957 T2DM Caucasian patients[5]. Cases with STDR were compared with controls with no DR or NPDR and no significant association between this promoter polymorphism and STDR risk was found. This finding is comparable to the results from our study, which evaluated differences between phenotypic extremes by comparing controls with no DR or minimal NPDR to cases with STDR. Furthermore, rs1800629 has been studied in smaller Chinese and Japanese cohorts with T2DM, again with no significant association found in either ethnic group[6,7]. Conversely, Sesti et al. recently reported a positive association between the A allele of rs1800629 and an increased risk of PDR in a cohort of Brazilian Caucasian patients [8]. To date no association has been found between rs361525 and DR, consistent with findings from the current study.

Functional studies have suggested a role for TNF as a biomarker for DR. Circulating levels of TNF are increased in the serum of patients with T1DM and PDR compared to patients with DM but without retinopathy[9]. mRNA expression of *TNF* as well as the level of soluble TNF receptors, are elevated in the vitreous of patients with PDR [10]. Furthermore, inhibition of TNF with angiopoietin-1 has shown promising outcomes in preventing early DR in a diabetic rat model [11].

There is increasing evidence to suggest that a complex interaction exists between *TNF, LTA* and other genes in the HLA class III region, and this may explain the discrepancy between genetic and functional work to date. Alleles within the MHC region are in strong linkage disequilibrium making a direct association between SNPs in this region and TNF phenotypes less likely. It has been postulated that alternative pathways may be responsible for TNF expression including regulation by linked genes, interaction between the 3'UTR outside the TNF promoter and the -308 element, and epigenetic control via TNF promoter methylation[3]. Further investigation of the relationship between these genes and factors modulating their expression is required to gain a better understanding of the role of these pro-inflammatory cytokines in the pathogenesis of DR.

Rs1800629 was significantly associated with DM type:

In our current study, the frequency of the rs1800629 A allele was significantly higher in patients with T1DM compared with patients with T2DM (MAF 23.5% in T1DM compared with 15.9% in T2DM) (OR 0.62; 95% CI 0.53-0.71; P<0.001), consistent with previously reported findings. Our study also showed that the MAF of rs1800629 was significantly higher in those without hypertension (OR 0.848; 95% CI 0.73-0.98; P=0.025), subjects of younger age (t=-4.0; P<0.001), and those with longer duration of diabetes (t=2.44; P=0.01) when all diabetics were analysed together. These differences reflect the risk factor profile of the T1DM cohort, and

are driven by the T1DM association. Each of the above mentioned significant associations survived Bonferroni correction (P<0.025). Rs361525 was not found to be associated with any covariates tested.

Resent research has shown that the TNF rs1800629 allele is in linkage disequilibrium with the MHC haplotype HLA-A1-B8-DR3 [12], which may explain the functional connection between the A allele, high TNF production and insulin dependent DM[3,13]. There is no clear evidence to suggest a direct role of SNPs in the *TNF/LTA* locus increasing T1DM susceptibility, independent of HLA DR- and DQ- haplotypes[13].

Conclusion:

In conclusion, the risk allele of rs1800629 (TNF -308A) is associated with T1DM. We found no association between either of the 2 polymorphisms in the promoter region of *TNF* and *LTA* and STDR, DME or PDR, in patients with either T1DM or T2DM. It is clear that increased levels of TNF, both locally and systemically, are associated with DR risk, however further investigation of the complex interplay between *TNF,* and *LTA* genes with regards to the HLA haplotypes, and epigenetic modifications is required to identify the specific effect of these variants on microvascular complications of DM, including DR, and determine the underlying biological drivers of these elevated levels.

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Figures and tables:

Table 1: Allelic association of rs1800629 (-308G>A) and rs361525 (-238G>A) with STDR, PDR and DME for T1DM and T2DM. Results for both unadjusted and adjusted analyses are presented. Uncorrected P-values are shown.

	T1DM				T2DM			
	Unadjusted		Adjusted		Unadjusted		Adjusted	
SNP	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value***	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value***
rs1800629								
STDR	1.08 (0.82-1.42)	0.590	0.95 (0.64-1.42)	0.812	0.99 (0.83-1.17)	0.864	0.95 (0.76-1.19)	0.672
PDR	1.18 (0.88-1.58)	0.257	1.02 (0.66-1.57)	0.944	0.94 (0.76-1.17)	0.579	0.91 (0.69-1.21)	0.536
DME	0.99 (0.70-1.41)	0.967	1.39 (0.84-2.30)	0.199	0.94 (0.78-1.14)	0.540	0.95 (0.75-1.21)	0.675
rs361525								
STDR	0.92 (0.58-1.44)	0.708	0.67 (0.33-1.35)	0.264	1.02 (0.78-1.33)	0.905	1.00 (0.69-1.43)	0.976
PDR	0.82 (0.50-1.35)	0.433	0.60 (0.27-1.32)	0.200	0.86 (0.61-1.21)	0.380	0.67 (0.40-1.13)	0.137
DME	1.15 (0.67-1.98)	0.616	0.83 (0.36-1.89)	0.653	0.97 (0.73-1.30)	0.834	0.90 (0.61-1.33)	0.602

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; STDR, sight threatening diabetic retinopathy; PDR, proliferative diabetic retinopathy; DME, diabetic macular edema; OR (95%CI), odds ratio with 95% confidence interval.
*Adjusted for age, sex, duration DM, HbA1c, hypertension, nephropathy.