Comparative analysis of the genetic associations of HLA-DR3 and tumour necrosis factor alpha with human IDDM

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Summary Insulin-dependent diabetes mellitus (IDDM) is associated with class II molecules of the MHC on chromosome 6, in particular HLA-DR and -DQ alleles, but a pathogenic role for TNF- α in the class III region of the MHC has also been implied. We therefore tested whether there was any independent association between a biallelic TNF polymorphism and IDDM. The TNF2 allele was present in 61 of 114 (54%) IDDM patients compared to 101 of 253 (40%) control subjects

(odds ratio 1.73; $p < 0.02$). Stratification analysis in individuals matched for HLA-DR3 revealed, however, that this association was not independent of HLA-DR3 and is most likely to be a result of linkage disequilibrium between these alleles. [Diabetologia (1994) 37: 500–503]

Key words IDDM, MHC, tumour necrosis factor alpha, polymorphism.

The central event in the pathogenesis of IDDM is the cell-mediated destruction of beta cells in pancreatic islets leading to reduced secretion of insulin, and eventually hyperglycaemia. The aetiology of the disease is complex but seems to involve an interaction between genetic and environmental factors. The strongest genetic associations of IDDM are with alleles of the MHC class II genes, located on chromosome 6p21 [1]. The DR3 and DR4 alleles of the HLA-DRB1 gene confer a high relative risk for IDDM, and DR3/DR4 heterozygosity increases susceptibility markedly compared with either allele alone [2, 3]. Nucleotide sequencing of HLA-DQB1 alleles has suggested that the presence of an aspartate residue at position 57 of the HLA-DQ β chain may protect against diabetes [4, 5]. However, this does not seem to be true in Japanese sub-

jects [6, 7], and more recent studies have indicated that both DR and DQ loci influence susceptibility to and protection from IDDM [8–10].

The high degree of linkage disequilibrium in the MHC region, and the large number of polymorphic loci, has complicated efforts to define specific susceptibility genes, but it is clear that genes outside of the class II region, carried on disease-associated haplotypes, could also be important. Several DR3- and DR4-containing ancestral haplotypes have been described that are associated with IDDM [11, 12]. Comparison of these haplotypes, which share a rare allele of the BAT 3 gene, in diabetic patients and control subjects has indicated that some of the genes relevant to IDDM may be located in the central MHC region between the BAT 3 gene and HLA-B [13]. Interestingly, this region contains the gene for TNF- α [14, 15], a potential molecular effector in the pathogenesis of IDDM [16, 17].

We have recently described a human TNF- α polymorphism which lies in the promoter region of the TNF- α gene and involves the substitution of guanine by adenine in the uncommon (TNF2) allele [18]. We also showed that TNF2 is strongly associated with the “autoimmune haplotype” HLA-A1-B8-DR3 in a northern European population [19]. We have now screened for TNF- α genotypes in HLA-DR typed

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; TNF- α , tumour necrosis factor alpha; PCR, polymerase chain reaction; SSCP, single stranded conformation polymorphism.

diabetic patients and control subjects to determine whether the TNF- α polymorphism may contribute to the MHC association with susceptibility to IDDM.

Subjects and methods

Patients and control subjects

The study group comprised 114 IDDM patients and 253 unrelated healthy control subjects. All study subjects were Caucasian and living in the north of England. The diabetic patients fulfilled World Health Organization criteria for diagnosis of IDDM [20]. The mean age of onset of IDDM was 25.64 ± 15.11 years. This study was approved by the South Sheffield Research Ethics Committee, and informed consent was obtained from all the subjects.

DNA preparation

Blood samples (20 ml) were collected in vacutainer tubes containing EDTA, and DNA was extracted essentially as described elsewhere [21]. Briefly, erythrocytes were lysed in buffer containing 1% Triton X-100; the remaining white cell pellet was treated with 0.5% SDS and digested with proteinase K (Promega, Southampton, UK) for 16 h at 37°C. The DNA was then extracted with phenol and chloroform and precipitated from ethanol.

PCR amplification of TNF- α

The TNF- α amplification was performed as previously described [19]. Genomic DNA (100 ng) was used for amplification, in a total volume of 50 μ l containing 0.2 μ mol/l concentrations of each primer, 1.25 units Taq DNA polymerase (Promega), 200 μ mol/l of each deoxynucleotide triphosphate (Promega), 10 mmol/l Tris-HCl pH 9.0, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, and 0.1% Triton X-100 (Promega). Negative controls without template DNA were performed in each experiment.

SSCP analysis

Of the PCR product 50 μ l were heated to 95°C for 5 min in the presence of 12 μ l 100% deionised formamide. The samples were loaded on a 9% polyacrylamide gel and electrophoresed at 4 V/cm, 4°C for 16 h. The gels were stained with ethidium bromide and visualised under ultraviolet light.

HLA DR typing

PCR amplification of DR β 1 alleles was carried out using the primers 5'-CCCCACAGCACGTTTCTTG-3' and 5'-CCGCTG CACTGTGAAGCTCT-3'. Amplified products were analysed by dot-blot hybridization using the British Society for Histocompatibility and Immunogenetics sequence-specific oligonucleotide probes according to the method of Vaughan et al. [22, 23]. Oligonucleotide probes were labelled with digoxigenin-ddUTP and detected using alkaline phosphatase conjugated antibody to digoxigenin followed by the chemiluminescent substrate 3-(2'-

Table 1. Genotype frequencies in IDDM patients and control subjects

Genotype	Patients	Control subjects	<i>p</i>	Odds ratio
<i>TNF</i>	<i>n</i> = 114	<i>n</i> = 253		
(1,1)	53 (46.5)	152 (60.1)	0.013 ^a	
(1,2)	48 (42.1)	89 (35.2)		
(2,2)	13 (11.4)	12 (4.7)		
(1,2) or (2,2)	61 (53.5)	101 (39.9)	0.015	1.73 (1.11–2.69) ^b
<i>DR3</i>	<i>n</i> = 66	<i>n</i> = 106		
(3,X) or (3,3)	42 (63.6)	40 (37.7)	0.001	2.89 (1.52–5.45) ^b

Numbers of individuals in each group with the given genotype, with percentages in parentheses. ^a Chi-square probability for the comparison of the three genotypes in patients and control subjects. ^b 95% confidence interval for the odds ratio (approximate relative risk) for carriage of TNF2 or DR3. X represents any DRB1 allele except DR3

Table 2. Analysis of DR3 and TNF2 matched individuals

	Patients		Control subjects		<i>p</i>
	TNF2 +	TNF2 –	TNF2 +	TNF2 –	
DR3 +	31	9	32	8	> 0.999
DR3 –	3	21	17	46	0.253
DR3/4	16	7	7	4	0.717

Numbers of patients and control subjects classified according to TNF2 and DR3 genotypes

spiroadamantane)-4-methoxy-4-(3'-phosphoryloxyphenyl)-1,2-dioxetane (AMPPD).

Statistical analysis

Associations between IDDM and genotype were analysed by the chi-square test, and Fisher's exact test was used to determine probability where appropriate. *P*-values are uncorrected. Odds ratios and 95% confidence limits were calculated on the rates of carriage of alleles as previously described [24].

Results

A significant difference in the frequency of the TNF2 allele was observed between the IDDM patients and the control subjects (32.4% and 22.3%, respectively; *p* = 0.004). Since we had previously shown that the TNF2 allele is strongly associated with HLA-DR3 [19], we carried out HLA-DR typing to allow comparison of the allele frequencies for TNF2 and DR3. As expected, a similar increase in allele frequency in diabetes was observed for DR3 (32.6% in patients and 18.9% in control subjects; *p* = 0.004). Table 1 shows the genotype frequencies for TNF and DR3, and indicates that there was also a significant difference in the frequencies of the TNF genotypes between patients and control sub-

Table 3. MHC haplotypes carrying the TNF2 allele

Cell line	DP	DQ	DR	Dw	TNF	B	C	A
STEINLIN	3/4	2	3	3	2	8		1
VAVY	1	2	3	3	2	8	7	1
COX	3	2	3	3	2	8	7	1
WT47	2	6	13	19	2	44	5	32
DKB	4	9	9	23	2	60	10	24
SPO010	2	5	11	DB2	2/1	44	5	2
BM16	2	7	12	DB6	2/1	18	7	2

Of 44 homozygous cell lines, the seven listed here carry the TNF2 allele. All others carried TNF1

jects ($p = 0.013$; Table 1). The odds ratio (approximate relative risk) for carriage of the TNF2 allele and DR3 alleles were 1.73 ($p = 0.015$; 95% confidence limits 1.11–2.69) and 2.89 ($p = 0.001$; 95% confidence limits 1.52–5.45), respectively (Table 1). There was no significant difference in the mean age of onset of disease between the three TNF genotypes.

Having established that TNF2, like DR3, conferred a risk for diabetes, we assessed whether these risks were independent using a stratification analysis. Control subjects and patients were classified into double positive (TNF2 + /DR3 +), positive/negative (TNF2 + /DR3 –), negative/positive (TNF2 – /DR3 +) and double negative (TNF2 – /DR3 –) genotypes. This analysis revealed that amongst individuals matched for their DR3 genotype, there were no significant differences in the proportions of patients and control subjects with respect to carriage of the TNF2 allele (Table 2). In addition, there was no significant difference in TNF2 carriage rate when HLA-DR3/4 heterozygous patients and control subjects were compared (Table 2). Therefore we found no evidence for a DR3-independent association of the TNF2 allele with diabetes.

These results indicated that the association of TNF2 with diabetes may be accounted for by linkage disequilibrium between the TNF and HLA-DR3 loci. We therefore analysed a panel of 44 cell lines homozygous at their MHC class I and class II loci to investigate which MHC haplotypes carried the TNF2 allele. The TNF2 positive cell lines are listed in Table 3. Of the 44 cell lines, three carried the HLA-A1, -B8, -DR3 haplotype and all of these also carried the TNF2 allele (Table 3). The TNF2 allele was not found on any other DR3 haplotypes, nor was it found on the one B8-positive DR3-negative haplotype, HLA-A26, -B8, -DR15. The TNF2 allele was found on four other haplotypes; these are also listed in Table 3.

Discussion

We found a strong association of the TNF2 allele with IDDM in a Caucasian population living in the north of England. However, the association was not independent of the association of HLA-DR3 with diabetes,

indicating that it is probably secondary to linkage disequilibrium with DR3. The linkage disequilibrium was confirmed by TNF2 typing of a panel of homozygous cell lines. This analysis showed that the HLA-A1, -B8, -DR3 haplotype always carried the TNF2 allele, in agreement with our previous association data [19]. The higher relative risk we observed for DR3 in diabetes may in part be accounted for by the absence of TNF2 on the B18, -DR3 haplotype which is also associated with diabetes. It is of interest to note that an independent study in a Danish population has also shown that the association between TNF- α and IDDM could not be separated from the DR3 association [25].

Since high TNF- α production levels have been associated with particular DR3 and DR4 haplotypes [26–28], it is tempting to speculate that TNF production phenotypes may be important determinants of the disease susceptibility conferred by these haplotypes. If this were the case, it would be expected that TNF region alleles would be markers for both TNF production and disease susceptibility. The TNF- α polymorphism described here lies in the promoter region of the gene, and therefore would be a strong candidate to play a functional role in TNF gene regulation. To address this question, we have analysed TNF- α production from lipopolysaccharide-stimulated human monocytes in relation to genotypes. We found that TNF1/2 heterozygous individuals had a slightly higher TNF response than TNF1/1 homozygotes, but this difference was not statistically significant [25]. Therefore, in support of the genetic data, we are not able to establish any functional role for this polymorphism at the present time.

There are several other polymorphic markers at the TNF locus, including five microsatellite markers and an Nco I restriction fragment length polymorphism [29–32], which have now been assigned to extended MHC haplotypes [33]. Certain TNF microsatellites have been shown to correlate with TNF- α production levels from stimulated monocytes, and to be more frequent in DR-matched patients compared to control subjects [27]. However, these do not map to regions likely to be involved in gene regulation; therefore it seems likely that they may be acting as markers for as yet undiscovered polymorphisms in this region of chromosome 6.

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