No evidence of a significant role for CTLA-4 in multiple sclerosis

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Abstract

Variation in the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) gene plays a significant role in determining susceptibility to autoimmune thyroid disease and type 1 diabetes. Its role in multiple sclerosis is more controversial. In order to explore this logical candidate more thoroughly we genotyped 771 multiple sclerosis trio families from the United Kingdom for the 3'untranslated region variable number tandem repeat, the CT60 single nucleotide polymorphism (SNP) and five haplotype-tagging SNPs. No individual marker or common haplotype showed evidence of association with disease. These data suggest that any effect of CTLA-4 on multiple sclerosis susceptibility is likely to be very small.

Key words: multiple sclerosis, cytotoxic T-lymphocyte-associated protein 4, CTLA-4, genetic association, disease severity

1. Introduction

Variation in the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) gene plays a significant role in determining susceptibility to autoimmune thyroid disease and type 1 diabetes (Ueda et al., 2003). In multiple sclerosis its influence on disease susceptibility (Ligers et al., 1999, Fukazawa et al., 1999, Harbo et al., 1999, Rasmussen et al., 2001, Dyment et al., 2002, Maurer et al., 2002, Kantarci et al., 2003, van Veen et al., 2003, Alizadeh et al., 2003, Teutsch et al., 2004) and course (Masterman et al., 2002, Maurer et al., 2002) has been studied in a variety of populations. A trend towards association with the common short (8 repeat) allele of the variable number tandem repeat (VNTR) polymorphism in the 3' untranslated region (3'UTR) of the gene has been reported (Kantarci et al., 2003). In nine other case control studies (Teutsch et al., 2004) no consistent results have emerged. We systematically studied the gene in a large cohort of multiple sclerosis trio families (an affected individual and both parents). We studied the 3'UTR VNTR, and six single nucleotide polymorphisms (SNPs): CT60, which was identified by Ueda *et al*. (Ueda et al., 2003) as showing the strongest linkage disequilibirum with type 1 diabetes and thyroid disease, and five haplotype tagging SNPs (htSNPs) (T-1722C, A-1661G, C-658T, C-319T and A49G) described by Johnson *et al*. (Johnson et al., 2001) See figure 1.

2. Patients and Methods

Patients

The 771 patients included in this study were recruited throughout the UK. All patients satisfied the Poser criteria (Poser et al., 1983) for definite (95%), or probable (5%) multiple sclerosis; 60% had relapsing, 31% secondary progressive and 9% primary progressive multiple sclerosis. The mean age at diagnosis was 30.9 years; female to male ratio was 3.0:1. All patients and their parents gave written informed consent.

Genotyping

All primers are listed in table 1.

3'UTR VNTR

The 3'UTR VNTR was genotyped according to previously described methods (Nistico et al., 1996). PCR was performed in 10 µl reactions using: 50 ng genomic DNA, 0.5 U *Taq* DNA polymerase, 200 µM dNTPs, 1.6x PCR Buffer and 2 mM $MgCl_2$, 0.125 µM primers. Reactions were carried out on an Applied Biosystems (ABI) 9700 Integrated Thermal Cycler using a 58 ºC touchdown protocol (Don et al., 1991). The products were separated on an ABI Prism 3700 DNA analyser. Genotyping was completed using ABI GENESCAN and GENOTYPER software.

CT60

The CT60 SNP was genotyped using a TaqMan Assay By Design performed according to the manufacturers standard conditions using ABI 9700 Integrated Thermal Cycler for PCR and a 7900 HT Sequence Detection System with Allelic Discrimination Software for genotyping.

Haplotype Tagging SNPs

The htSNPs were genotyped using a specifically designed multiplex *SNaPshot* assay performed according to the manufacturers standard conditions. Two primary PCR fragments were amplified the first encompassing SNPs T-1722C and A-1661G, and the second SNPs C-658T, C-319T and A49G (figure 1). The products from these amplifications were combined in equal volumes prior to primer extension. Primer extension products were separated and sized on a 3700 DNA analyser

Statistical analysis

Evidence for association with susceptibility was sought using the TRANSMIT program (Clayton, 1999) to perform transmission/disequilibirum testimg (TDT). Chi square was used to compare haplotype frequencies between primary progressive and bout onset patients. Disease severity was measured using the Multiple Sclerosis Severity Score (MSSS) (Roxburgh et al., 2005), and multiple linear regression was used to search for evidence of association with severity. Prior to analysis, Hardy Weinberg equilibrium was confirmed

for each marker using the PEDSTATS program; genotyping errors were identified and excluded using the PEDCHECK program (O'Connell and Weeks, 1998).

3. Results

Disease Susceptibility

TDT analysis revealed no statistically significant evidence for association of any of the SNPs or haplotype tagging haplotypes (table 2). There was a trend to over- transmission of the 8 repeat allele of the 3'UTR VNTR and under transmission of the CT60 G allele over-represented in Graves' disease and type 1 IDDM over. The six common haplotypes occurred in a similar frequency to that previously reported (Johnson et al., 2001) and accounted for 99.7% of observed haplotypes.

We also analysed the data from all seven markers combined using TRANSMIT. No statistically significant evidence for association was seen with any of the common haplotypes or in a global analysis. A trend towards over- transmission of both haplotypes containing the A allele of CT60 (undertransmitted in IDDM and autoimmune thyroid disease) was seen. These haplotypes also contain the 8 repeat allele of the 3'UTR VNTR.

Alizadeh *et al*. (Alizadeh et al., 2003) have suggested an interaction between the third htSNP (C- 658T) and HLA DR15 status with over-

transmission of the C allele only amongst DR15 positive patients. We stratified the available data for this marker in the 716 (93%) families where the DR15 status of the index case was known. Transmission of the C allele at –658 was not significantly distorted and, in fact, we observed a trend toward *under*-transmission of this allele, which was similar in both the HLA DR15 positive and negative patients.

Disease Course

Disease course was known in 744 patients. We could deduce precise htSNP haplotypes in 43/72 patients with primary progressive disease and 550/672 patients with bout onset disease. Using these data we were unable to confirm the previous finding of an association between haplotypes containing the C allele at -C319G and the G allele at A49G (allele 1 of htSNPs 4 & allele 2 of htSNP5) with primary progressive disease (Masterman et al., 2002, Maurer et al., 2002): proportions of such haplotypes were 37.7 %amongst primary progressive patients and 36.2% amongst bout onset cases (p=0.40). Homozygosity for these haplotypes was similarly non-discriminative, occurring in 14.3% of primary progressive and 13.3% of bout onset patients (p=0.84).

Disease Severity

Excluding the 61 patients whose disability was assessed at a time of relapse (n=59) or within the first year from disease onset (n=2) the average EDSS in the remaining 710 index cases was 4.5 and the mean duration of disease 12.6 years. Within the cohort, the mean MSSS

(Roxburgh et al., 2005) was 5.36. None of the individual polymorphisms or common haplotypes formed by the htSNPs was associated with disease severity ($p>0.36$, multiple linear regression).

4. Discussion

We have comprehensively analysed the variation from the CTLA-4 gene in a large cohort of multiple sclerosis trio families and found no evidence for association with any marker or common marker haplotype. A trend towards over-transmission of the 8 repeat allele from the 3'UTR VNTR (Kantarci et al., 2003) was seen. Other previously described findings of an epistatic interaction between the htSNP C-658T and HLA DR15, and association between haplotypes of the -C319G and A49G SNPs and disease course, were not confirmed. Analysis of the CT60 SNP revealed a trend to under transmission of the common G allele in contrast to the data from Graves' disease and type 1 diabetes. These results suggest that any role of CTLA-4 in multiple sclerosis is likely to be very small, and further suggest that if such an effect exists it is not determined by the G allele of the CT60 variant which is so important for other autoimmune diseases. This interpretation is consistent with the results of a meta-analysis of previous studies of the A49G SNP (Teutsch et al., 2004).

If the trend towards over transmission of the A allele from CT60 were confirmed this would indicate that different variants of CTLA-4 may

predispose to different autoimmune diseases: G allele haplotypes to thyroid disease and diabetes; and A allele haplotypes to multiple sclerosis. This would not be the first time that a dichotomous relationship between thyroid disease and multiple sclerosis has been reported. One third of patients with multiple sclerosis treated successfully with CAMPATH 1H (a monoclonal antibody to CD52) go on to develop Graves' disease. This switch in autoimmune response reflects a change from a Th1 to a Th2 (Coles et al., 1999) cytokine profile. A hypothesis worthy of consideration is that patients who do and do not make this switch differ at the CTLA-4 locus (especially CT60).

Although the use of carefully developed tagging SNPs has allowed us to analyse all the common haplotypes, it is still possible that others not directly tested are relevant and have been missed. Future studies of this gene in multiple sclerosis will need to be very much larger than the study presented here if they are to have power to demonstrate relevance of this gene.

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Titles and Legends to Tables

Table 1 Primer Sequences for CTLA-4 analysis.

3'UTR VNTR = 3' untranslated region variable number tandem repeat. HtSNP = haplotype tagging single nucleotide polymorphism

Table 2 Susceptibility to multiple sclerosis and transmission of 3'UTR VNTR and CT60 alleles

3'UTR VNTR = 3' untranslated region variable number tandem repeat. $p_{\text{uncorrected}}$ = p value uncorrected for multiple testing; SNP = Single nucleotide polymorphism; htSNP = haplotype tagging SNP. In the haplotype list 1 indicates the commoner allele of each htSNP (i.e. respectively T,A,C,C,A); and 2 indicates the minor allele (i.e. respectively C,G,T,TG).

Titles and Legends to Figures

Figure 1

Schematic structure of CTLA-4 gene showing position of polymorphisms and primary PCR amplimers.

Primary PCR amplimer 1 is 211 bp in length and spans the first two htSNPs; Primary PCR amplimer 2 is 840 bp in length and spans htSNP 3, 4 and 5. htSNP = Haplotype tagging single nucleotide polymorphism, VNTR = Variable tandem repeat, bp = base-pair

Table 1

		Primer Sequence (5' –3')	Product Size (bp)	
3'UTR VNTR		FAM- GTG ATG CTA AAG GTT GTA TTG C AAA ACA TAC GTG GCT CTA TGC AC	84- 132	
CT60		Forward: TGG AAG GTA TCC ATC CTC TTT CCT Reverse: CAT GCC AAT TGA TTT ATA AAG GAC TGC TA Probes: A/TTT GGG ATA TAA CGT GGG TTA		
SNaPshot				
Primary PCR 1		CCA CTG GCT TCT GCT CCT AGC TCA AGC GCC AAC AAG	211	
Primary PCR 2		GGG TTG GCT TTT CTT TGG ACC TTT GCA GAA GAC AGG GA	880	
Interrogating Primers:			Product Size (bases)	Concentration in extension step
htSNP1: - 1722	T>C	TTT TTT TTT TTT TTC TAT CAT GAT CAT GGG TTT AGC TG	38	0.3 µM
htSNP2: - 1661	A>G	TTT TTT TCA GGA ACA TTT GTT TTT CAC TTT TT	32	0.3 µM
htSNP3: - 658	C>T	CTT CTG CAA AAC CAG AGG CAG CTT CTT TTC	30	3.0 µM
htSNP4: - 319	C>T	AGT CTC CAC TTA GTT ATC CAG ATC CT	26	3.0 µM
htSNP5: +49	G>A	TTT TTT TTT TTT TTT TTT TTC ACA AGG CTC AGC TGA ACC TGG CT	44	3.0 µM

Table 2

	Genotype Success Rate N (%)	Het (%)	Observed	Expected	χ^2	$p_{uncorrected}$
3'UTR VNTR	740 (96)	0.63				
8 repeat allele					2.85	0.09
17 repeat allele					1.22	0.27
Other alleles					0.77	0.38
SNPs: (Transmission of Common allele)						
CT60	724 (94)	0.50	765	784	1.95	0.16
htSNP1	735 (95)	0.13	1427	1418	1.27	0.26
htSNP2	735 (95)	0.28	1273	1268	0.22	0.64
htSNP3	616 (80)	0.14	1166	1175	1.95	0.16
htSNP4	616 (80)	0.15	1175	1177	0.09	0.76
htSNP5	611 (79)	0.47	804	787	2.34	0.13
htSNP haplotypes						
1.1.1.1.1			570	557	1.3	0.24
1.1.1.1.2			458	468	0.76	0.35
1.2.1.2.1			118	119	0.02	0.89
1.1.2.1.1			119	111	1.6	0.22
2.1.1.1.2			93	99	0.99	0.37
1.2.1.1.1			118	122	0.23	0.60
Global					4.2 (5 d.f.)	0.52