Polymorphisms of the TAP1 and TAP2 genes in human alveolar echinococcosis

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Summary

We postulated that TAP genes may influence the susceptibility of some individuals to *Echinococcus multilocularis* infection. Six coding region variants (codons 333 and 637 in TAP1, and 379, 565, 651 and 665 in TAP2) were typed in 94 patients and 100 controls. Thr/Thr homozygosity at TAP2/665 was more prevalent in patients than in controls [64% vs. 45%, respectively; odds ratio (OR) = 2.1 (95% confidence interval (CI) 1.1; 2.7)] and Thr/Ala heterozygozity was less prevalent (32% vs. 50%, respectively) ($P = 0.014$). Of the 38 patients with progressive lesions, 76% were Thr/Thr, as compared with 55% of patients without progressive lesions and 45% of controls ($P = 0.058$ and 0.02, respectively), independent of HLA status. To determine whether this association is functionally relevant, functional analyses and/or confirmation in distinct populations of patients with alveolar echinococcosis would be required.

Introduction

Human alveolar echinococcosis (AE) is a parasitic disease caused by intrahepatic growth of the larvae of *Echinococcus multilocularis*. It is prevalent mainly in the northern hemisphere. The adult worm develops in the small intestine of a definitive host, usually the fox, and disperses eggs which are then eaten by an intermediate host, for example a wild rodent. The hexacanth embryo is released and penetrates through the wall of the intestine into the liver, where it grows in an infiltrating manner to form a mass of small vesicles surrounded by a granulomatous, fibrous infiltrate. When the rodent falls prey to a fox, the life cycle of *E. multilocularis* is complete. Humans can be infected by eating contaminated fruit or vegetables, or by touching foxes. Cell-mediated immunity plays a major role in the protective immune mechanism against the larva of *E. multilocularis* (Gottstein, 1992).

Epidemiological surveys have indicated that only 10–30% of people exposed to infection will actually develop AE. Thus, about 70–90% of these individuals will never develop the disease (Gottstein, 1992). Humans can be susceptible or resistant to AE. Disease progression can be affected either by immunogenetic factors and the immune status of the host, or by immunomodulation induced by the parasite. Previous studies in susceptible and resistant strains of mice (Bresson-Hadni *et al*., 1990) and in patients with active progressive or abortive lesions (Vuitton *et al*., 1989) have demonstrated that the relative ratio of T-lymphocyte subsets in the liver affects the efficiency of the granuloma. A relatively high number of CD4+ T cells within the granuloma is correlated with abortive or slow progression of the larvae, while CD8+ T cells and macrophages are predominant in ineffective granulomas in severe cases. Ineffectiveness of granuloma could be mediated by interleukin (IL)-10, as an abnormal spontaneous secretion of IL-10 by circulating mononuclear cells has been demonstrated (Wellinghausen *et al*., 1999; Godot *et al*., 2000a), and CD8+ T cells and non-T non-B cells have been shown to be involved in its synthesis (Godot *et al*., 1997). Recent studies found that loci within the major histocompatibility complex (MHC) were associated with AE. Severe case were associated with HLA-DR3, -DQ2 (Eiermann *et al*., 1998), and secretion of the anti-inflammatory cytokine IL-10 was shown to be significantly higher in those patients who had the HLA-DR3, -DQ2 haplotype (Godot *et al*., 2000b). HLA-DP 0401 was associated with susceptibility.

The TAP1 and TAP2 genes are two MHC genes located in the class II region (Spies *et al*., 1990). They encode a heterodimer, termed 'transporter associated with antigen processing' (TAP), located on the membrane of the endoplasmic reticulum (ER). It transports peptides from the cytoplasm into the ER, where they are assembled with HLA class I molecules and are subsequently presented to CD8+ T cells (Spies *et al*., 1990). TAP

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molecules also contribute to epitope selection, which is critical for CD8+ T-cell clone generation. The TAP1 and TAP2 genes are polymorphic, and their polymorphisms have been shown to influence the selection of peptide epitopes in rats (Momburg *et al*., 1994). Thus, the function of the TAP molecules is believed to be important in the initiation and regulation of the immune response.

In the light of the predominance of CD8+ T cells infiltrating the periparasitic granuloma in progressive cases of AE, the location of the TAP1 and TAP2 genes, and the roles of their products in antigen processing and presentation, we postulated that TAP genes may influence the susceptibility of some individuals to *E. multilocularis* infection and/or disease progression. Polymorphisms of the TAP1 and TAP2 genes were thus investigated in 94 patients originating from the same endemic area.

Materials and methods

Subjects

The 94 patients (45 women and 49 men), with a mean age (± SD) of 61.9 (± 16.4) years at the time of the study and with clinical AE follow-up of at least 2 years (median: 11 years; range: 2–33 years), originated from regions in which AE is endemic in the French and German Jura mountains. Diagnosis was based on computed tomography scans and enzyme-linked immunosorbent assay (ELISA)-based detection of serum antibodies directed against a purified *E. multilocularis* carbohydrate antigen or recombinant antigens and/or histological examination of intrahepatic lesions. Patients were considered to have a progressive form of the disease in cases of an increase in the size of the lesions, severe complications, relapses or metastasis formation. Classification of AE patients was established according to Eiermann *et al*. (1998). One hundred unrelated healthy controls were recruited from the same geographical areas. All were tested for the presence of anti-*E. multilocularis* antibodies and were found to be negative. All patients and controls had the same ethnic background. All of them lived in rural areas of the Jura mountain region where population migration is very limited.

TAP genotyping

Polymorphic positions at codons 333 and 637 in the TAP1 gene and codons 379, 565, 651 and 665 in the TAP2 gene were analysed, covering the most common variations described to date. Each position is dimorphic: TAP1/333 (A → G, Ile → Val, exon 4), TAP1/637 (A → G, Asp → Gly, exon 10), TAP2/379 (G → A, Val → Ile, exon 5), TAP2/565 (G → A, Ala → Thr, exon 9), TAP2/651 (C → T, Arg → Cys, exon 9), and TAP2/665 (A → G, Thr → Ala, exon 11).

Polymorphisms in the TAP genes were analysed using a variation of the amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) technique (Newton *et al*., 1991). PCR was performed in a 50-μl final volume containing 100–300 ng DNA, 1.5–2.5 mM $MgCl_2$, 200 μM of each dNTP, 5 μl of 10 × gold PCR buffer (150 mM Tris-HCL, pH 8.0, and 500 mM KCL), 7.5 mM $(NH_4)_2SO_4$, 0.2–0.75 μg of each primer and 1.25 units of Taq DNA polymerase. Reaction conditions were 95 °C for 10 min, followed by 85 °C for 5 min, 30 (TAP2/651) to 35 cycles of 94 °C for 30 s, 58–62 °C for 30 s and 72 °C for 30 s, 72 °C for 7 min and then cooling to 4 °C. The annealing temperature was optimized as follows: 62 °C for TAP1/333, 58 °C for TAP1/637, TAP2/379 and TAP2/565, and 60 °C for TAP2/651 and TAP2/665. Ten μl of PCR products was separated by 2% agarose gel electrophoresis and stained with ethidium bromide. Primers for TAP1 codons and for TAP2/379, 565 and 665 codons were designed according to Powis *et al*. (1993) with minor modifications to the control primers: for TAP2/379, the 5′ and 3′ flanking sequences were ggaagtgcttcgggagatccaggatgcagt and ttaaaaagaacaaataaagcccaaggcc, respectively; for TAP2/565, the 3′ flanking sequence was agctacagggacacgacct tcaccactaag; for TAP2/665, the 3′ flanking sequence was ggtcctggagacgcccctgagaagaggg. Primers for the TAP2/651 codon polymorphism were designed following Penfornis *et al*. (2002).

Statistical analysis

Chi-squared tests were used for comparisons, except in cases where Fisher's exact test was appropriate. The level of significance was set at a P value of 0.05.

In order to investigate linkage disequilibrium between HLA-DRB1 alleles and TAP amino acid variants, a stratified analysis was performed according to the expression of DR3, as this HLA-DRB1 allele has been reported to be significantly associated with progressive AE (Eiermann *et al*., 1998). The data were analysed in two-by-two tables involving stratification of each factor (DR3 and Thr/Thr homozygosity at TAP2/665) against the other, according to the method proposed by Svejgaard & Ryder (1994). For this analysis, the statistical significance was established by Fisher's exact test.

Results

TAP allele assignment

TAP1 and TAP2 alleles were defined according to the nomenclature used by Powis *et al*. (1993) and Jackson & Capra (1995), respectively, as defined in Zhang *et al*. (2002), as the WHO nomenclature does not take into account all the TAP1 alleles or the TAP2 codon 651 polymorphism (Teisserenc *et al*., 1997). Because the polymorphism site TAP2/687 is in absolute linkage with that at TAP2/665 in the TAP2 gene, as reported elsewhere (Caillat-Zucman *et al*., 1993, 1995; Rønningen *et al*., 1993; Jackson & Capra, 1995), we only needed to examine the change at codon 665 to determine the TAP2 alleles.

Table 1 Comparison of TAP1 and TAP2 polymorphism (a) and genotype (b) distributions between AE subjects and controls

(a)

TAP1			TAP2		
Polymorphism	AE subjects, *n* (%)	Controls, *n* (%)	Polymorphism	AE subjects, *n* (%)	Controls, *n* (%)
Codon 333	*n* = 94	*n* = 100	Codon 379	*n* = 93	*n* = 100
Ile/Ile	62 (66)	66 (66)	Val/Val	69 (74)	78 (78)
Ile/Val	21 (22)	29 (29)	Val/Ile	20 (21)	20 (20)
Val/Val	11 (12)	5 (5)	Ile/Ile	4 (4)	2 (2)
Codon 637	*n* = 94	*n* = 100	Codon 565	*n* = 93	*n* = 100
Asp/Asp	64 (68)	70 (70)	Ala/Ala	80 (86)	86 (86)
Asp/Gly	28 (30)	27 (27)	Ala/Thr	13 (14)	14 (14)
Gly/Gly	2 (2)	3 (3)	Thr/Thr	0	0
			Codon 651	*n* = 88	*n* = 100
			Arg/Arg	73 (83)	89 (89)
			Arg/Cys	15 (17)	11 (11)
			Cys/Cys	0	0
			Codon 665	*n* = 91	*n* = 100
			Thr/Thr	58 (64)	45 (45)
			Thr/Ala	29 (32)	50 (50)
			Ala/Ala	4 (4)	5 (5)

(b)

TAP1			TAP2		
Genotype	AE subjects, *n* (%)	Controls, *n* (%)	Genotype	AE subjects, *n* (%)	Controls, *n* (%)
A/A	62 (66)	64 (64)	A/A	28 (33)	28 (28)
A/B or C/D	19 (20)	24 (24)	A/B	19 (22)	39 (39)
A/C	2 (2)	5 (5)	A/C	6 (7)	4 (4)
A/D	0	2 (2)	A/D or C/E	7 (8)	5 (5)
B/B	2 (2)	3 (3)	A/E	1 (1)	0
B/C	9 (10)	1 (1)	A/F	6 (7)	2 (2)
C/C	0	1 (1)	A/G or B/C	3 (3)	2 (2)
Total	94	100	B/B	3 (3)	5 (5)
			B/D or E/G	2 (2)	4 (4)
			B/F	6 (7)	4 (4)
			C/C	3 (3)	1 (1)
			C/D	1 (1)	1 (1)
			C/F	0	1 (1)
			D/F	1 (1)	3 (3)
			Total	86	99

All comparisons are non-significant except at codon 665 ($P = 0.014$).

We observed the seven possible allelic combinations as previously reported (Jackson *et al*., 1995). Because of high frequencies of heterozygosity at two or more amino acid variants or because of technical failures, some allele combinations could not be distinguished with absolute confidence. In these cases, in determining the haplotypes, we considered only the seven previously published haplotypes (TAP2A to TAP2G) (Jackson *et al*., 1995). Even so, TAP2B/C could not be distinguished from A/G in two controls and three patients, A/D could not be distinguished from C/E in five controls and seven patients, and B/D could not be distinguished from E/G in four controls and two patients. These samples were excluded from the TAP2 haplotype analysis. Similarly, we could not separate TAP1A/B from C/D in the TAP1 gene in 24 controls and 19 patients.

Distribution of TAP1 and TAP2 variant amino acids (Table 1)

ARMS-PCR based genotyping was carried out in 94 patients and 100 healthy controls at TAP1/333 and TAP1/637. No significant difference in the variant distribution at the two sites was found between patients and controls. Because of the paucity of DNA in some samples, the TAP2/651 polymorphism site was determined in 88 patients, TAP2/665 in 91 patients, and the other two TAP2 polymorphism positions in 93 patients. A different pattern of distribution between patient and control groups was observed only for TAP2/665. Thr/Thr homozygosity was significantly more prevalent in patient subjects than in controls

Table 2. Comparison of TAP1 and TAP2 allele distributions between AE subjects and controls

	TAP1 [*n* (%)]		TAP2 [*n* (%)]	
Allele	AE subjects	Controls	AE subjects	Controls
A	126 (84)	135 (89)	88 (59)	101 (57)
B	13 (9)	7 (5)	31 (21)	53 (30)
C	11 (7)	8 (5)	13 (9)	8 (5)
D	0	2 (1)	2 (1)	4 (2)
E			1 (1)	0
F			15 (10)	10 (6)
Total	**150**	**152**	**150**	**176**

[64% vs. 45%, respectively; odds ratio (OR) = 2.1 (95% CI 1.1; 2.7)] while Thr/Ala heterozygozity was less frequent (32% vs. 50%, respectively) ($P = 0.014$). The other polymorphism sites showed similar frequencies of amino acid substitution in the AE and control groups.

Distribution of TAP1 and TAP2 alleles and genotypes (Tables 1 and 2)

The TAP1B/C genotype was more frequent in the AE group than in controls, but the frequencies of the TAP1A, B, C and D alleles were not significantly different between patient and control groups. The TAP2A/B genotype and the TAP2B allele were decreased in the AE group, but these differences were not significant. The other TAP2 allele and genotype distributions were not different between patient and control groups.

Association between TAP genes and clinical severity of AE

Of the 38 patients with progressive lesions, 76% were Thr/Thr homozygous at TAP2/665, compared with only 55% of patients without progressive lesions and 45% of healthy controls ($P = 0.058$ and 0.02, respectively). As DR3 has been reported to be significantly associated with progressive AE (Eiermann *et al*., 1998), we first checked for this association in our study: indeed, 15 out of 36 patients (42%) with a progressive disease were DR3 vs. 10 of 53 patients (19%; $P = 0.053$) without progressive lesions and 21 of 100 controls (21%; $P = 0.04$). We then assessed whether the effect of the TAP2/665 dimorphism on disease severity was independent of DR3 or was the result of linkage disequilibrium between the TAP2/665 and DR3 loci. We used a simple method based on two-by-two tests of various components of a two-by-four table (Svejgaard & Ryder, 1994). Data comparing Thr/Thr homozygosity at TAP2/665 in DR3 patients and controls are summarized in Table 3a. Tests 1 and 2 indicated that both DR3 and TAP2/665/Thr-Thr confer susceptibility to the progressive form of AE (OR = 2.69 and 4.28, $P = 0.03$ and 0.0014, respectively). A stratification was then carried out in order to test whether each factor deviated in the presence or absence of the other. Tests 3 and 4 showed that DR3 was not associated with the progressive form of AE independently of the TAP2/665 dimorphism (OR = 2.13 and 1.96, $P = 0.20$ and 0.60 in subjects who were and were not Thr/Thr homozygous at TAP2/665, respectively). Conversely, the Thr/Thr homozygosity susceptibility effect was significant in DR3-negative subjects (test 6), although not in DR3-positive subjects (test 5) (OR = 3.64 and 4.00, $P = 0.023$ and 0.14, respectively). Test 7 showed that there was no difference in the susceptibility effect of DR3 and Thr/Thr homozygosity for the progressive form of AE (OR = 0.53, $P = 0.70$), whereas test 8 suggested a significant additive effect of the two factors on susceptibility to the disease (OR = 7.83, $P = 0.0005$). Finally, in tests 9 and 10, no linkage disequilibrium was found between DR3 and TAP2/665/Thr-Thr, either in patients or controls (OR = 2.60 and 2.39, $P = 0.42$ and 0.13, respectively). No association was found between the non-progressive form of AE on the one hand and HLA-DR3 status or Thr/Thr homozygosity at TAP2/665 on the other hand (Table 3b).

Discussion

We observed a significant increase in the frequency of Thr/Thr homozygosity at codon 665 of TAP2 in patients with AE compared with controls. This difference seemed mainly to be attributable to the strong association between Thr^{665} homozygosity and the severe forms of AE. Results of a previous study indicated that HLA-DR11 may reduce the risk of developing the disease after *E. multilocularis* infection and that HLA-DP 0401 may increase this risk (Eiermann *et al*., 1998). In the same study, the severe forms of AE were significantly associated with the HLA-DR3, -DQ2 haplotype. The TAP genes lie between the HLA-DQ and -DP loci centromeric to HLA-DR (Spies *et al*., 1990). A strong linkage disequilibrium has been found between TAP2 and the DQ-DR loci in several studies: the DQ2-DR3 haplotype was found to be in positive linkage with Thr^{665} (Rønningen *et al*., 1993) or with TAP2A, the short allele of TAP2, which includes a Thr at codon 665 (Caillat-Zucman *et al*., 1995). Our findings could be explained by this linkage disequilibrium. However, in our study, the association between Thr/Thr^{665} homozygosity and severe forms of AE remained significant even after stratification for DR3. Alternatively, severe forms of AE could be linked to another HLA class II gene in linkage disequilibrium with both the DQ-DR and the TAP2 loci.

Another possible explanation is that there are extended HLA class II haplotypes of susceptibility to severe forms of AE, including both the DQ-DR and the TAP2 loci. The additive effect of both DR3 and TAP2 Thr/Thr^{665}

Table 3. DRB1 and TAP2 associations with AE in patients with (a) and without (b) progressive lesions

(a)

Factor A DR3	Factor B TAP2/665/Thr-Thr	Patients with progressive lesions (*n*)	Controls (*n*)
+	+	13	13
+	–	2	8
–	+	15	32
–	–	6	47

Comparison	OR	*P* value	Test	Conclusion
A vs. non-A	2.69	0.03	1. A associated?	A associated with progressive AE
B vs. non-B	4.28	0.0014	2. B associated?	B associated with progressive AE
+ + vs. – +	2.13	0.20	3. A associated in B-positives?	A not associated in B-positives
+ – vs. – –	1.96	0.60	4. A associated in B-negatives?	A not associated in B-negatives
+ + vs. + –	4.00	0.14	5. B associated in A-positives?	B not associated in A-positives
– + vs. – –	3.67	0.023	6. B associated in A-negatives?	B associated in A-negatives
+ – vs. – +	0.53	0.70	7. Difference between A and B associations?	No difference between A and B associations
+ + vs. – –	7.83	0.0005	8. Combined A–B association?	Additive effect of DRB1 and TAP2
Association between A and B in patients	2.60	0.42	9. Linkage disequilibrium in patients?	No linkage disequilibrium between A and B in patients
Association between A and B in controls	2.39	0.13	10. Linkage disequilibrium in controls?	No linkage disequilibrium between A and B in controls

(b)

Factor A DR3	Factor B TAP2/665/Thr-Thr	Patients without progressive lesions (*n*)	Controls (*n*)
+	+	9	13
+	–	1	8
–	+	19	32
–	–	23	47

Comparison	OR	*P* value	Test	Conclusion
A vs. non-A	0.91	0.96	1. A associated?	A not associated with non-progressive AE
B vs. non-B	1.42	0.39	2. B associated?	B not associated with non-progressive AE
+ + vs. – +	1.17	0.97	3. A associated in B-positives?	A not associated in B-positives
+ – vs. – –	0.36	0.34	4. A associated in B-negatives?	A not associated in B-negatives
+ + vs. + –	3.99	0.23	5. B associated in A-positives?	B not associated in A-positives
– + vs. – –	1.21	0.76	6. B associated in A-negatives?	B not associated in A-negatives

homozygosity on the susceptibility to progressive AE lesions favours this hypothesis. This would suggest a functional influence of TAP2 polymorphisms on AE progression. Although no effect of natural TAP2 polymorphisms on the repertoire of peptides available to be presented by class I molecules has been reported to date (e.g. Momburg *et al*., 1994), these studies used non-specific random sequence peptide libraries. It is possible that some TAP2 polymorphisms could have a specific effect on the binding and translocation of specific antigenic peptide(s) involved in an infectious or autoimmune disease, while not modifying the spectrum of non-specific peptides presented by HLA molecules on the cell surface. Of particular interest is the observation of the predominance of CD8+ T cells infiltrating the periparasitic granuloma in the more severe forms of AE (Vuitton *et al*., 1989; Bresson-Hadni *et al*., 1990), the activation of CD8+ T cells as measured by intracellular cytokine staining (Kilwinski *et al*., 1999), and their suspected role in the secretion of immunomodulatory IL-10 cytokine (Godot *et al*., 1997). Thr/Thr^{665} homozygosity of TAP2 could favour the presentation of parasitic peptides which would strongly stimulate CD8+ T-cell proliferation (Manfras *et al*., 2002) and/or selected immunosuppressant cytokine secretion. Unfortunately, immunodominant cytotoxic T lymphocyte (CTL) epitopes of *E. multilocularis* have not yet been identified and cannot be specifically studied in TAP binding and translocation assays. However, preliminary studies suggest that specific antigens (such as *E. multilocularis*-specific alkaline phosphatase) do have special properties in inducing particular cytokine profiles from peripheral blood mononuclear cells (PBMC) (Godot *et al*., 2000a,b).

Previous studies of TAP2 gene polymorphisms have also shown associations with the progression and/or the

phenotype of other immunologically mediated diseases or conditions, either autoimmune or infectious, such as insulin-dependent diabetes mellitus (Caillat-Zucman *et al*., 1993), human immunodeficiency virus (HIV)-I infection (Kaslow *et al*., 1996), Sjögren's syndrome (Kumagai *et al*., 1997), inflammatory bowel diseases (Heresbach *et al*., 1997), response to live measles vaccine virus (Hayney *et al*., 1997) and hepatitis C virus infection (Kuzushita *et al*., 1999). It is also noteworthy that all but one (Kumagai *et al*., 1997) of these studies (including ours) involve either the most frequent TAP2 allele, TAP2A (which differs from the second most frequent allele, TAP2B, at codon 665), or the Ala/Thr dimorphism at codon 665. However, this residue is located in the COOH-terminal part of TAP2, in the ATP-binding domain, not in the peptide-binding domain (Nijenhuis & Hämmerling, 1996), and so is less likely to be responsible for any functional effect on the peptide translocation specificity. A possible explanation is that there is linkage disequilibrium between the TAP2/665 dimorphism and another, still unidentified, polymorphism located in the peptide-binding site.

Finally, linkage disequilibrium could also have occurred with a non-HLA gene, such as the tumour necrosis factor (TNF) gene, located in the same area. However, preliminary studies of polymorphisms in the promoter of the TNF gene, which is a logical candidate as TNF has been associated with resistance/susceptibility in AE, in humans (Bresson-Hadni *et al*., 1994) as well as in experimental animals (Amiot *et al*., 1999), failed to find any differences between AE patients and controls (S. Harraga *et al*., unpublished data). Studies designed to search for other candidate genes outside the MHC should also be conducted, as a study in mice indicated that AE susceptibility genes appeared to be located in the non-H2 region (Emery *et al*., 1997).

In conclusion, we observed a positive association between Thr/Thr homozygosity at codon 665 of TAP2 and severe forms of AE. To determine whether this association is functionally relevant, functional analyses and/or confirmation in distinct populations of patients with AE would be required.

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