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The most severe forms of type I autoimmune hepatitis are associated with genetically determined levels of TGF-β1

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HLA associations

Abstract We previously reported that paediatric (PAH) and adult (AAH) forms of type I autoimmune hepatitis (AH) have different HLA-associations and clinical outcome. In the present study we investigated the role of TGF-β1 genetic polymorphisms in the different outcome of PAH and AAH. We found a significant increase of "high producer" 25GG genotype in PAH and 10CC in AAH. Low inflammation and low fibrosis in AAH was associated with the increase of codon 10CC (high producer) and codon 25CC (low producer) genotypes. The analysis in AAH of the two positions-haplotypes revealed that combined presence of 25GG and 10CC seems to neutralize the 10CC effect which remained in AAH having the $10CC^{+}$–$25GG^{-}$ haplotype. Altogether these results may explain, at least partially, the different clinical outcome of AAH and PAH.
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Introduction

Type I autoimmune hepatitis (AH) is a progressive liver disease affecting predominantly women and characterised by the presence of circulating anti-nuclear and/or anti-smooth muscle autoantibodies, hypergammaglobulinemia and response to immunosuppressive treatment [1]. In a previous report we demonstrated that the paediatric (PAH) and adult forms (AAH) differ in their clinical characteristics and MHC associations [2]; whereas AAH is associated with HLA-DR3 and HLA-DR4 [3,4], PAH is strongly associated with the HLA-DRB1*1301-DQB1*0603 haplotype [2,5–7].

Transforming growth factor-β1 (TGF-β1) a 25 kDa homodimeric protein secreted by several cell types [8] and its anti-inflammatory effect is required to maintain immune homeostasis and to prevent autoimmune diseases [9]. In addition, TGF-β1 promotes hepatic fibrogenesis by several mechanisms which include the production of extracellular matrix proteins and their receptors and inhibition of the synthesis of matrix-degrading proteolytic enzymes [10,11]. Moreover, several experimental models [12–14] and pathologies [8,15,16], showing an aberrant expression of TGF-β1, are accompanied by development of hepatic fibrosis.

In addition to many post-translational regulation points, production of TGF-β1 is significantly associated with genetic polymorphisms. Several single-nucleotide polymorphisms (SNP) have been identified in the human TGF-β1 gene [17]. Two are located in exon 1 and change the amino acid sequence of the signal peptide, nucleotide +869 (codon 10, T→C, Leu→Pro) and nucleotide +915 (codon 25, G→C, Arg→Pro). The TGF-β1 T allele at codon 10 have been associated with decreased levels of TGF-β1 [18–20]. Additionally, in comparison with heterozygous (GC) individuals, lymphocytes from individuals with the homozygous GG genotype at codon 25 produced significantly higher levels of TGF-β1 [21].

Because high producer genotypes have been associated with fibrotic progression and a more severe disease [21,22], the present study was designed to evaluate if functional genetic polymorphisms of codons 10 and 25 of TGF-β1 gene may explain the more severe outcome of PAH.

Patients and methods

Study populations

This study included 117 PAH and 80 AAH Latin American Caucasian patients who fulfilled the clinical, laboratory, histological and immunologic criteria for the diagnosis of AH [23] recruited from the Hepatology Outpatient Clinics of the J. P. Garrahan National Paediatric Hospital, R. Gutiérrez Children's Hospital, F. J. Muñiz Infectious Diseases Hospital, José de San Martín Clinical Hospital, Bonorino Udaondo Gastroenterology Hospital, Buenos Aires, Gastroenterology Section Medicine School, Rosario, Argentina. All patients were tested and found to be negative for infectious (HIV, HBV, HCV, cytomegalovirus and Chagas disease) and Wilson's disease. Patients were compared with 209 ethnicity- and sex-matched healthy controls (HCs). Informed consent was obtained from each patient and control. The study protocol conforms to the ethical guidelines of each author's institution.

Histological parameters used to evaluate disease severity at diagnosis

The histologic activity index (HAI) assigned according to the Knodell scoring system ranged between 1 and 18. Fibrosis was staged with the Ishak scale (ranging from 0 = no fibrosis to 6 = cirrhosis).

DNA extraction and polymerase chain reaction

Blood was collected in EDTA-containing tubes. Genomic DNA for cytokine and HLA typing was obtained by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. The HLA typing was performed in our HLA Laboratory following previously reported methodology [2].

DNA was amplified in 30 μl reaction mixture containing 100–300 ng of the DNA sample, 1.5 (TGF-β1) or 3 (IL-10) mM $MgCl_2$, 600 μM each primer [Table 1 [24]] and 1 U Taq polymerase in a PTC-100 thermocycler, following manufacturer's specifications (Inbio-Highway, Buenos Aires, Argentina). PCR consisted of 40 cycles of 30 s at 94 °C, 30 s at 56 °C (TGF-β1) or at 51 °C (IL-10) and 1 min at 72 °C. The amplified TGF-β1 fragment of 274 bp (amplified region: +691 to +965) or IL-10 fragment of 587 pb (amplified region: −1115 to −528) were electrophoresed in a 2% agarose gel stained with ethidium bromide (0.5 μg/ml).

Sequence-specific oligonucleotide probing (SSOP)

Two 5′ dioxigenin-labelled probes were used to positively identify each SNP by a dot blot assay [Table 1 and Ref. [24]] by using 800 pmol of the specific probe, as described previously [25]. Allele-specific probes were used to determine each TGF-β1 and IL-10 SNP genotypes and the IL-10 promoter haplotypes were inferred after compound analyze of the 3 SNPs results.

Statistical analysis

The homozygous versus non-homozygous genotypes and the allelic frequency were analysed by contingency tables with a two-sided Fisher's exact test. We applied the Bonferroni's correction (p_c) by 3 when total number of PAH or AAH patients were compared. Alternatively, p value was corrected by 6 when patients were further divided into different histological subgroups. p values <0.05 were considered statistically significant, $p<0.01$ very significant and $p<0.001$ extremely significant. The odds ratio (OR) with a 95% confidence interval (CI) was calculated to evaluate the relative risk in each patient group.

Results

TGF-β1 allelic and genotypic frequencies in different populations

We first explored in 189 control individuals from an Argentinean Caucasian population, the functional genetic polymorphisms in the TGF-β1 gene at codon 10 (T/C) and

Table 1 Primer and probes sequence used to IL-10 and TGF-β1 PCR-SSOP technique.

Gene	Primer	Primer sequence (5′→3′)	Annealing temperature
IL-10	sense	ATCCAAGACAACACTACTAA	51 °C
	antisense	TAAATATCCTCAAAGTTCC	
TGF-β1	sense	CTTCACCAGCTCCATGTCGATAG	56 °C
	antisense	ACTGCGCCCTTCTCCCTG	
SNP	**allele**	**Primer sequence (5′→3′)**	**Wash temperature**
IL-10			
−1082	G	TTCTTTGGGAGGGGGAAG	51 °C
	A	ACTTCCCCTTCCCAAAGAA	58 °C
−819	C	GAGGTGATGTAACATCTCTGTGC	66 °C
	T	GCACAGAGATATTACATCACCTGT	62 °C
−592	C	CCGCCTGTCCTGTAGGAA	58 °C
	A	TTCCTACAGTACAGGCGGG	58 °C
TGF-β1			
Codon 10	T (Leu)	GCTGCTGCTGCTGCTGCT	59 °C
	C (Pro)	GCTGCTGCCGCTGCTGC	57 °C
Codon 25	G (Arg)	GCCTGGCTGGCCGGCCG	61 °C
	C (Pro)	GCCTGGCCCGCCGGCCG	56 °C

codon 25 (G/C). The allelic and genotypic frequencies of these SNPs are quite similar to the previously reported frequencies in other populations, with some minor ethnic differences (Table 2). For instance, the frequency of the TGF-β1 10T allele in the Dutch population was 67% [26], whereas in the remaining populations varied between 50% and 59% [17,19,27]. Similarly, the frequency of the 10TT homozygous genotype [28] in Germany was higher than in the other populations [45% vs. 23–36% [17,19,27,29,30]], and the frequency of TGF-β1 10CC genotype in North American individuals was low [11–12% [29,30]], intermediate in European populations [16–17% [17,28]] and high in Asian populations [23–27% [19,27]]. The frequency of TGF-β1 10CC homozygous genotype in Argentinean population was 21% which is closer to the observed in the Asian population. The incidence of the 25C allele was low in almost all populations investigated [5–9% [17,26]] and was not detected in a Chinese cohort [27]. The analysis in both codons of allelic or genotypic frequencies according to gender did not show differences.

Functional polymorphisms of TGFβ1 in AH

The polymorphism of TGF-β1 codon 10 (T/C) and 25 (G/C) was successfully genotyped in 104 PAH and 74 AAH patients. Patients data at presentation, including presence of autoantibodies and the HLA-DR*1301 allele associated with PAH, are shown in Table 3.

Table 2 TGF-β1 allele and genotype frequencies in different populations.

Population	Ref	*n*	Codon 10					Codon 25				
			Allele frequency[a]		Genotype frequency			Allele frequency[a]		Genotype frequency		
			C	T	C/C	T/C	T/T	G	C	G/G	G/C	C/C
Argentinean		189	46%	54%	21%	50%	29%	91%	9%	82.5%	17%	0,5%
Male		106	44%	56%	21%	47%	32%	90%	10%	81%	19%	0%
Female		76	48%	52%	23%	51%	27%	91%	9%	84%	15%	1%
North-Americans	29	37	–	–	11%	57%	32%	–	–	92%	8%	0%
North-Americans	30	707	–	–	12%	52%	36%	–	–	86%	13%	7%
Germany	28	97	–	–	16%	38%	45%	–	–	91%	7%	2%
The Netherlands	26	61	33%	67%	–	–	–	92%	8%	–	–	–
Belfast	17	144	41%	59%	17%	47%	36%	95%	5%	92%	8%	
French	17	485	41%	59%	17%	47%	36%	92%	8%	85%	15%	
Chinese	27	104	49%	51%	25%	47%	28%	100%	0%	100%	0%	0%
Japanese males	19	289	50%	50%	27%	46%	27%	–	–	–	–	–
Japanese females	19	302	50%	50%	23%	54%	23%	–	–	–	–	–

n: number of individuals.
[a] The number of alleles is 2n.

Table 3 Data at presentation of children and adult patients with type I autoimmune hepatitis.

Group of AH patients	*n*	Age (years) median (range)	Male:female	% Autoantibodies positives patients (SMA/ANA)	% HLA-DR1301
PAH	117	9 (1–17)	22:85	78%/49%	54%
AAH	80	42 (18–85)	12:68	66%/61%	19%

PAH: type I autoimmune hepatitis in paediatric patients; AAH: type I autoimmune hepatitis in adult patients SMA: anti-smooth muscle antibodies: ANA: anti-nuclear antibodies.

In PAH patients, codon 25 showed both an increased frequency of the "high producer" G allele as well as an increased frequency of the GG homozygous genotype. The TGF-β1 25G allele was detected in 96% of PAH patients and in 83% of AAH patients (p_c=0.0006, OR=4.7, CI=2–10.9, Table 4). Similarly, the presence of the 25GG genotype was detected in 94% of the PAH patients and 76% of the AAH (GG^+ vs. GG^-: p_c=0.005, OR=4.9, CI=1.8–13.2) and 82.5% of the HCs (p_c=0.019, OR=5.3, CI=1.35–8.3). Conversely, the "low producer" 25CC genotype was observed in 9% of the AAH patients and in only 0.5% of the HCs (CC^+ vs. CC^-: p_c=0.005, OR=0.05, CI=0.006–0.46).

A different picture was obtained from the analysis of codon 10. The high producer TGF-β1 10C allele was present in 61% of AAH patients versus 46% in controls (p_c=0.006, OR=1.9, CI=1.27–2.8) and the 10CC genotype in 43% AAH patients and 21% in HCs (CC^+ vs. CC^-: p_c=0.0015, OR=2.9, CI=1.7–5.3). The differences observed in the frequency of the C allele and the CC genotype between the paediatric and adult forms of AH did not reach statistical significance.

Interestingly, the analysis of the codon 10 and 25 two-position haplotype demonstrated that only PAH patients showed an increase in the combined incidence of 10CC and 25GG genotypes. Both high producer genotypes were detected in 30% of PAH patients and 14% of the HCs ($10CC^+$–$25GG^+$ vs. others genotypes: p_c=0.0051, OR=2.6, CI=1.4–4.7, Table 5). Additionally, confirming the increase of the TGF-β1 10CC genotype in AAH patients, we detected in these patients a frequency of 19% for the $10CC^+$–$25GG^-$ genotype, which contrasted with the 7% frequency in the HCs ($10CC^+$–$25GG^-$ vs. others genotypes: p_c=0.025, OR=3.2, CI=1.4–7) and with the 4% frequency in the PAH (p_c=0.009, OR=6, CI=1.8–18).

For the analysis of the histological findings, we subdivided AH patients in those with a HAI below 9 (minimal and mild chronic hepatitis) or above 9 (moderate and severe chronic hepatitis). Fibrosis was staged between 0 and 3 (low), 4 and 6 (high). Additionally, we compared cirrhotic versus non-cirrhotic patients. AAH patients showed a strong increase of the codon 10CC genotype in patients with low inflammatory reaction, low fibrosis stage and as expected, in those non-developing cirrhosis (Table 5). AAH patients with an activity index (HAI) below nine, presented a highly significant increase of TGF-β1 codon 10CC genotype ($10CC^+$ vs. $10CC^-$: AAH vs. HC: p_c<0.0006, OR=0.05, CI=0.014–0.17). The same strong increase of 10CC was observed in AAH with a fibrosis stage between 0 and 3 (p_c<0.0006, OR=0.07, CI=0.024–0.26) as well as in AAH non cirrhotic vs. HC (p_c<0.0006, OR=0.09, CI=0.04–0.21). The analysis of codon 25 in the same group of patients showed a strong increase of the low producer genotype CC ($25CC^+$ vs. $25CC^-$: AAH HAI<9 vs. HC: p_c<0.006, OR=0.02, CI=0.002–0.2; AAH non cirrhotic vs. HC: p_c<0.0006, OR=0.02, CI=0.003–0.2).

In PAH patients the described increase of the 25GG genotype was only detected in children presenting the highest inflammatory reaction. However, this difference

Table 4 TGF-β1 allele and genotype frequencies of codons 10 and 25 in HA patients.

TGF-β1 gene polymorphism	*n*	Codon 10					Codon 25				
		Genotype frequency (*n*) %			Allele frequency[a] (*n*) %		Genotype frequency (*n*) %			Allele frequency[a] (*n*) %	
		C/C	T/C	T/T	C	T	G/G	G/C	C/C	G	C
Healthy controls	189	(39) 21	(95) 50	(55) 29	(173) 46	(205) 54	(156) 82.5	(32) 17	(1) 0.5	(344) 91	(34) 9
Male HC	106	(22) 21	(50) 47	(34) 32	(94) 44	(118) 56	(86) 81	(20) 19	(0) 0	(192) 90	(20) 10
Female HC	76	(17) 23	(38) 50	(21) 27	(72) 47	(80) 53	(64) 84	(11) 15	(1) 1	(139) 91	(13) 9
PAH patients	104	(35) 34	(38) 37	(31) 30	(108) 52	(100) 48	(98) 94**#	(4) 4	(2) 2	(200) 96***	(8) 4
Male PAH	18	(7) 39	(6) 33	(5) 28	(20) 56	(16) 44	(17) 95	(1) 5	(0) 0	(35) 97	(1) 3
Female PAH	83	(28) 34	(30) 36	(25) 30	(86) 52	(80) 48	(78) 94	(3) 4	(2) 2	(159) 94	(7) 4
AAH patients	74	(32) 43##	(27) 37	(15) 20	(91) 61#	(57) 39	(56) 76	(11) 15	(7) 9##	(123) 83	(25) 17
Male AAH	10	(5) 50	(4) 40	(1) 10	(14) 70	(6) 30	(8) 80	(1) 10	(1) 10	(17) 85	(3) 15
Female AAH	61	(23) 38	(25) 41	(13) 22	(71) 58	(51) 42	(47) 77	(10) 16	(4) 7	(104) 85	(18) 15

Codon 25: **p_c=0.005 and ***p_c=0.0006 PAH vs. AAH and #p_c=0.019 PAH vs. HC, ##p_c=0.005 AAH vs. HC. Codon 10: #p_c=0.006, ##p_c=0.0015 AAH vs. HC. Fisher exact test by 2×2 contingency tables ($10CC^+$ vs. $10CC^-$, C^+ vs. C^-, $25GG^+$ vs. $25GG^-$, $25CC^+$ vs. $25CC^-$, $25G^+$ vs. $25G^-$). Bonferrroni's correction was applied to *p* value (p_c) for the number of patient's groups (3). PAH: type I autoimmune hepatitis in paediatric patients; AAH: type I autoimmune hepatitis in adult patients; HC: healthy controls.

[a] The number of alleles is 2n. *n*: number of individuals.

Table 5 TGF-β1 genotype frequencies in AH patients: association with histological features.

TGF-β1 gene polymorphism	n	Codon 10 Genotype frequency (*n*) %			Codon 25 Genotype frequency (*n*) %			Codons 10 and 25 two-position Haplotype frequency (n) %			
		C/C	T/C	T/T	G/G	G/C	C/C	10C/C$^+$– 25G/G$^+$	10C/C$^+$– 25G/G$^-$	10C/C$^-$ 25G/G$^+$	10C/C$^-$ 25G/G$^-$
HC	189	(39) 21	(95) 50	(55) 29	(156) 82,5	(32) 17	(1) 0.5	(26) 14	(13) 7	(130) 69	(20) 11
PAH patients	104	(35) 34	(38) 37	(31) 30	(98) 94$^{**\#}$	(4) 4	(2) 2	(31) 30$^{\#\#}$	(4) 4	(66) 63	(3) 3
PAH HAI>9	71	(20) 28	(30) 42	(21) 30	(67) 94†	(4) 6	(2) 3	(18) 25†	(2) 3	(49) 69	(2) 3
PAH HAI<9	13	(4) 31	(4) 31	(5) 38	(11) 85	(2) 15	(0) 0	(2) 15	(2) 15	(9) 69	(0) 0
PAH cirrhotic	42	(11) 26	(17) 40	(14) 33	(38) 90	(2) 5	(2) 5	(10) 24	(1) 2	(28) 67	(3) 7
PAH non cirrhotic	32	(13) 41$^{\#\#}$	(8) 25	(11) 34	(29) 91	(3) 9	(0) 0	(10) 31†	(3) 9	(19) 59	(0) 0
AAH patients	74	(32) 43$^{\#\#}$	(27) 37	(15) 20	(56) 76	(11) 15	(7) 9$^{\#\#}$	(17) 23	(14) 19$^{**\#}$	(40) 54	(3) 4
AAH HAI>9	49	(15) 31	(20) 41	(14) 29	(39) 80	(7) 14	(3) 6	(7) 14	(8) 16	(32) 65	(2) 4
AAH HAI<9	14	(10) 71$^{\#\#\#}$	(2) 14	(2) 14	(8) 57†	(3) 21	(3) 21$^{\#\#}$	(5) 36†	(5) 36$^{\#}$	(3) 21$^{\#\#}$	(1) 7
AAH E 4–6	46	(14) 30	(19) 41	(13) 28	(36) 78	(5) 11	(5) 11	(6) 13	(8) 17	(30) 65	(2) 4
AAH E 0–3	16	(10) 63$^{\#\#\#}$	(3) 19	(3) 19	(10) 63	(3) 19	(3) 19	(5) 31	(5) 31$^{\#}$	(5) 31$^{\#\#}$	(1) 6
AAH cirrhotic	31	(6) 19	(16) 52	(9) 29	(27) 87	(2) 6	(2) 6	(4) 13	(2) 6	(23) 74	(2) 6
AAH non cirrhotic	33	(19) 71$^{\#\#\#}$	(6) 18	(8) 12	(21) 53†	(6) 24	(6) 24$^{\#\#\#}$	(8) 29	(11) 41$^{\#\#\#}$	(13) 24$^{\#\#}$	(1) 6

$^{**}p_c<0.01$ and $^{***}p_c<0.001$ PAH vs. AAH. $^{\#}p_c<0.05$, $^{\#\#}p_c<0.01$, $^{\#\#\#}p_c<0.001$, $^{\dagger}p<0.05$ PAH or AAH vs. HC. Histologic activity index (HAI) ranged of 1–18 (Knodell scoring system) and the fibrosis grade (Ishak scale, E) ranged of 0–6, 6=cirrhosis. PAH: type I paediatric autoimmune hepatitis; AAH: type I adult autoimmune hepatitis; HC: healthy controls.

lost their statistical significance when we applied the Bonferroni's correction, possible as a reflection of the lower number of patients available for complete clinical assessment. The 10CC genotype also seems to have anti-cirrhotic properties in PAH. This genotype was increased in PAH non-cirrhotic individuals (10CC$^+$ vs. 10CC$^-$: PAH non cirrhotic vs. HC: $p_c=0.006$, OR=0.18, CI=0.08–0.4).

Interestingly, the analysis of the two position-haplotypes revealed that combined presence of 25GG and 10CC seems to neutralize the effect observed with 10CC in AAH patients. This effect remained in AAH with the haplotype 10CC$^+$–25GG$^-$(10CC$^+$–25GG$^-$ vs. others genotypes: AAH HAI<9 vs. HC: $p_c=0.02$, OR=0.13, CI=0.04–0.4; AAH non cirrhotic vs. HC: $p_c<0.0006$, OR=0.15, CI=0.06–0.37).

Because PAH is strongly associated with the HLA-DRB1*1301-DQB1*0603 haplotype [2,5], we analysed the frequencies of these HLA alleles in relation to the TGF-β1 genotypes. The HLA-DRB1*1301 frequency was similarly increased in PAH patients irrespective of the TGF-β1 genotype (Fig. 1A). The frequency of HLA-DR3 or DR4 in AAH showed no significant difference among the different TGF-β1 genotypes (Figs. 1B and C).

IL-10 functional genetic polymorphisms in AH

In a previous report of PAH patients [31] we found that expression levels of IL-10 mRNA were not significantly different from HCs. The present study analyzed the functional IL-10 polymorphisms located at the promoter region [32] in PAH, AAH and HCs individuals. In particular, the SNPs at positions −1082 (G/A), −819 (C/T) and −592 (C/A) from the transcriptional start site, which are in linkage disequilibrium, and they are responsible for three different haplotypes: GCC, ACC and ATA. There is a correlation between IL-10 genotype and cytokine production, i.e., ACC/ACC, ACC/ATA and ATA/ATA (designated the AA genotype) are associated with low IL-10 production, GCC/ACC and GCC/ATA (GA genotype) are considered intermediate producers, and GCC/GCC (GG genotype) is considered a high producer.

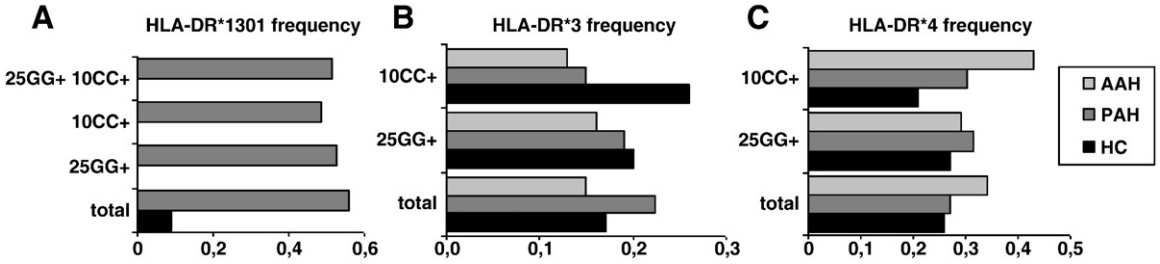

Figure 1 Association of HLA-susceptibility alleles and TGF-b1 genetic polymorphisms in AH. (A) The frequency of HLA-DRB1*1301 in all PAH patients (*n*=139) and HCs (lower bar, *n*=362) or in PAH patients carrying the TGF-β1 25GG$^+$–10CC$^+$ (*n*=31), 25GG$^+$ (*n*=95) or 10CC$^+$ (*n*=33) genotype. The same analysis was performed for individuals carrying HLA-DR3 (B) or HLA-DR4 (C) (total: AAH: *n*=74; 25GG$^+$: AAH: *n*=51, HCs: *n*=153; 10CC$^+$: AAH: *n*=23, HCs: *n*=38). Fisher's exact test by 2×2 contingency tables is not significant.

Table 6 IL-10 promoter haplotype and genotype frequencies in HA patients.

IL-10 promoter haplotypes	N	Haplotype frequency[a] (*n*) %			Genotype frequency (*n*) %		
		GCC	ACC	ATA	G/G	G/A	A/A
Healthy controls	209	(73) 35%	(82) 39%	(54) 26%	(21) 10%	(104) 50%	(84) 40%
PAH patients	117	(37) 32%	(39) 33%	(41) 35%	(14) 12%	(47) 40%	(56) 48%
AAH patients	80	(25) 31%	(30) 38%	(25) 31%	(10) 12%	(31) 39%	(39) 49%

n: number of individuals. PAH: type I autoimmune hepatitis in paediatric patients; AAH: type I autoimmune hepatitis in adult patients.
[a] The number of alleles is 2n.

The retrospective analysis of PAH, AAH patients and healthy controls, did not show differences in haplotype and genotype frequencies (Table 6). These results provide further support to our previous findings [31] concerning a lack of involvement of IL-10 in the pathogenesis of autoimmune hepatitis.

Discussion

We have proposed that paediatric (PAH) and adult forms (AAH) of type I autoimmune hepatitis may represent different clinical entities [2]. In addition to a different and very strong HLA-susceptibility association, the paediatric form has a more severe outcome that requires a more aggressive immunosuppression regimen. In spite of a stronger immunosuppression, in comparison with AAH, PAH patients progress to cirrhosis and liver transplantation with higher frequency [2]. TGF-β1 is believed to be the most potent master cytokine in the induction of hepatic fibrosis, and a correlation between the liver expression and serum level of TGF-β1 with disease activity has been reported [8]. The present study demonstrates differences in TGF-β1 gene polymorphisms that may explain, at least partially, the different outcomes of AH. When compared with AAH and HCs, the PAH patients presented a higher frequency of the high producer allele G and the GG genotype at codon 25. When compared with the controls, the AAH patients had an increased frequency of the codon 10 high producer allele C and the CC genotype. An interesting feature of AAH patients arises when TGF-β1 genotypes were compared according to histological parameters. The strong increase of the 10CC genotype was present in patients having low inflammatory reaction, low fibrosis stage and as expected, did not developed cirrhosis. The same group of patients also showed a strong increase of the low producer genotype 25CC. Interestingly, the analysis of haplotypes revealed that combined presence of 25GG and 10CC in AAH patients seems to neutralize the protective effect of 10CC genotype. This effect remained in AAH with the haplotype $10CC^{+}$–$25GG^{-}$ In contrast, PAH patients showed an increase of the codon 25GG genotype restricted to those children presenting the highest inflammatory reaction.

It was previously reported that serum levels of TGF-β1 were increased in patients with autoimmune hepatitis [33]. In line with this report, the current study seems to support a role for TGF-β1 in autoimmune hepatitis. However, we found a differential role for the two polymorphic TGF-β loci. The 10CC genotype seems to be associated with anti-inflammatory mechanisms. In contrast, the presence of the 25GG genotype was found associated with a more aggressive form of the disease, that can even neutralize the effect of the 10CC genotype. Several reports have assigned codon 10 CC genotype as a high producer genotype. For example, transfection constructs containing either T or C alleles revealed that the C variant leads to a much greater increase of TGF-β1 secretion than the T allele [18]. Moreover, serum levels and mRNA expression in PBMC resulted higher in $10CC^{+}$ individuals than in CT or TT samples [19,20,34]. However, other studies reported that codon 10 T allele is the one associated with higher levels of cytokine [21,35]. Taking together these contradictory results, further studies should be made to clarify if the different association we found with codon 10 and codon 25 in AAH and PAH reflects just a different production of TGF-β1 or may represent different biological effect.

In chronic infection with hepatitis C virus (HCV), high-producer TGF-β1 alleles have also been associated with progression to liver cirrhosis [22,36–38]. A differential association of TGF-β1 with the level of fibrosis or with inflammation could explain results obtained in other autoimmune disease. For instance, patients with Hashimoto's disease in whom the inflammatory component predominate over the development of fibrosis, more severe forms of the disease were associated with higher frequency of the TGF-β1 10T allele and 10TT genotype [39]. Similarly, an increased frequency of 10T allele has been reported in rheumatoid arthritis and systemic sclerosis [40,41]. The association of TGF-β1 low-producer alleles with autoimmune disease may be caused by the requirement of its anti-inflammatory effect to maintain immune homeostasis and to prevent autoimmune diseases [9].

In a previous study we reported that frequency of high producers IL-10 genotypes were higher in noncirrhotic female chronically infected with HCV [32]. It is also known that chronically HCV-infected patients who receive a short treatment with recombinant IL-10 showed a decreased liver fibrosis [42]. In this sense, results from the present study seem to exclude the participation of IL-10 polymorphism in the more sever outcome of PAH.

We can conclude that a protective role of 10CC observed in AAH can explain at least partially the different clinical outcome of AAH and PAH. This is supported by our observation that the combined presence of 25GG and 10CC seems to neutralize the protective effect of 10CC and in consequence a more aggressive clinical outcome in PAH patients.

Although it is recognised that the mechanisms underlying the pathogenesis of AAH and PAH are heterogeneous, the differential role of the TGF-β1 codon 10/codon 25 polymorphisms may be an additional tool in the prediction of disease severity in autoimmune hepatitis.

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