Brief Genetics Report

CTLA4 Gene Haplotypes Cannot Protect From IDDM in the Presence of High-Risk *HLA DQ8* or *DQ2* Alleles in German Families

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IDDM results from the autoimmune destruction of insulin-producing β-cells (1), a process presumably caused by a lack of tolerance against diabetogenic peptides (2) and genetic susceptibility conferred by major histocompatibility complex class II alleles (3–5). The cytotoxic T lymphocyte–associated molecule 4 (*CTLA4*) gene region, among others, also confers genetic susceptibility to IDDM, as identified at the *IDDM12* locus and confirmed in several studies (6–9). *CTLA4* belongs to the immunoglobulin superfamily and shares many features with the *CD28* molecule (10,11). Both *CD28* and *CTLA4* bind to the same ligand, but they carry out different functions. Binding of the costimulatory molecule *B7* to *CD28* activates T-cells by inducing the expression of several cytokines, cytokine receptors, and regulatory genes (12), whereas *B7* binding to *CTLA4* leads to T-cell apoptosis (13). In contrast to *CD28*, which is expressed both on resting and activated T-cells (12), *CTLA4* expression is restricted to activated T-cells (14). The affinity of *B7* molecules is at least 100-fold higher to *CTLA4* than to *CD28* (15,16), which allows termination of the immune response by T-cell apoptosis in spite of the stimulating *CD28* molecules (13,15,17). Therefore, *CTLA4* expression may play a key role in autoimmunity (16,18,19).

The *CTLA4* gene contains three polymorphic sites (10,11,20,21): *1*) at position 642 of the 3′ untranslated region of exon 4: AT_n repeat, 84– to 134–base pair (bp) fragment (Genbank no. M37243, locus HUMIGCTL3); *2*) at position 49 of exon 1: adenine^{49} or guanine^{49} (Genbank no. M74363); and *3*) at position –318 of the promoter region: cytosine^{-318} or thymine^{-318} (Genbank no. M74363). Because of their close linkage, these markers are expected to show similar associations with IDDM but may also define subgroups at higher risk. We therefore investigated the transmission of all three polymorphisms in 109 families with at least one offspring with IDDM to evaluate their contribution to disease susceptibility, as well as their interaction with predisposing *HLA DQ* alleles.

The diagnosis of IDDM was based on World Health Organization criteria, and all patients were from the Rhein-Main region. The transmission distortion test was calculated as follows: transmitted haplotypes (transmitted from heterozygous parents to affected offspring) were compared with the expected random transmission frequency for the polymorphisms, applying a χ^2 test, and significance was defined at $P < 0.05$. Combining the polymorphic sites, we also constructed *CTLA4* extended haplotypes and calculated delta values (Δ) (22).

Genomic DNA was prepared from peripheral blood by standard methods. The *CTLA4* exon 1 dimorphism was assigned by single-strand conformation polymorphism (9), and the promoter polymorphism at position –318 was defined by restriction enzyme analysis with *Mse*I (21). Microsatellite polymorphisms at position 642 of the 3′ untranslated region flanking exon 4 were studied by amplifying a DNA fragment with primer A, 5′ GTGATGCTAAAGGTTGTATTGC 3′ (labeled with hexachloro-6-carboxyfluorescein), and primer B, 5′ GTGATGCTAAAGGTTGTATTGC 3′. Amplification was performed with 0.3 µg genomic DNA, 1 U Taq Polymerase (Gibco BRL, Eggenstein, Germany), 10 pmol of each primer, and 8 mmol/l dNTP in a final volume of 50 µl, under the following conditions: initial 94°C for 4 min, followed by 27 cycles 94°C for 45 s, 56°C for 45 s, and 72°C for 45 s, with a final extension of 72°C for 4 min. Amplified fragments varied from 84–134 bp in length, as detected using Genescan 672 software (Applied Biosystem Division, Perkin-Elmer, Foster City, CA) on a 373A automated DNA sequencer (Perkin-Elmer).

The distribution of the *CTLA4* microsatellite, exon 1, and the promoter polymorphism on transmitted haplotypes derived from 109 families with at least one offspring with IDDM is shown in Table 1. Haplotypes with a fragment length of 84 bp at the microsatellite locus were significantly less common in transmitted (36%), particularly maternal transmitted (26%), haplotypes, $P < 0.05$ and $P < 0.02$, respectively. In contrast, the 118-bp microsatellite allele was found more often in transmitted (87%) haplotypes, $P < 0.04$. The 102 bp allele is also more frequent in transmitted (63%) haplotypes, although not significantly. Guanine^{49} was found significantly more often on transmitted (71%) haplotypes, $P < 0.04$. Pro-

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bp, base pair.

TABLE 1
Transmission of *CTLA4* polymorphisms from heterozygous parents to children with IDDM

	Maternal transmitted	Maternal not transmitted	*P* values	Paternal transmitted	Paternal not transmitted	*P* values	All transmitted haplotypes	All non-transmitted halplotypes	*P* values
CTLA4 microsatellite marker									
84 bp	15 (26)	43 (74)	<0.02	37 (54)	32 (46)		42 (36)	75 (64)	<0.05
102 bp	32 (71)	13 (29)		23 (53)	20 (47)		55 (63)	33 (37)	
118 bp	5 (71)	2 (29)		8 (100)	—		13 (87)	2 (13)	<0.04
CTLA4 exon 1 polymorphism									
Adenine^{49}	17 (29)	41 (71)	<0.04	22 (42)	30 (58)		39 (35)	71 (65)	<0.05
Guanine^{49}	41 (71)	17 (29)	<0.04	30 (58)	22 (42)		71 (65)	39 (35)	<0.05
CTLA4 promoter polymorphism									
Cytosine $^{-318}$	8 (50)	8 (50)		11 (46)	13 (54)		19 (47)	21 (53)	
Thymine $^{-318}$	8 (50)	8 (50)		13 (54)	11 (46)		21 (53)	19 (47)	
CTLA4 microsatellite marker combined with adenine^{49} at the exon 1 locus									
84 bp	15 (26)	42 (74)	<0.02	27 (46)	32 (54)		42 (36)	74 (64)	<0.05
102 bp	2 (50)	2 (50)		1 (20)	4 (80)		3 (33)	6 (67)	
118 bp	4 (80)	1 (20)		7 (100)	—		11 (92)	1 (8)	<0.04
CTLA4 microsatellite marker combined with cytosine^{-318} at the promoter locus									
84 bp	15 (28)	39 (72)	<0.03	27 (47)	30 (53)		43 (38)	69 (62)	
102 bp	32 (71)	13 (29)		22 (54)	19 (46)		54 (63)	32 (37)	
118 bp	5 (71)	2 (29)		4 (100)	—		9 (82)	2 (18)	
CTLA4 extended haplotypes: promoter, exon 1, and microsatellite markers									
c^{-318}, g^{49}, 102 bp	30 (73)	11 (27)		22 (58)	16 (42)		52 (66)	27 (34)	
c^{-318}, a^{49}, 84 bp	15 (28)	38 (72)	<0.04	27 (47)	30 (53)		42 (38)	68 (62)	
t^{-318}, a^{49}, 84 bp	1 (17)	5 (83)		2 (33)	4 (67)		3 (25)	9 (75)	
Combined transmission of *CTLA4* microsatellite markers and *HLA DQA1*0501 DQB1*0201*									
84 bp	15 (60)	10 (40)		12 (67)	6 (33)		27 (63)	16 (37)	
102 bp	12 (92)	1 (8)	<0.03	7 (78)	2 (22)		19 (86)	3 (14)	<0.03
All	37 (73)	14 (27)	<0.04	26 (76)	8 (24)	<0.05	63 (72)	24 (28)	<0.004
Combined transmission of *CTLA4* microsatellite markers and *HLA DQA1*0301 DQB1*0302*									
84 bp	6 (60)	4 (40)		16 (80)	4 (20)		22 (73)	8 (27)	
102 bp	11 (79)	3 (21)		12 (75)	4 (25)		23 (77)	7 (23)	
All	30 (77)	9 (23)	<0.03	44 (80)	11 (20)	<0.002	74 (79)	20 (21)	<0.0001

Data are number of alleles (frequency of alleles). A total of 109 families were studied. Transmitted and nontransmitted alleles were compared with the random transmission of 50% by χ^2 test. a, adenine; c, cytosine; g, guanine; t, thymine.

moter polymorphisms are nearly equally distributed in all haplotypes. The combination of adenine^{49} and the 118-bp allele is significantly increased in transmitted (92%) haplotypes, $P < 0.04$. Conversely, adenine^{49} in combination with the 84-bp microsatellite allele is significantly reduced in transmitted (36%) and maternal transmitted haplotypes, $P < 0.05$ and $P < 0.02$, respectively. The promoter allele cytosine^{-318} combined with the 84-bp microsatellite is significantly less frequent in maternal transmitted (28%) haplotypes, $P < 0.03$, as are the extended haplotype cytosine^{-318}, adenine^{49}, and the 84-bp allele, $P < 0.04$. In combination with the haplotype *HLA DQA1*0501 DQB1*0201* on chromosome 6, no more significant difference was found for the protective 84-bp microsatellite allele. Also, when transmitted together with the haplotype *HLA DQA1*0301 DQB1*0302*, the 84-bp microsatellite allele does not confer protection.

Our analysis demonstrates a strong linkage of promoter and exon 1 polymorphisms of the *CTLA4* gene. Cytosine^{-318} is common in both exon 1 alleles, but thymine^{-318} is strongly linked to adenine^{49}, $\Delta = 0.033$. Within our families, the promoter alleles show similar frequencies in affected versus control haplotypes and therefore do not add to IDDM risk (Table 1). As shown by other studies (7,8), exon 1 and microsatellite polymorphisms (adenine^{49} and allele 8 [84 bp], as well as guanine^{49} and allele 17 [102 bp]) are also strongly linked. In our families, 176 (95%) of 185 haplotypes with 84 bp at the microsatellite locus were adenine^{49} positive, whereas 95 (91%) of 104 haplotypes with the 102-bp microsatellite allele had guanine^{49} in exon 1, $\Delta = 0.2$. The 84-bp microsatellite marker, as well as the extended haplotype with cytosine^{-318} and adenine^{49}, is reduced in transmission from mothers. If confirmed in other studies, this may be another example of parental imprinting of a susceptibility gene (23). In this study, we have identified the 118-bp microsatellite allele as an additional susceptibility marker in IDDM. Of 15 118-bp microsatellite alleles, 12 are linked to adenine^{49}, which is not associated with IDDM. This points to the microsatellite as the primary and most informative susceptibility marker on this haplotype. As reported previously in our case-control study, we confirm that guanine^{49} (alanine at codon 17) is associated with IDDM (9), although the significance is only marginal.

Combined transmission analysis of *CTLA4* and *HLA*

DQA1 DQB1 haplotypes reveals a modulation of the genetic protection conferred by the *CTLA4* 84-bp microsatellite. This allele confers protection by itself; however, when transmitted in combination with susceptible *HLA DQ* haplotypes, a non-significant positive association is observed. Thus, the high-risk *HLA DQA1*0301 DQB1*0302* and *DQA1*0501 DQB1*0201* haplotypes are dominant over the protective *CTLA4* 84-bp allele. This interaction may be a chance observation, however, and needs to be confirmed in a larger data set. A functional explanation for the *CTLA4* association with IDDM cannot be offered at present. The microsatellite polymorphism maps closely to an intracellular localization motif that is situated in the middle of exon 4 (24). A short half-life of *CTLA4* molecules at the cell surface has been reported (14), as well as a higher level of intracellular *CTLA4* molecules. Also, a phosphorylation signal for the SH2 domain containing tyrosine phosphatases (SYP) is located within exon 4 (25). The T-cell receptor and *CTLA4* both interact with this signal transduction pathway, but no studies relating these data to *CTLA4* polymorphisms are available at present.

Therefore other genes in the vicinity of the *CTLA4* locus may also contribute to the reported associations.

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