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Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection

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Abstract The natural outcome and response to treatment in hepatitis C virus (HCV) infection varies between individuals. Whereas some variation may be attributable to viral and environmental variables, it is probable that host genetic background also plays a significant role. Interleukin (IL)-10 has a key function in the regulation of cellular immune responses and in the suppression of pro-inflammatory cytokine secretion. Functional polymorphisms in the IL-10 gene have been described. We investigated the role of these polymorphisms in the outcome of HCV infection, treatment response and development of fibrosis in a case-control association study. Self-limiting infection was associated with the IL-10 (–592) AA genotype (OR=2.05; $P=0.028$). Persistent infection was associated with the IL-10 (–1082) GG genotype (OR=0.48; $P=0.018$). Sustained response to interferon therapy was associated with the IL-10 (–1082) GG genotype (OR=2.28; $P=0.005$) and the haplotype GCC (OR=2.27; $P=0.020$). The IL-10 (–1082) AA genotype and the ATA/ATA and ACC/ACC homozygous haplotypes were more frequent among patients with rapid fibrosis. Furthermore, the microsatellites IL-10.R and IL-10.G were associated with interferon response with IL-10R.2 conveying susceptibility (OR=1.80; $P=0.034$), and IL-10R.3 and IL-10.G13 being protective (OR=0.47; $P=0.003$ and OR=0.59; $P=0.042$, respectively). We

conclude that polymorphisms in the IL-10 promoter appear to have some influence on the outcome of HCV infection, treatment and development of fibrosis.

Keywords Self-limiting infection · Interferon · Fibrosis · Genotype · Disease association · Hepatitis C

Introduction

Infection with the hepatitis C virus (HCV) is characterized by a broad spectrum of possible outcomes. Infection is self-limited in a fortunate minority, while the majority of patients develop persistent (chronic) infection (Alter et al.1992; Seeff et al.1992; Tong et al.1995). Among those individuals with persistent HCV infection, the majority develop chronic hepatitis and progressive fibrosis (Marcellin1999). However, the rate at which fibrosis develops varies substantially between individuals, with some progressing to cirrhosis within 10 years and others never progressing to any significant liver disease within their natural lifespan (Poynard et al.1997; Kenny Walsh1999). Similarly, the outcome of interferon-based anti-HCV therapies varies, with only about 15% achieving sustained virologic remission with interferon monotherapy (Hoofnagle et al.1986; Davis and Lau1997; Westendorp et al.1997; Touzet et al.2000) and 40% achieving sustained response rates with combination therapy including ribavirin (McHutchison et al.1998; Manns et al.2001).

The vigour of the immune response is likely to play an important role in mediating the natural history of HCV. Strong CD4+ T-helper (Th) and CD8+ cytotoxic T-lymphocyte (CTL) responses are likely to be crucial in both clearance of acute viraemia and the clearance of viraemia in the context of therapy (Diepolder et al.1996; Cramp et al. 1998a, 1998b; Thursz et al.1999; Godkin et al.2001). Immunological responses may also drive HCV-mediated liver fibrogenesis (Poynard et al.1997) acting through cytokines that stimulate extracellular matrix deposition, such as tumor necrosis factor alpha (TNF α) and transforming growth factor beta (TGF β).

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The cytokine interleukin-10 (IL-10) has pleiotropic effects on a number of different cell types. In general it is regarded as a suppressor of immune responses because it inhibits the secretion of pro-inflammatory and antiviral cytokines such as TNF α and interferon- γ (IFN γ) (Moore et al.1993). In addition IL-10 inhibits the development and activation of CD4+ T-helper lymphocytes with a Th1 (IFN γ -secreting) phenotype (Moore et al.1993).

Evidence for a strong genetic contribution to IL-10 production is well established (Westendorp et al.1997; Reuss et al.2002). A number of polymorphisms appear to control the level of secretion of the cytokine. There are three single nucleotide polymorphisms (SNPs) at positions -1082, -819 and -592 with respect to the transcription initiation site (Turner et al.1997). In addition two dinucleotide repeat polymorphisms are located approximately 1 kb (IL-10.G) (Eskdale and Gallagher 1995) and 4 kb (IL-10.R) (Eskdale et al.1996) upstream from the transcription initiation site. Studies on the functional characteristics of these polymorphisms suggest that they influence the level of secretion of the cytokine (Eskdale et al.1999).

In this study we investigated the role of IL-10 gene promoter variants in determining the outcome of HCV infection, treatment response and development of fibrosis and compare our results with previous studies.

Materials and methods

Patients

A total of 659 White Caucasian patients were recruited in nine liver centres across Europe and by the UK Blood Transfusion Service. Patients were recruited randomly or sequentially in each centre in order to minimize selection bias. Each patient gave informed consent and ethical approval was obtained from the local research ethics committee at each centre. Patients were classified into the following groups: (1) self-limiting HCV infection: individuals with antibodies to HCV who had no evidence of viraemia on at least two occasions and who had persistently normal liver transaminase levels; (2) persistent HCV infection: individuals who had evidence of viraemia for at least 6 months; (3) sustained treatment response (SR): patients with persistent HCV infection treated with IFN α alone who had normal liver transaminases and no evidence of viraemia 6 months after the end of treatment; (4) response-relapse (RR): patients with persistent HCV infection treated with IFN α who had normal liver transaminases and no evidence of viraemia at the end of treatment, but in whom viraemia returned during the follow-up period; (5) non-response (NR) to treatment: patients with persistent HCV infection treated with IFN α who never lost viraemia during treatment; (6) fast fibrosis: patients who would be expected to reach cirrhosis in 20 years or less; (7) slow fibrosis: patients who would not expect to reach fibrosis for at least 30 years. An initial response (IR) group was defined as a combination of the RR and the SR groups.

HCV antibodies and viraemia

Antibodies to HCV antigens were detected with both an enzyme-linked immunoassay (EIA) or a recombinant immunoblot assay (RIBA) containing four HCV antigens on a cellulose acetate strip used according to the manufacturer's instructions (Abbott Laboratories, North Chicago, Ill.). The presence or absence of viral

particles in serum was determined by reverse transcription polymerase chain reaction using a commercially available assay (Amplicor, Roche) with a sensitivity of approximately 200 genomes/ml.

Liver histology and rate of fibrosis

All liver biopsies were reported by a single pathologist without knowledge of the patients' clinical details or where they came from. Each biopsy was stained using haematoxylin and eosin and a reticulin stain. Biopsies were assessed as adequate if there were three or more portal tracts. They were scored using the modified Histological Activity Index semi-quantitative scoring system (Ishak et al.1995). Two percent of biopsies were re-scored, without knowledge of the initial score to assess intra-observer error. (The kappa statistic for the two assessments was 0.94).

Estimates of the duration of infection were obtained. In patients acquiring their infection through transfusion of blood or blood products the date of blood transfusion was taken as the onset of infection. In patients acquiring their infection through intravenous (IV) drug use the date of first IV drug use was assumed to be the onset of infection, because it is known that the majority of IV drug users become infected during the first months of abuse.

The rate of fibrosis was calculated from the histological stage and duration of infection using the formula: Fibrosis rate=Fibrosis score/Years since infection.

DNA extraction and genotyping

Genomic DNA was extracted from a 5 ml sample of whole blood collected into EDTA. Extraction was performed using a commercial kit (Nucleon II, Scotlabs, UK) according to the manufacturer's instructions.

IL-10 (-1082) and IL-10 (-592) genotyping

The IL-10 (-592) locus was genotyped with two of the following primers: either 5'-CAGAGACTGGCTTCCTACAGT-3' or 5'-AGAGACTGGCTTCCTACAGG-3' (reverse allele specific primers, and 5'-GGGTAAAGGAGCCTGGAACACAT-3' (forward common primer); the IL-10 (-1082) locus with either 5'-CTACTAAGGCTTCTTG-GGAG-3' (forward allele specific primers), and 5'-GGAGGTCCCTTACTTTCCTCTACCTA-3' (reverse common primer).

All PCR reactions were performed using 5–100 ng DNA in a total volume of 20 μ l. Each reaction comprised 0.2 μ M of each of the two primers; 2.5 units of Stoffel Gold Polymerase (gift from David Birch, RMS); 1 \times Stoffel Gold buffer (10 mM Tris-HCl, 10 mM KCl, pH 8.0); an additional 30 mM KCl for a final concentration of 40 mM; 3 mM MgCl₂; 50 μ M each dATP, dCTP, and dGTP; 25 mM TTP; 75 mM dUTP; 2 units of uracil DNA glycosylase (UNG) (PE); 0.2 \times SybrGreen I (Roche Molecular Probes); 2 μ M ROX (Roche Molecular Probes); 5% DMSO; and 2.5% glycerol (Germer et al.2000).

Kinetic PCR reactions were performed on a GeneAmp 5700 Sequence detection System (Perkin Elmer Applied Biosystems). An initial incubation step of 2 min at 50 $^{\circ}$ C, (to allow UNG-mediated elimination of carryover PCR product contamination), and an enzyme heat activation step of 12 min at 95 $^{\circ}$ C were followed by 40 two-step amplification cycles of 20 s at 95 $^{\circ}$ C for denaturation and 20 s for 58 $^{\circ}$ C for annealing and extension, and a final 5 min product extension step at 72 $^{\circ}$ C.

The GeneAmp software was used to define a threshold value for the emergence of PCR products at the beginning of the linear phase of amplification. The software was then used to determine the threshold cycle (C_T) at which the reaction crossed the threshold and genotypes were assigned by comparison of C_T values between parallel reactions performed with allele-specific primers. Genotype results were confirmed by sequencing of 1% of samples.

IL-10.R and IL-10.G dinucleotide repeat genotyping

Sample genotypes for the two promoter microsatellites IL-10.R and IL-10.G were analysed by a fluorescence-based semi-automated genotyping method. Briefly, PCR products spanning each microsatellite were generated under standard conditions in a 15 µl reaction containing 1× reaction buffer, 1.5 mM MgCl₂, 0.32 mM dNTP, 1 unit TaqGold polymerase (all reagents Perkin Elmer Applied Biosystems, Warrington, UK), 0.12 µM of forward and reverse primer respectively, 50 ng DNA and dH₂O. Cycling parameters were 94 °C for 14 min followed by 35 cycles of 94 °C for 15 s, 58 °C and 53 °C for 30 s for IL-10.R and IL-10.G, respectively, and 72 °C for 30 s, followed by a final step of 72 °C for 7 min. PCR primer sequences for IL-10.R were: forward 5'-GAATGCACCCCTCCAAAATCTAT-3', HEX-labelled, and reverse 5'-GAAGCTCCGCCAGTAAGTT-3'. PCR primer sequences for IL-10.G were: forward 5'-GTCCTTCCCCAGGTAGAGCAA-CACTCC-3', FAM-labelled, and reverse 5'-CTCCAAAGAAGCCTTAGTAGTGTT-3'. 0.5 µl of each PCR amplification product was run on an ABI prism 373 sequencer (Perkin Elmer Applied Biosystems) and sample genotypes analysed using GENESCAN and Genotyper software programs (Perkin Elmer Applied Biosystems, Warrington, UK). The allele specifications for the two microsatellite polymorphisms are in accordance with Eskdale and colleagues (Eskdale and Gallagher1995; Eskdale et al.1996).

Statistical analyses

Univariate analyses were performed by comparison of allele and genotype frequencies for each locus using standard contingency tables for the following patient groups: (1) self-limiting versus persistent infection; (2) SR versus NR to interferon; (3) IR versus NR to interferon; (4) RR versus SR to interferon (5) rapid versus slow fibrosis. Microsatellite data was analysed using CLUMP software, to account for the presence of alleles of low frequency (Sham and Curtis1995).

To facilitate categorical analyses, continuous variables were categorized as follows: viral genotypes were dichotomized into genotype 1 and non-1.

All univariate and multivariable calculations, including odds ratios (OR), 95% confidence intervals (95%CI), and *P* values (both maximum likelihood and Fisher's exact where appropriate) were conducted using the SPSS (version 10) or Epistat software.

Haplotypes

Simple haplotypes involving the three single nucleotide polymorphisms were assigned by inference from the genotype data for IL-10 (-592) and IL-10 (-1082), and close linkage of IL-10 (-819) and IL-10 (-592) (Turner et al.1997; Edwards-Smith et al.1999; Gibson et al.2001).

Results

Characteristics of patients are given in Table 1. Female gender was associated with self-limiting infection: 61.9% of the subjects with self-limiting infection were female in comparison to 42.8% of patients with persistent infection (OR=2.17; 95%CI: 1.29–3.41; *P*=0.0003). Gender showed no effect on interferon response. Male gender was associated with rapid fibrosis: of those classified as fast fibrosers, 67.6% were male compared to 43.5% in the slow fibrosis group (*P*=0.005). As expected, viral genotype1 was found more frequently amongst NR (60.0%) compared to SR (33.1%): OR=2.2; 95%CI: 1.28–3.86; *P*=1×10⁻⁷.

Age at the time of infection had the expected effect on rate of fibrosis with 97.5% of individuals classified as having slow fibrosis being infected under the age of 40, compared to 64.7% of those with fast fibrosis: *P*=8×10⁻⁸. Age at the time of infection had no effect on the outcome of interferon therapy. Source of infection had no demonstrable impact on the outcome of hepatitis infection.

The distribution of genotypes in our HCV cohorts conformed to Hardy-Weinberg equilibrium.

Self-limiting versus persistent infections

At the IL-10 (-592 locus), the AA homozygous genotype was found more frequently in subjects with self-limiting infection compared to those with persistent infection:

Table 1 Characteristics of White Caucasian hepatitis C virus (HCV) patient cohort. *IVDU* Intravenous drug user, *SD* standard deviation

	Self-limiting infection <i>n</i> =105 (%)	Persistent infection <i>n</i> =554 (%)	Sustained responder <i>n</i> =154 (%)	Relapsed responder <i>n</i> =119 (%)	Non-responder <i>n</i> =150 (%)	Fast fibrosis <i>n</i> =71 (%)	Slow fibrosis <i>n</i> =62 (%)
Gender							
Male	40 (38.1)	317 (57.2)	89 (57.8)	70 (58.8)	89 (59.3)	48 (67.6)	27 (43.5)
Female	65 (61.9)	237 (42.8)	65 (42.2)	49 (41.2)	61 (40.7)	23 (32.4)	35 (56.4)
Viral genotype							
1	5 (4.7)	255 (46.0)	51 (33.1)	56 (47.1)	90 (60.0)	19(26.8)	25 (40.3)
Non-1	6 (5.7)	124 (22.4)	45 (29.2)	32 (26.9)	14 (9.3)	22 (31.0)	9 (14.5)
Unknown	94 (89.5)	175 (31.6)	58 (37.7)	31 (26.0)	46 (30.7)	30 (42.2)	28 (39.4)
Source of infection							
IVDU	20 (19.1)	121 (21.8)	37 (24.0)	25 (21.0)	33 (22.0)	20 (28.1)	30 (48.4)
Blood products	25 (23.8)	124 (22.4)	41 (26.6)	21 (17.6)	45 (30.0)	28 (39.4)	24 (38.8)
Miscellaneous	2 (1.9)	15 (2.7)	6 (3.9)	5 (4.2)	2 (1.3)	0 (0)	3 (4.8)
Unknown	58 (55.2)	294 (53.1)	7 (45.4)	68 (57.1)	70 (46.7)	23 (32.4)	5 (8.0)
Average age at infection ± SD							
	25.6±12.6	29.3±13.8	29.6±12.9	27.4±12.4	29.4±14.8	36.2±14.2	21.0±8.6

Table 2 Differential distribution of IL-10 alleles and genotypes among individuals with self-limiting HCV infection compared to individuals with persistent HCV infection

Locus	Genotype	Self-limiting <i>n</i> (%)	Persistent <i>n</i> (%)	OR	95% CI	<i>P</i> -value
IL-10 (-592)		<i>n</i> =105	<i>n</i> =545			
	AA	14 (13.3)	38 (7.0)	2.05	1.01–4.11	0.028
	AC	42 (40.0)	207 (38.0)	1.09	0.69–1.70	0.697
	CC	49 (46.7)	300 (55.0)	0.71	0.46–1.11	0.115
IL-10 (-1082)		<i>n</i> =94	<i>n</i> =577			
	AA	27 (28.7)	183 (31.7)	0.87	0.52–1.44	0.562
	AG	54 (57.4)	250 (43.3)	1.77	1.11–2.81	0.010
	GG	13 (13.8)	144 (25.0)	0.48	0.25–0.92	0.018

Table 3 Differential distribution of IL-10 genotypes with sustained response (SR) to interferon therapy, with relapsed response (RR) and with non-response to anti-HCV therapy (NR)

Locus	Genotype	SR (%)	RR (%)	NR (%)	OR	95% C.I.	<i>P</i> -value
IL-10 (-592)		<i>n</i> =154	<i>n</i> =115	<i>n</i> =149			
	AA	14 (9.1)	6 (5.2)	11 (7.5)	1.25 (SR vs NR)	0.51–3.08	0.589
	AC	63 (40.9)	47 (40.8)	50 (34.2)	1.37 (SR vs NR)	0.84–2.25	0.186
	CC	77 (50.0)	62 (53.9)	88 (58.2)	0.69 (SR vs NR)	0.43–1.12	0.113
IL-10 (-1082)		<i>n</i> =148	<i>n</i> =119	<i>n</i> =141			
	AA	51 (34.5)	35 (29.4)	43 (30.1)	1.23 (SR vs NR)	0.73–2.06	0.423
	AG	57 (38.6)	44 (37.0)	80 (55.9)	0.49 (SR vs NR)	0.30–0.81	0.003
	GG	40 (26.9)	40 (33.6)	20 (14.0)	2.28 (SR vs NR)	1.21–4.32	0.005

13.3% versus 7.0% (OR=2.05; 95%CI: 1.01–4.11; *P*=0.028) (Table 2). The IL-10 (-1082) GG genotype was more frequent in individuals with persistent infection, 144 out of 577 (25.0%) compared to those with self-limiting infection, 13 out of 94 (13.8%); OR=0.48; 95%CI: 0.25–0.92; *P*=0.018. The IL-10 (-1082) AG genotype was significantly more frequent in individuals with self-limiting infection (OR=1.77; 95%CI: 1.11–2.81; *P*=0.010), possibly indicating a heterozygous effect. No association was identified for self-limiting infection with the microsatellite polymorphisms IL-10.G and IL-10.R.

Response to antiviral treatment

Patients with SR to interferon therapy showed a significantly increased frequency of the IL-10 (-1082) GG genotype compared to patients with NR to interferon; 26.9% versus 14.0% (OR=2.28; 95%CI: 1.21–4.32; *P*=0.005) (Table 3). In addition, in patients who had an IR to interferon therapy there was an increased frequency of the GG genotype compared to NR to interferon treatment; 29.9% versus 14.0% (OR=2.63; 95%CI: 1.49–4.69; *P*=0.003) (data not shown in table). IL-10 (-1082) heterozygotes were more frequent in the group of NR (OR=0.49; 95%CI: 0.30–0.81; *P*=0.003), suggesting a heterozygous effect.

At the IL-10.R MS allele 2 was increased in NR (71 out of 92 or 77.2%) compared to SR (30 out of 46 or 65.2%) indicating a susceptibility allele (OR=1.80; 95%CI: 1.00–3.24; *P*=0.034). IL-10.R allele 3 was found in 15 out of 46 (32.6%) treatment-responders compared to 15 out of 92 (16.8%) NR, suggesting a protective effect of this allele (OR=0.47; 95%CI: 0.22–0.78; *P*=0.003). These results were very similar when stratifying the cohort

according to initial response (OR=1.47 for IL-10.R2 and OR=0.57 for IL-10.R3). The frequency of IL-10.G allele 13 was higher in IR compared to NR (18.7% versus 12.0%) and showed borderline significance between the two groups (OR=0.59; 95%CI: 0.34–1.01; *P*=0.042).

Fibrogenesis

Although the differences in genotype and haplotype frequencies between fast and slow fibrosers did not reach statistical significance, the genotype IL-10 (-1082) AA was more abundant in the fast fibrosers (39.2% vs 23.8%, OR=1.82; 95%CI: 0.83–4.00; *P*=0.103 (Table 4).

Other comparisons did not show any significant associations (*P*<0.05).

Haplotypes

There are three simple haplotypes in the promoter region of the IL-10 gene extending over the three single nucleotide polymorphisms at positions -1082, -819 and -592 with respect to the transcriptional initiation site. The -819 and -592 loci are in tight linkage disequilibrium allowing inference of the haplotype from the genotype data at the -1082 and -592 loci (Table 5). Individuals carrying two copies of the GCC haplotype were more likely to respond to interferon therapy than those with other haplotypes (OR=2.27; 95%CI: 1.07–4.88; *P*=0.021 for SR vs NR; OR=2.49; 95%CI: 1.21–5.19; *P*=0.001 for initial response). In the fast fibrosers the homozygous haplotypes ACC/ACC and ATA/ATA were more abundant (9.2% vs 6.1%, 16.9% vs 6.1%, respectively), but

Table 4 Differential distribution of IL-10 genotypes among individuals with fast progression of fibrosis compared to individuals with slow progression of fibrosis

Locus	Genotype	Fast fibrosis <i>n</i> (%)	Slow fibrosis <i>n</i> (%)	OR	95% CI	<i>P</i> value
IL-10 (-592)	AA	<i>n</i> =71 6 (8.6)	<i>n</i> =62 5 (8.1)	1.05	0.27–4.24	0.935
	AC	20 (28.2)	25 (40.3)	0.58	0.26–1.27	0.139
	CC	45 (63.4)	32 (51.6)	1.62	0.77–3.45	0.170
IL-10 (-1082)	AA	<i>n</i> =74 29 (39.2)	<i>n</i> =65 17(23.8)	1.82	0.83–4.00	0.103
	AG	29 (36.5)	32(49.2)	0.66	0.32–1.38	0.234
	GG	16 (24.3)	16(26.9)	0.84	0.36–2.00	0.676

Table 5 Frequency of IL-10 haplotypes.

Haplotype	Self-limiting infection <i>n</i> =64 (%)	Persistent infection <i>n</i> =383 (%)	Sustained- responder <i>n</i> =132 (%)	Relapsed- responder <i>n</i> =78 (%)	Non- responder <i>n</i> =109 (%)	Fast fibrosis <i>n</i> =65 (%)	Slow fibrosis <i>n</i> =49 (%)
GCC	53 (41.4)	259 (33.8)	120 (45.5)	77 (49.4)	91 (41.7)	53 (40.8)	47 (47.9)
ATA	33 (25.8)	192 (25.1)	78 (29.5)	35 (22.4)	53 (24.3)	29 (22.3)	26 (26.5)
ACC	38 (29.7)	217 (28.3)	84 (31.8)	35 (22.4)	75 (34.4)	48 (36.9)	27 (27.5)
GCC/GCC	9 (14.1)	82 (21.4)	31 (23.5)*	22 (28.2)*	13 (11.9)*	12 (18.5)	10 (20.4)
ATA/ATA	5 (7.8)	24 (6.3)	13 (9.8)	3 (3.8)	6 (5.5)	6 (9.2)	3 (6.1)
ACC/ACC	7 (10.9)	34 (8.9)	14 (10.6)	7 (8.9)	9 (8.2)	11 (16.9)	3 (6.1)

* SR vs NR (GCC/GCC): OR=2.27 (1.07–4.88) *P*=0.021. SR+RR=IR vs NR (GCC/GCC): OR=2.49 (1.24–5.09) *P*=0.001

without reaching statistical significance due to low numbers (Table 5).

We were not able to analyse extended haplotypes including the two haplotypes due to the lack of complete genotyping data for all markers within our cohort.

Stratification of the data according to viral genotype (divided into 1 and non-1), gender or age of infection resulted in reduced numbers for the analysis, but did not introduce any significant changes into the presented results (data not shown).

Discussion

There is compelling evidence that IL-10 plays a role in HCV disease pathogenesis; however, contradictory reports exist as to the exact effect of the IL-10 promoter polymorphisms on the natural outcome of HCV infection treatment response (Edwards-Smith et al.1999, Powell et al.2000; Yee et al.2001) and development of fibrosis during HCV infection (Powell et al.2000). We set out to analyse the role of IL-10 promoter polymorphisms IL-10 (-592) and IL-10 (-1082) in HCV infection, employing a large multi-centre cohort. We assessed IL-10 haplotypes including a SNP at position (-819), which could be inferred from our data, since it is in tight linkage disequilibrium with the (-592) variant. We also investigated the potential influence of two microsatellite markers, IL-10.R and IL-10.G on the outcome of HCV infection.

Our results suggest that spontaneous resolution of HCV infection may be influenced by the two SNPs in the promoter region of the IL-10 gene we investigated. In particular the AA genotype at the IL-10 (-592) locus was

weakly associated with self-limiting infection, whereas persistent infection was associated with the GG genotype at the IL-10 (-1082) locus. The association of the IL-10 (-592) AA genotype with self-limiting HCV infection has not been observed previously (Vidigal et al.2002; Constantini et al.2002), whereas the association of the IL-10 (-1082) GG genotype with persistent infection confirms an earlier report, although this was based on a comparison with healthy controls rather than patients with self-limiting infection (Vidigal et al.2002). A further small study on Sicilian samples showed the IL-10 GG genotype to be highest in a very small number of patients with self-limiting infection compared to those with persistent infection and these presented with an increased frequency compared to healthy controls (18, 42 and 135 individuals in each group) (Lio et al.2003). A plausible explanation for the association of the IL-10 (-1082) GG genotype with persistent infection is derived from experiments showing that the IL-10 (-1082) G allele produces higher levels of the cytokine, which may compromise the cellular immune response to the virus (Turner et al.1997; Eskdale et al.1998; Edwards-Smith et al.1999; Reuss et al.2002). There was no association with the two microsatellite markers and outcome of infection.

In our population the IL-10 (-1082) genotype appears to influence the outcome of interferon therapy. The GG genotype was found at approximately twice the frequency in SR compared to patients with NR. Furthermore, homozygosity for the GCC haplotype was associated with a 2.5 fold higher rate of response to interferon therapy. This contrasts with previous reports, which identified an association of the IL-10 (-592) locus with treatment response. Edwards-Smith et al. (1999) showed an association of the IL-10 (-592) CC genotype with NR

in 43 patients compared with initial response and an association of the corresponding GCC haplotype (41 patients) with NR. Yee et al. (2001) reported an association of the IL-10 (-592) AA genotype with SR in a study of 49 SR and 55 NR who had been treated with interferon and ribavirin combination therapy. However, Vidigal et al. (2002) did not observe any associations with the IL-10 promoter polymorphisms and the outcomes of combination therapy in a study of 37 SR and 38 NR. Possible explanations for these different results are outlined in further detail below. In addition to the IL-10 (-1082) association we found significant difference in the frequency of IL-10.R and IL-10.G alleles and response to interferon treatment. IL-10.R2 was shown to render individuals more susceptible to NR, whereas allele R3 appeared to have a beneficial effect with regard to treatment response. Furthermore, the frequency of IL-10G.13 was increased in IR patients, but not SR compared to NR, thus suggesting a less strong effect on susceptibility to failure to respond to treatment.

Additional SNPs have been identified in the IL-10 promoter region (D'Alfonso et al.2000; Gibson et al.2001). However, many of these are rare polymorphisms, which fall into four major haplotypes (Eskdale et al.1999). Unfortunately we were not able to accurately construct extended haplotypes including the three SNPs and the two microsatellites from our data, due to the limitation in sample numbers for which genotyping data for all five markers was available.

The heterozygous effect seen for IL-10 (-1082) AG in association with self-limiting infection and NR is more difficult to explain in biological terms, but could suggest a possible underlying heterozygous advantage. In some publications (Edwards-Smith et al.1999; Yee et al.2001) the frequencies of heterozygotes are not mentioned, but are apparent in other studies (Vidigal et al.2002; Lio et al.2003).

Fibrosis is a further outcome measure of HCV infection. The study of Powell et al. (1999) did not show a correlation between stages of fibrosis and IL-10 promoter polymorphisms. However, it has been suggested that the distinction between fast and slow fibrosis is a clearer measure for disease progression than stages of fibrosis (Wright et al.2003). We analysed our data accordingly and the results showed a higher frequency of the low IL-10 producing genotype [IL-10 (-1082) AA], and haplotypes (ACC/ACC and ATA/ATA) in the fast fibrosers. This is in agreement with the anti-fibrotic role of IL-10, although our results only reached borderline significance and sample numbers for this comparison are small; thus these findings should be interpreted with caution. There was no evidence of an influence of the microsatellites on fibrosis in our cohort.

Conflicting results between studies can be a reflection of one or several factors, such as differences in sample size, the selection of subjects for the study, differences in analysis parameters, genetic heterogeneity of various populations and different gene-gene or gene-environment interactions. For instance, in the Edwards-Smith study

(Edwards-Smith et al.1999) the number of subjects analysed with respect to interferon response was rather small, a factor which is known to give rise to spurious and unreproducible results (Ioannidis et al.2001), and a later study by the same group showed no significant differences in IL-10 promoter polymorphism frequencies between HCV patients and a healthy control population (Powell et al.2000). In the study conducted by Yee and colleagues (2001) the patients were treated with combination therapy. Ribavirin is thought to promote a desirable Th1 (IFN γ -secreting) phenotype of Th lymphocytes whereas IL-10 suppresses Th1 lymphocytes (Lau et al.2002). It is therefore plausible that the combination of ribavirin with a host genotype associated with low IL-10 production would favour the elimination of HCV. Therefore, the differences observed between our results and those presented by Yee and colleagues (2001) could be explained by the different mechanisms of viral elimination when combination therapy is used. Furthermore, findings by Yee and colleagues (2001) were not reproduced by Vidigal et al. (2002), even though they, too, studied patients (37 SR and 38 NR) treated with combination therapy and no associations were observed for the outcome of infection and interferon response in two further independent cohorts (Constantini et al.2002).

Underlying differences in ethnicity of patients investigated for the various studies are also likely to affect the observed differences in findings between studies, which makes comparisons very difficult. However, the IL-10 genotype frequencies in our multi-centre European population are consistent with those published elsewhere (Powell et al.2000), but differ from other IL-10 genotype profiles (Vidigal et al.2002). We found no difference in genotype frequencies in individuals from Northern and Southern European origin within our own study (data not shown).

An alternative explanation for the discrepancies in published results could be the consequence of different environmental/cultural backgrounds. For instance smoking and a decreasing body mass index appear to decrease IL-10 production (Reuss et al.2002). However, such additional environmental confounders are often difficult to record and are not easily controlled for. Multivariate analysis according to viral genotype, gender and age of infection in our cohort resulted in very small subgroups and stratification did not have a significant effect on the presented data. We thus chose to present our data in an unstratified manner for these parameters.

Some reports describe the application of the Bonferroni correction to adjust for multiple comparisons (Constantini et al.2002). A number of statistical experts have addressed this issue (Rothman 1990) and most agree that the Bonferroni correction is too conservative for this type of disease association study. We thus chose not to apply the Bonferroni correction to our data. An alternative approach to Bonferroni correction is replication of results in an independent cohort. However, despite using larger numbers of patients in our study compared to some others, we were unable to resolve the discrepancies

between early studies and between those studies and our own.

Functional studies of the effect of promoter variants on IL-10 secretion are also contradictory. Several publications have indicated that a G allele at the -1082 position and the GCC haplotype are associated with increased levels of IL-10 secretion in transfected monocytic cell lines as well as in LPS and concanavalin A-stimulated PBMC cells (Turner et al.1997; Edwards-Smith et al.1999). It has been shown that differences in IL-10 promoter activity between these haplotypes are caused by differential binding activity of the IL-10 (-1082) and IL-10 (-592) alleles to the transcription factor PU.1 (Reuss et al.2002). However, the IL-10 promoter haplotypes did not show a correlation with the production of IL-10 in LPS-stimulated whole blood cells (Reuss et al.2002). Another study demonstrated that the A allele at position -1082 was more transcriptionally active through a reporter gene assay (Rees et al.2002). It is difficult to draw any firm conclusions from these reports as the systems used to investigate IL-10 secretion have been heterogeneous. Cell type and the immunological context of IL-10 signalling have major effects on cytokine transcription, which may lead to these apparent inconsistencies.

Excess secretion of IL-10 is likely to inhibit immunological responses which favour viral elimination such as Th1 T-helper lymphocyte responses and the secretion of pro-inflammatory and antiviral cytokines such as TNF α and IFN γ . It is likely that the IL-10 (-592) AA genotype is associated with low levels of IL-10 secretion, which facilitates viral elimination both at the time of initial infection, as demonstrated by our data, and when patients are treated with interferon and ribavirin combination therapy.

In summary, whereas some of our results are in agreement with earlier publications, other results from our study stand in contrast to previous findings on IL-10 promoter polymorphism and HCV infection in other populations. As such it is difficult to ascertain the magnitude of effect of genetic polymorphisms on disease outcome and the exact mechanisms underlying these processes remain poorly understood. In addition, the existence of IL-10 homologues and different IL-10 binding receptors (Fickenscher et al.2002) are likely to complicate the determination of levels of IL-10 expression in vitro. The interaction with these molecules could affect the correlation between IL-10 promoter polymorphisms and outcome of HCV infection. Further research on the functional implications, specifically in relation to the immune response to HCV, are clearly warranted.

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