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TAP1 alleles in insulin-dependent diabetes mellitus: A newly defined centromeric boundary of disease susceptibility

(antigen presentation/*TAP* genes/peptide transporters)

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ABSTRACT **It has been previously demonstrated that individuals with certain *DR* alleles have an increased relative risk of developing insulin-dependent diabetes mellitus (IDDM). The disease association is even stronger with certain *DQ* alleles but there is little association with *DP* providing a boundary of disease association to the 430 kb between *DQ* and *DP*. The recently described *TAP* (transporter associated with antigen processing) genes have been mapped approximately midway between *DP* and *DQ*. Therefore, it was of interest to determine if any *TAP* alleles were associated with IDDM. In addition to the alleles of *TAP1* that have been described, others were identified during this study. Diabetics and normal controls were screened for *TAP1* using single-stranded conformational polymorphism and relative risk was determined. In the same population group we have studied extensively in the past, we found a higher association of a *TAP1* allele with IDDM than with any single *HLA-DP* allele but the risk was lower than with *HLA-DQB1*0302*. These data provide new limits for IDDM susceptibility to the 190-kb interval between *TAP1* and *HLA-DQB1*.**

The recently identified TAP1 and TAP2 (transporter associated with antigen processing) proteins share homology with such proteins as MDR (multi-drug resistance) protein and CFTR (cystic fibrosis transmembrane conductance regulator) protein and other members of the ABC superfamily of proteins, which transport materials across membranes (1–4). Although the actual function of the *TAP* genes is in question, indirect evidence has been presented to suggest that TAP proteins function as a heterodimer to transport peptides across the endoplasmic reticulum membrane, where the peptides associate with assembling class I molecules (5–9). Mutant cell lines that lack expression of either *TAP* gene fail to express normal levels of class I on the cell surface, but normal class I surface expression is restored after transfection with the appropriate *TAP* cDNA (8, 10). Transgenic mice with a disrupted *TAP1* gene express severely reduced levels of surface class I molecules and are depleted of $CD8^+$ T cells (11). These mice appear unable to present cytosolic antigens to class I-restricted cytotoxic T cells. Expression of *TAP1* and *TAP2* is then apparently necessary for normal class I surface expression.

Recently Powis *et al.* (12) reported that polymorphisms in the rat *TAP2* allele affects T-cell recognition of class I RT1.A^a molecules. Differences in RT1.A^a between strains that carry different *TAP2* alleles can also be distinguished by antibodies. These results suggest that the products of different transporter alleles supply different subsets of peptides in the different strains, generating class I–peptide complexes with altered conformations (12). It has also been observed that distinct human cell lines expressing identical HLA-B27 molecules can be distinguished by their ability to present certain viral epitopes in the context of HLA-B27 (13). This has led to the speculation that genetic variation in the transporter genes might be responsible for the observed difference in HLA-B27 recognition. Such genetic variability among the TAP proteins could be implicated in certain class I-mediated autoimmune diseases.

A variety of autoimmune disorders, including insulin-dependent diabetes mellitus (IDDM), are known to be associated with major histocompatibility complex (MHC) genes (14). It is not clear whether the destruction of the β-islet cells of the pancreas observed in IDDM is mediated through a class I or class II event. It has been observed that the majority of T lymphocytes that infiltrate the pancreas at the onset of IDDM are $CD8^+$ (15, 16). An increase in class I expression on islet cells has also been observed. Since the TAP proteins have been implicated in class I expression and since allelic differences in the *TAP* genes could affect the types of peptides presented by class I molecules, an investigation of the *TAP* genes in relation to IDDM seems warranted (12). Nonobese diabetic (NOD) mice have been shown to harbor a rare variant of *TAP1* using restriction fragment length polymorphism analysis (17, 18). It has been suggested that this *TAP1* variant might be associated with aberrant class I expression on islet cells in the NOD mice leading to the destruction of the β cells, resulting in disease (17). A subsequent investigation confirmed that the NOD mice carried an unusual allele of *TAP1* but reported no variation in the *TAP* or class I mRNA levels between NOD mice and other strains; however, variation in peptide loading of class I by variant TAPs was not discounted (18).

The *TAP* genes are located within the 430-kb region between *DQ* and *DP* and it has not been firmly established whether they are in linkage disequilibrium with *DQ* or *DP* (1, 2, 19, 20). Several groups, including our own, have demonstrated an association of the *DQB1*0302* allele with an increased risk for the development of IDDM, whereas other alleles (i.e., *DQB1*0602*) were associated with protection (21–26). In subsequent studies it was determined that there was little association with known *DP* alleles and susceptibility to IDDM (25). *DP* is in only weak linkage disequilibrium with *DQ*, whereas there is strong linkage disequilibrium between *DQ* and *DR* alleles (27). It is not clear where within the class II region the linkage disequilibrium observed in the HLA complex breaks down. Restriction fragment length polymorphism analysis of a limited number of homozygous cell lines suggested that recombination hot spots may exist between *TAP1* and *DP* and between *TAP1* and *DQ* (19). Thus, although the apparent relative risk for IDDM appears to peak at the *DQ* locus, it is possible that a more significant asso-

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; SSCP, single-stranded conformational polymorphism; MHC, major histocompatibility complex.

ciation might be observed at the *TAP* locus, which is located more centromeric in the MHC.

At the present time four alleles of *TAP1* have been identified (28, 29). One of the *TAP1* alleles that was not observed during this study was identified using the technique of amplification refractory mutation system polymerase chain reaction (29). The identification of three other *TAP1* alleles was originally facilitated by the technique of single-stranded conformational polymorphism (SSCP). This technique relies on the differential mobility of single-stranded DNA fragments due to their nucleotide sequence under nondenaturing conditions and has been successfully used to identify a variety of genetic variants (28–32). Although SSCP was used to identify the *TAP* variants, only one of the three alleles of *TAP1* has been completely sequenced (1, 2, 28). The other *TAP1* alleles were identified in homozygous cell lines, suggesting that the amino acid substitutions appeared within a single allele (28). During the course of our investigation four distinct *TAP1* alleles were identified by sequencing *TAP1* cDNA clones generated from several individuals. A fifth allele was identified utilizing SSCP analysis of genomic DNA. Since variation was only noted in the 3′ half of the *TAP1* genes, we sequenced the 3′ 1300 bp of several *TAP* cDNA clones and confirmed that specific amino acid changes are common. These polymorphisms have been studied in a large population of normal controls and patients with IDDM, most of whom have been the subject of extensive investigations into the MHC-associated genes in this autoimmune disease.¶

MATERIALS AND METHODS

Patients and Normal Controls. One hundred and ninety-nine unrelated Caucasian patients with IDDM as defined by the criteria of the National Diabetes Data Group were studied. One hundred and forty random unrelated Caucasian individuals served as normal controls. Other details of the patient and control population are as described (23, 25).

RNA Isolation. RNA was isolated from peripheral blood lymphocytes or Epstein–Barr virus-transformed B cells from normal control individuals and diabetic patients using the one-step method of Chomczynski and Sacchi (33).

PCR Reactions. PCR primers used to amplify cDNA or genomic DNA were designed based on published sequences (1) and were synthesized on an Applied Biosystems model 394 RNA/DNA oligonucleotide synthesizer. PCR oligonucleotides used for amplification of the 3′ half of *TAP1* were either 5′-ACCCTGATCACCCTGCCTCTGCTTTTC-3′ (1201–1227 bp) or 5′-TTCTCTGAGTGAGAATCTGAGCTT-3′ (1101–1124 bp) as 5′ primers and 5′-TTTCATTCTGGAGCATCTGCAGGA-3′ (2436–2459 bp). The 3′ primer amplifies from the stop translation codon so that the fragment includes only coding sequence. General PCR conditions were 94°C/90 sec, 58°C/120 sec (annealing temperatures varied with primers), and 72°C/3 min for 30 cycles with a 30-min extension at 72°C. Reaction mixtures included 0.4 μM of each primer, 200 μM dNTPs, and 1 unit of Taq polymerase and 10× Taq buffer.

SSCP Analysis. SSCP oligonucleotides (8 μg) were labeled in a 50-μl reaction mixture with 300 μCi of ^{32}P (specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq) using 16 units of T4 polynucleotide kinase (Promega) for 1 hr at 37°C. Primer pairs used to amplify genomic SSCP fragments are 5′-CATCATGTCTCGGGTAACAGAGGA-3′ and 5′-ACTGAGTCTGCCAAGTCT-3′ for region 1, 5′-GAATCCTCTACATTGGTGGGC-3′ and 5′-ACACTGGGGAGTGAAGGTGGAGGGACC-3′ for region 2, and 5′-CATCTTGGCCCTTTGCTCTGCAG-3′ and 5′-CTGTAACTGGCTGTTTGCATC-3′ for region 3.

SSCP Gels. SSCP gels were prepared using 2× MDE acrylamide. MDE (mutation detection enhancement) is a reagent manufactured by J. T. Baker in a 2× concentration. A final concentration of either 0.70× (2110–2230/intron), 0.5×, or 0.45× (1501–1840 bp, 900–1240 bp, 1880–2200 bp, and 1058–1260/intron) MDE in 0.6× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) was used for identification of alleles. Gels were run at 10 W constant power for 9–24 hr. SSCP-PCR reaction mixtures were diluted 1:10 in 95% formamide/10 mM NaOH/0.025% bromphenol blue/0.025% xylene cynol. Samples were heated to 95°C for 5 min and then placed on ice for 1–2 min prior to loading. Three sets of reactions were used to determine the allele type. Gels were dried for 1 hr under vacuum and placed under film for 4–24 hr.

Cloning of PCR Products. Initially PCR products were generated from the 3′ half of *TAP1* (1300–1250-bp coding sequence) cDNAs prepared from normal controls and diabetic patients. The products were directly ligated into T-tailed pBluescript (34). Variants were confirmed by direct sequencing of PCR products from both cDNA and genomic DNA.

Statistical Analysis. Gene frequencies were calculated from the frequencies at which the alleles were observed using the formula $g = 1 - (1 - f)^{1/2}$, where f is the observed allele frequency and g is the gene frequency. Since a large number of unrelated individuals were tested, no distinction was made for homozygosity for a particular allele. The significance of the differences in allele frequencies was determined by χ^2 analysis. Relative risk was calculated by the method of Woolf (35) and Haldane (36).

RESULTS

Identification of Four Distinct Alleles of *TAP1*. Previous studies had suggested that polymorphisms of the *TAP* genes were localized to the C-terminal half of the molecule (4, 28). Genomic sequencing in our laboratory confirmed that the 5′ half of the *TAP1* gene was nonpolymorphic. Thus we concentrated most of our investigation on the 3′ half of the gene. *TAP1* cDNA clones were generated from the lymphocytes of 5 diabetics and 11 nondiabetics. Sequencing 1250–1300 bp of 5–10 clones from these 16 individuals resulted in the identification of four unique sequences, which are shown in Fig. 1, with the resulting amino acid substitutions summarized in Table 1. Nucleotide numbering in Table 1 is based on Trowsdale *et al.* (1) and the amino acid designation is based on the second ATG in that sequence being the initiation codon. *TAP1*0101*‖ was observed in all but one of the individuals studied by sequencing. One diabetic was homozygous for *TAP1*0301*, which contains a Val-333 resulting from an A → G substitution at nucleotide 1207. Two nondiabetics were found to be heterozygous for *TAP1*0301*. *TAP1*02011* was identified in one individual. In addition to the Val-333 amino acid substitution observed in *TAP1*0301*, *TAP1*02011* contains an A → G base change at nucleotide 2120, which results in the amino acid corresponding to position 637 changing from an Asp to a Gly. Sequence analysis of clones generated from 10 members of two families resulted in the identification of *TAP1*0401*, which contains the Val-333, the Gly-637, and two additional unique changes, a G → T base

¶The sequences reported in this paper have been deposited in the GenBank data base [accession nos. L21204 (*TAP1*0101*), L21206 (*TAP1*02011*), L21205 (*TAP1*02012*), L21208 (*TAP1*0301*), and L21207 (*TAP1*0401*)].

‖The names *TAP1*0101*, *TAP1*02011*, *TAP1*0301*, *TAP1*0401*, and *TAP1*02012* have been officially assigned by the WHO (World Health Organization) Nomenclature Committee in July 1993. This follows the agreed policy that, subject to the conditions stated in the most recent Nomenclature report (37), names will be assigned to new sequences as they are identified. Lists of such new names will be published in the following WHO Nomenclature report.

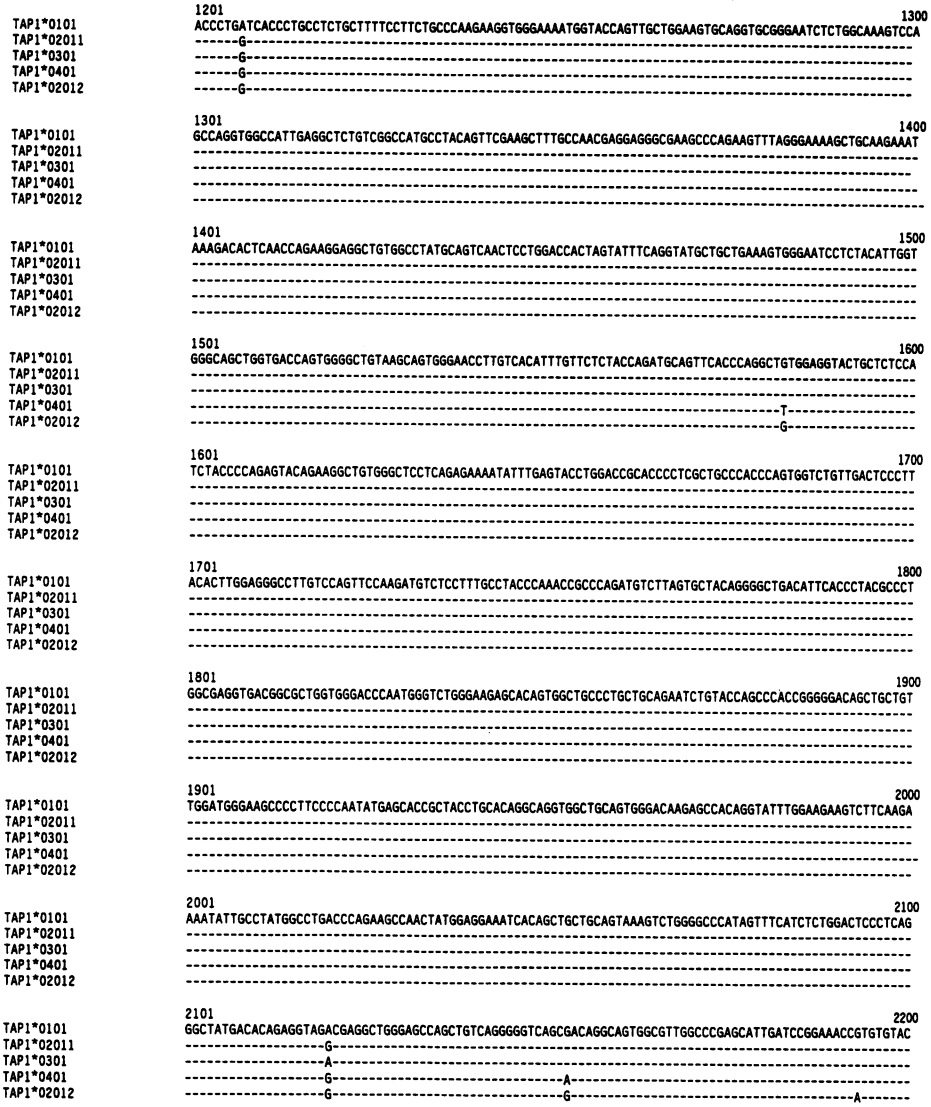

FIG. 1. Nucleotide sequence comparison of four *TAP1* alleles. Dashes depict identical sequence. Single base changes are indicated as compared to *TAP1*0101*.

change resulting in a Val → Leu substitution at amino acid 458 and a G → A base change, which results in an Arg → Gln substitution at position 648. At least 50% of the clones from five members of the two families possessed these four amino acid changes. By sequencing the entire 1300-bp fragment of the cDNA clones, it was possible to determine that these four amino acid changes were present within one allele. This polymorphism did not correspond to any published variant (28). Direct sequencing of PCR products generated from both cDNA and genomic DNA confirmed that the results from the clones were not due to Taq polymerase or reverse transcriptase errors. An additional (sixth) allele referred to as *TAP1 1C* by Powis *et al.* (29) was not observed during this investigation.

Table 1. Amino acid substitutions in *TAP1* alleles

Amino acid	333	458	637	648
Nucleotide	1207	1582	2120	2153
	ATC GTC	GTG TTG	GAC GGC	CGA CAA
*TAP1*0101*	Ile	Val	Asp	Arg
*TAP1*02011*	Val	Val	Gly	Arg
*TAP1*0301*	Val	Val	Asp	Arg
*TAP1*0401*	Val	Leu	Gly	Gln

Nucleotide numbering is based on the cDNA sequence of Trowsdale *et al.* (1); amino acid numbering is based on Colonna *et al.* (28). *TAP1*02012* contains the same amino acid changes as *TAP1*02011* but also contains an additional silent nucleotide change (see Fig. 1).

Using this sequence information, SSCP analysis was used to screen cDNA of other IDDM patients and normal controls. Oligonucleotide primers derived from cDNA sequence were generated to produce 300- to 400-bp fragments covering the entire human *TAP1* cDNA. Analysis of fragments generated from the 5′ half of the cDNA indicated that there were no variations in this region. Sequence analysis of the SSCP fragment (900–1240 bp) verified that the observed SSCP pattern was always due to the Ile → Val change at amino acid 333. SSCP analysis of two specific families documented that Val-333, Leu-458, Gly-637, and Gln-648 always segregated together. No other variants were apparent from SSCP analysis of cDNA fragments derived from amplification of other regions of *TAP1*.

The *TAP1* Alleles Segregate with *HLA* Genes. Members of several families previously typed at *DRβ1*, *DQα1*, *DQβ1*, and *DPβ1* by oligonucleotide typing techniques (23, 25, 27) were studied in detail. We found that in all circumstances the alleles of human *TAP1* segregated with the *HLA* genes. Even the less frequently represented alleles were found associated with multiple *DQ* and *DP* allele combinations within these families. Due to the small number of groups studied no significant information on linkage disequilibrium could be obtained in this analysis.

The *TAP1* Alleles Provide a Newly Defined Centromeric Boundary for IDDM Disease Susceptibility. Ninety-seven patients with IDDM who had been previously typed at *DRβ1*,

DQα1, *DQβ1*, and *DPβ1* and an additional 102 diabetic DNA samples typed at *DRβ1*, *DQβ1*, and *DQα1* but not *DPβ1* were screened for *TAP1* using genomic SSCP. Genomic DNA samples from 140 normal Caucasians were also screened for *TAP1* alleles by SSCP. The *TAP1* allele was identified by analysis of three genomic SSCP fragments (see *Materials and Methods*). Analysis of the third fragment (2110–2230) identified a fifth *TAP1* allele, which was designated *TAP*02012*. *TAP1*02012* contains Val-333, Gly-637, and an additional G → A nucleotide change at 2193 bp that is silent. Gene frequencies for the five *TAP1* alleles are summarized in Table 2. The gene frequency of *TAP1*0301* is ≈7-fold increased in patients with IDDM and is therefore associated a relative risk of 7.7. The gene frequency of *TAP1*0201* is slightly increased in the diabetic population and is associated with a risk of 2.1.

Homozygotes of *TAP1*0301* and *TAP1*02011* were observed in the diabetic population but not in the normal population. The *TAP1*0301/TAP1*0301* homozygous genotype was observed in four diabetics (2.0%) and is associated with a relative risk of 6.3 (Table 3). The *TAP1*0301/TAP1*0101* heterozygous genotype is associated with a significant relative risk (4.5) but represents only 9% of the diabetics. An excess in observed *TAP*0101* homozygotes in the diabetic population over the expected number based on calculated gene frequencies was also observed. The normal population was found to be in Hardy–Weinberg equilibrium with respect to *TAP1*.

DISCUSSION

This study addresses the question of whether or not alleles at the *TAP1* locus are associated with IDDM. The availability of a large population of normal controls as well as diabetics who had been previously completely or nearly completely typed for all alleles within the class I and class II regions of the human MHC and the possibility that the *TAP1* gene product may play a significant role in class I-restricted antigen presentation provided the background for this analysis. The possibility that recognition of class I-restricted peptides by $CD8^+$ cytotoxic T cells results in the destruction of islet cells in IDDM provided additional impetus for the study. To accomplish these ends we needed to understand the genomic structure of the *TAP1* locus, determine the extent of polymorphism in our population by nucleotide sequence analysis, establish a convenient means for allele-specific typing, document segregation within families, and perform the analysis on a large group of normal controls and diabetics. In a large population of previously well-characterized patients, our results demonstrate that although there is a modest association with certain *TAP1* alleles with IDDM, the association is not profound and is most likely due to extended haplotypes described previously (29).

Among Caucasians, IDDM is associated with *HLA-DR3*, *HLA-DR4*, or *HLA-DR3,4*. Indeed, in several studies, it has been documented that virtually 95% of Caucasians with IDDM contain one or both of these alleles (14, 38–44). Extensive analysis at the *DR* locus has shown that the relative risk associated with these alleles is ≈4–6. When the analysis is performed at the *DQ* locus, two alleles seem associated with disease: *DQw2* (*DQB1*0201*) and *DQw8* (*DQB1*0302*) (22, 26, 44, 45). In previous studies we demonstrated that approximately one-third of our patients with IDDM are positive only for *DQB1*0201*, approximately one-third are positive for *DQB1*0302*, and ≈25% are positive for both (23). Several analyses both in our own and other laboratories provide a relative risk of ≈6 with the *DQB1*0302* allele whether present in the homozygous or heterozygous state. Approximately two-thirds of all North American Caucasians with IDDM are positive for *DQB1*0302* (23).

We as well as other laboratories have shown that the *DP* locus provides a centromeric boundary for disease (25, 46–49). By oligonucleotide typing of >20 *DP* alleles no allele was associated with significant levels of protection or susceptibility. Though there were a few *DP* alleles that seem highly associated with disease, they represented a relatively small etiologic fraction (25).

The IDDM association observed with *TAP1* parallels the results obtained for the *DP* locus. Although *TAP1*0301* is associated with a high relative risk (7.7), the etiologic fraction of individuals with *TAP1*0301* is relatively small (9%). The other *TAP1* alleles either are associated with moderate relative risk or are protective. *TAP1*0301* does not group any specific diabetics from the perspective of their *DQα* or *β* type or their *DPβ* type. Specifically, had our analysis identified, for example, that 5% of Caucasians who are neither *DR3* nor *DR4* or the group who are not *DQB1*0302* or *DQB1*0201*, this might have been of considerable interest. On the other hand, analysis of *TAP1*0301* individuals shows that their distribution of *DQβ* alleles is no different than the distribution of the more common *TAP1*0101* individuals (data not shown). These results suggest that although there may be an association of IDDM with *TAP1*0301*, it is most likely the result of a passenger effect related to a gene that has yet to be identified located between *TAP1* and *DQβ1* or that *DQβ1* itself is responsible for the HLA-associated susceptibility to IDDM. It has been shown through studies of deletion mutant cell lines that at least one unknown gene in the vicinity of the *TAP* genes is needed for the intracellular production of class II–peptide complexes (50, 51). Such a gene or genes could

Table 2. *TAP1* gene frequencies

TAP1 allele	Normal†	IDDM‡	Relative risk
*TAP1*0101*	138 (88.0)	184 (71.0)	0.2*
*TAP1*02011*	17 (6.27)	44 (11.6)	2.1
*TAP1*0301*	3 (1.07)	29 (7.5)	7.7**
*TAP1*0401*	11 (4.00)	7 (1.8)	0.4
*TAP1*02012*	10 (3.63)	6 (1.5)	0.4
Blank	— (−2.9)	— (6.6)	

Gene frequencies were calculated using the formula $g = 1 - (1 - f)^{1/2}$, where g = gene frequency and f = observed allele frequency. Homozygotes were not considered in the calculation. *, $\chi^2 = 6.8729$, $P < 0.05$; **, $\chi^2 = 13.418$, $P < 0.0001$.
†n = 140; alleles typed, 179.
‡n = 199; alleles typed, 270.

Table 3. Frequencies of *TAP1* alleles in normal subjects and patients with IDDM

TAP1 genotype	Normal†	IDDM‡	Relative risk
0101/0101	100	122	0.6
0101/0301	3	18	4.5*
0101/02011	16	34	1.6
0101/0401	10	5	0.3
0101/02012	9	5	0.1
0301/0301	0	4	6.3
02011/0301	0	6	—
0301/0401	0	1	—
0301/02012	0	0	—
02011/02011	0	2	—
02011/0401	0	1	—
02011/02012	1	1	—
0401/0401	1	0	—
0401/02012	0	0	—
02012/02012	0	0	—

A dash indicates too few normals/patients to calculate. *, $\chi^2 = 7.978$, $P = 0.005$.
†n = 140.
‡n = 199.

dramatically impact expression of self-reactive peptides presented by class II and could be tightly linked to any disease association related to class II expression. In a recent study of *TAP2* alleles, Ronningen *et al.* (52) showed that a certain *TAP2* allele (665 Thr; 687 stop) was in linkage disequilibrium with both *DR4-DQ8* and *DR3-DQ2* and that this extended haplotype accounted for any association of *TAP2* with IDDM. Powis *et al.* (29) have also suggested the existence of extended haplotypes in the MHC that include *TAP2*.

Colonna *et al.* (28) found little association of *TAP1* alleles and IDDM based on a study of 50 diabetics and 37 normal controls. *TAP1*02011* was present in 40% (20 of 50) in the diabetics and 27% (10 of 37) in the normal controls. We observed a similar ratio of *TAP1*02011* in our diabetic and normal populations but our results suggest a lower *TAP1*02011* gene frequency. *TAP1*0401* and *TAP1*02012* would be included in the Colonna analysis as *TAP1*02011* and this would be reflected in the differences seen in gene frequencies of *TAP1*02011* between the two studies.

*TAP1*0101* and *TAP1*0301* differ in one amino acid. Based on the proposed model of TAP1 protein structure (1, 2) this amino acid is likely present in a transmembrane region of the protein. The other amino acid changes seen in the *TAP1* alleles occur in the cytoplasmic domain of the protein. A single amino acid interchange of a glycine to a valine in the cytoplasmic domain of P glycoprotein (MDR-1) results in an altered drug efflux activity (53). Amino acid variations in the cytoplasmic domain of CFTR protein have been linked to altered anion selectivity (54). It has been suggested that such an amino acid change in the MDR protein actually affects drug binding, which would result in altered efflux kinetics in the mutant protein. Amino acid variations in the *TAP1* alleles could result in altered binding affinities for certain peptides and ultimately could affect the presentation of peptides by class I molecules. Although there is no significant number of diabetics whose disease can be strongly associated with any *TAP1* allele without the contribution of *DQβ1*, it is possible that there is some contribution of *TAP1* to the disease process in a subset of patients. Nonetheless, this investigation provides a newly defined boundary for IDDM disease association to that region between *TAP1* and *DQβ1*.

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