

Association of low-density lipoprotein receptor polymorphisms and outcome of hepatitis C infection

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The low-density lipoprotein receptor (LDLR) has been proposed to promote hepatitis C virus endocytosis and the cell membrane protein CD81 may also promote HCV host cell entry. The CD81 gene was sequenced to screen for novel polymorphisms, but no SNPs were identified. Polymorphisms within the LDLR gene are associated with the pathogenesis of familial hypercholesterolemia, atherosclerosis and obesity. We therefore studied genetic variation within the LDLR gene and clinical features of hepatitis C infection. An amino acid change in exon 8 was associated with severity of fibrosis; a SNP in exon 10 correlated with viral clearance and overall inflammation, and a SNP in the 3' UTR appeared to influence treatment response. There were no other significant associations between any of the SNPs studied and the clinical measures of hepatitis C infection. We furthermore report on linkage disequilibrium within the gene and haplotype frequencies in our population. Our findings support a possible role for the LDLR in the modulation of disease progression by affecting immune responses, rather than functioning as receptor for HCV.

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Introduction

Hepatitis C is a disease with a worldwide prevalence of approximately 3%.¹ An estimated 80% of individuals infected by the hepatitis C virus (HCV) develop persistent infection, and these patients are likely to develop chronic liver disease leading to cirrhosis and/or cancer of the liver.² The rate of disease progression varies widely and it is not known what precisely determines the clinical outcome of the disease long term. Furthermore, the mechanism of viral entry into the host hepatocyte is unknown.

In recent years many efforts have been made to identify receptors involved in viral entry into host cells. Two molecules have been proposed to function as HCV receptors, namely the low density lipoprotein receptor (LDLR) and CD81.^{3–5} The LDLR gene family has been shown to function as a receptor for the minor-group common cold virus and for the subgroup A Rous sarcoma virus previously.^{6,7} The LDLR is known to play an important role in cholesterol homeostasis and lipid transport by mediating the cellular uptake of plasma LDL.^{8,9} It has been firmly established that mutations and polymorphisms in the LDLR gene are associated with familial hypercholesterolemia (FH), obesity and atherosclerosis.^{10–12} FH is an autosomal dominant inherited condition charac-

terized by raised levels of LDL-cholesterol leading to increased risk of coronary heart disease. Changes in lipid metabolism during the acute phase response in the liver due to infection and inflammation also suggest the involvement of the lipoprotein system in innate immune responses.¹³

CD81 is a member of the tetraspanin or transmembrane 4 superfamily (TM4SF) and like other members of its family is involved in signal transduction, cell–cell adhesion and cellular activation or development.¹⁴ In particular, the large extracellular loop (LEL) of the CD81 molecule appears to be involved in the binding between HCV envelope glycoprotein E2 and CD81.^{5,15} Thus, CD81 is thought to play a role in HCV endocytosis, although receptor downregulation after binding has not been observed.

The outcome of a large number of infectious diseases is affected by a multitude of factors including environmental factors, interactions with pathogens and host factors, particularly genetic variability. Disease association studies based on polymorphisms are widely used in the elucidation of host genetic factors influencing multifactorial diseases and may help in identifying individuals at risk of developing a disease or a specific disease outcome.¹⁶ The clinical outcome of hepatitis C infection and response to current treatment is variable and remains poorly understood. Only approximately 20–30% of infected individuals will spontaneously clear the virus (self-limiting infection), whereas the remaining 70–80% proceed to chronic HCV infection.² If treated, patients generally now receive a combination therapy with interferon- α and Ribavirin for 6 or 12 months. Patients can

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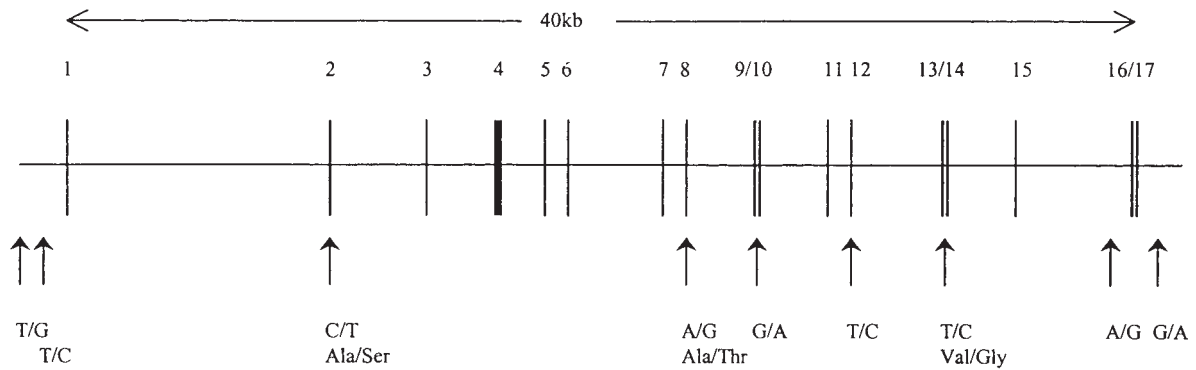


Figure 1 Structure of LDLR gene and position of SNPs under investigation. This illustrates the structure of the LDLR gene indicating the location of the nine SNPs investigated. The base change and, if present, amino acid change for each SNP is shown. All polymorphisms were genotyped and analysed except for the two promoter polymorphisms, which were monomorphic in our population.

be classified into three groups according to the response. Sustained responders (SR) are those who have normal liver transaminases and undetectable viraemia 6 months after completion of therapy. Relapsers (RL) are those who initially respond to treatment but at 6 months post-treatment viraemia has recurred. Non-responders (NR) are those patients who never lose their viraemia.¹⁷ Chronic hepatitis C is characterised by varying degrees of hepatic inflammation and fibrosis. Approximately one-third of carriers will develop cirrhosis and/or cancer of the liver in the long term.¹⁸ The degree of inflammation, the severity of fibrosis and response to therapy are likely to be influenced by host factors. Increasing research activity focuses on the elucidation of host genetic factors affecting disease progression in hepatitis C.^{19,20} Case-control studies have identified associations with HCV persistence and polymorphisms in histocompatibility antigens (HLA),^{20,21} cytokine genes such as TNF- α , IL-10 and TGF- β ^{22–24} as well as the hemochromatosis (HFE) gene^{25–27} have been correlated with parameters of HCV infection.

Due to its proposed function as receptor for HCV, its role in immune responses and the presence of known polymorphisms, the LDLR provides an important candidate for the study of genetic susceptibility to hepatitis C. We report here on the investigation of nine single nucleotide polymorphisms (SNPs) in the LDLR gene and their possible influence on clinical parameters of HCV infection in a case-control study. Specifically, we genotyped SNPs in the promoter region, exons 2, 8, 10, 12, 13; intron 15 and the 3'UTR (see Figure 1). Possible associations between genetic variation and viral clearance, overall inflammation, severity of fibrosis and response to treatment were assessed. In addition we report on the screening of the coding region of the putative HCV co-receptor, CD81, for polymorphisms that might affect the binding affinity between CD81 and virus particles.

Results

Patient's characteristics

In the current study a total number of 837 HCV patients was available. The sex was known for 599 (71.6%) patients, the sex ratio was 54.8% male to 45.2% females. We had data on ethnicity for 583 (69.7%) of patients; 91% of these were of white Caucasian, 5.5% of Asian and 4.5% of Afro-Caribbean origin, respectively. Both sex and

ethnicity were accounted for by logistic regression. Age data was available for 385 (46.0%) patients, covering an age range of 18 to 84 years. 87% of patients with known age were <40 and the remaining 13% >40 years old. Taking the age cut-off of 40 years²⁸ into account by logistic regression in addition to sex and ethnicity halved the numbers of samples available for the analysis and thus drastically reduced the statistical power, although the odds ratio (OR) for the associations between genotype and disease parameters observed did not change significantly (data not shown). Furthermore, genotype frequencies for all seven SNPs investigated did not vary significantly between different age groups (0–20, 21–40, 41–60 and >60 years, data not shown). Interferon response data was available for 50.4% of patients with persistent HCV infection. However, data on regime of anti-viral treatment (IFN monotherapy or combination treatment) was only obtainable for a subset of these, comprising 110 samples. Similarly, viral genotype was available for a limited number of patients ($n=180$); in this subgroup we observed viral genotypes 1, 2, 3, 4 and 5 with a frequency of 63.4, 9.4, 23.3, 3.3 and 0.6%, respectively. Due to the limitations of our data with respect to age, type of anti-viral therapy and viral genotype we did not account for these factors in the analysis, however, we corrected for sex and ethnicity by logistic regression.

Genotyping analysis in LDLR

All available genotype data was analysed with respect to the following comparisons: self-limiting *vs* persistent HCV infection; treatment responders (SR) *vs* non-responders (NR); sustained treatment responders and relapsers (RL) combined *vs* non-responders; mild *vs* severe fibrosis; and mild *vs* severe inflammation (as measured by necro-inflammatory score). Table 1 shows a summary of overall *P*-values for all SNPs tested, corrected for ethnicity and sex by logistic regression. The number of samples available for each comparison is given in parentheses. Table 2 gives the results for presence/absence of specific genotypes and alleles, with the risk associated with carriage of specific alleles given as an OR.

Two SNPs in the promoter region were shown to be monomorphic in our population. The codon change in exon 8 at position 11429 (Ala/Thr) was found to be associated with severity of fibrosis ($P=0.022$, $\chi^2=7.676$). This is due to an increased frequency of the G allele (Ala),

Table 1 Analysis of genotype frequencies

SNP Position in AF217403	Self-limiting vs persistent infection	SR vs NR	SR+RL vs NR	Mild vs severe fibrosis	Mild vs severe inflammation
Exon 2 C → T at 39 Ala → Ser	NS (139 vs 506)	NS (58 vs 99)	NS (153 vs 99)	NS (196 vs 66)	NS (170 vs 98)
Exon 8 A → G at 11429 Thr → Ala	NS (128 vs 402)	NS (46 vs 85)	NS (122 vs 85)	P = 0.022 (143 vs 58) $\chi^2 = 7.676$	P = 0.048 (132 vs 79) $\chi^2 = 6.086$
Exon 10 G → A at 13397	P = 0.013 (147 vs 426) $\chi^2 = 8.653$	NS (53 vs 76)	NS (140 vs 76)	NS (161 vs 57)	P = 0.025 (132 vs 89) $\chi^2 = 7.364$
Exon 12 T → C at 16730	NS (127 vs 464)	NS (52 vs 91)	NS (140 vs 91)	NS (175 vs 64)	NS (154 vs 93)
Exon 13 C → T at 20001 Val → Gly	NS (129 vs 466)	NS (54 vs 89)	NS (144 vs 89)	NS (172 vs 61)	NS (151 vs 91)
Intron 15 A → G at 26857	NS (138 vs 515)	NS (58 vs 101)	NS (151 vs 101)	NS (196 vs 70)	NS (167 vs 106)
3'UTR G → A at 31125	NS (85 vs 428)	NS (45 vs 93)	P = 0.023 (128 vs 93) $\chi^2 = 7.560$	NS (190 vs 65)	NS (163 vs 98)

Table 2 shows the results for the genotype distribution for all SNPs investigated with overall *P*-values and number of individuals analysed in each clinical category in parentheses. All results were corrected for ethnic origin and sex by logistic regression. A *P* value of <0.05 was considered as significant and significant results are shown in bold.

Table 2 Genotype analysis

	Overall genotype distribution n (%)			HWE	<i>P</i> -value (χ^2 value)	Comparison of specific genotypes	<i>P</i> -value (χ^2 value)	OR 95% CI
	GG	GA	AA					
Exon 8 (A → G)								
Mild fibrosis	4 (2.80)	30 (20.98)	109 (76.22)	0.56	0.022	GG + GA vs AA	0.006	2.68
Severe fibrosis	4 (6.90)	21 (36.20)	33 (56.90)	0.97	(7.676)		(7.623)	1.38–5.20
Exon 8 (A → G))								
<5 NI score	102 (77.27)	24 (18.18)	6 (4.55)	0.03	0.048	AG vs AA + GG	0.014	0.43
>5 NI score	52 (65.82)	24 (30.38)	3 (3.80)	0.99	(6.086)		(6.086)	0.22–0.84
Exon 10 (G → A)								
Self limiting	17 (11.56)	47 (31.97)	83 (56.46)	0.05	0.013	AG vs AA + GG	0.005	0.46
Persistent	31 (7.28)	178 (41.78)	217 (50.94)	0.79	(8.653)		(7.852)	0.27–0.78
Exon 10 (G → A)								
<5 NI score	5 (3.79)	61 (46.21)	66 (50.00)	0.13	0.025	GG + GA vs AA	0.041	0.31
>5 NI score	10 (11.24)	45 (50.56)	34 (38.20)	0.69	(7.364)		(4.166)	0.10–0.94
3' UTR (G → A)								
SR+RL	67 (52.34)	55 (42.97)	6 (4.69)	0.45	0.023	GG + GA vs AA	0.006	3.58
NR	43 (46.24)	35 (37.63)	15 (16.13)	0.25	(7.560)		(7.554)	1.07–9.87

Table 3 shows the results for the genotype frequencies, Hardy-Weinberg-equilibrium (HWE) and presence/absence of specific genotypes for SNPs that showed a significant overall genotype distribution. All data was corrected for sex and ethnicity by logistic regression. Odds ratio (OR) and 95% CI indicate the risk associated with the carriage of specific alleles.

rendering patients carrying this allele more susceptible to severe fibrosis (OR 2.68). The exon 8 polymorphism furthermore showed borderline significance with overall inflammation with a potentially beneficial effect of heterozygote status (OR 0.43). The G → A change at position 13397 in exon 10 was shown to be associated with viral clearance ($P = 0.013$, $\chi^2 = 8.653$), which appears to be a heterozygote effect. The SNP in exon 10 furthermore showed a correlation with overall inflammation ($P = 0.025$, $\chi^2 = 7.364$). Upon stratification of the alleles it was shown that the frequency of the G allele is higher in patients with a necro-inflammatory score <5, carriage of this allele thus appears to have a protective effect (OR 0.31). The SNP in the 3'UTR was correlated with response to treatment ($P = 0.023$, $\chi^2 = 7.560$). Presence of the G allele was increased in the sustained responder + relapser group, indicating that individuals carrying the G allele are more likely to respond to treatment than those carrying the A allele (OR 3.58). There were no other significant results between any of the SNPs studied and the clinical parameters of HCV infection.

Linkage disequilibrium (LD) across LDLR locus

The analysis of LD was carried out using the GOLD programme and included all SNPs studied, except for the two promoter SNPs, spanning a region of approximately 32 kb. The following pairwise combinations were shown to be in significant LD: exons 2 and 10 ($D' = 0.532$, $P = 0.00834$); exon 2 and intron 15 ($D' = 0.894$, $P < 0.0001$); exons 10 and 13 ($D' = 0.534$, $P < 0.0001$); exons 12 and 13 ($D' = 0.528$, $P < 0.0001$); intron 15 and 3'UTR ($D' = 0.820$, $P < 0.0001$).

LDLR haplotype analysis

A total of 25 haplotypes was observed in a subgroup of samples ($n = 91$) for which data of all SNPs was available (excluding the two promoter SNPs). Three of these are relatively common with a frequency of >10%. Haplotypes that were present with a frequency of more than 5% are listed in Table 3. A common pattern for haplotypes appearing with a frequency of >5% was seen with the haplotype C-A-G-(T/C)-(T/C)-G-G comprising a collective frequency of 64.28%.

CD81 sequencing

The coding region of the CD81, including exons 5 to 7 which encode the large extracellular loop, was found to

be highly conserved in the 35 patients sequenced, indicating that novel polymorphisms were not present in this region.

Discussion

Genetic variation in the LDLR gene has been studied intensively over the past two decades or more. Most research has concentrated on the involvement of LDLR defects in FH but, more recently, the analysis of LDLR polymorphisms has been applied to the study of obesity, atherosclerosis and HIV infection.^{11,12,29,30} The data reported here provides evidence for an involvement of LDLR polymorphism in the pathogenesis and/or progression of hepatitis C. Within the LDLR gene there are abundant polymorphisms, which have been well characterized and studied intensively (details found at <http://www.ucl.ac.uk/fh>). For the present study we selected nine SNPs as shown in Figure 1, based on likely functionality and reported prevalence.

We showed a negative correlation between the SNP in exon 8 and severity of fibrosis as well as a protective effect on inflammation. Correlations were seen between the SNP in exon 10 and viral clearance as well as inflammation and finally an association between the SNP in the 3'UTR and response to treatment. Of these only the polymorphism in exon 8 leads to an amino acid change from alanine to threonine. In our population carriage of the G allele (Ala) for this polymorphism appeared to render patients more susceptible to develop severe fibrosis (OR 2.68). Heterozygosity at this SNP appeared to have a protective effect against inflammation (OR 0.43), as it did at the SNP in exon 10 with respect to viral persistence (OR 0.46). Heterozygous effects are difficult to interpret and it may be that these results have arisen by chance as the P -values of 0.014 and 0.041 respectively become non-significant if corrected for the number of multiple comparisons. Borderline significance was also found with carriage of the G allele for the exon 10 polymorphism and association with severity of inflammation (OR 0.31). Finally, patients carrying the G allele at the SNP in the 3'UTR were shown to be less likely to respond to interferon therapy than those carrying the A allele (OR 3.58). Neither SNP in exon 10 or in 3'UTR lead to amino acid changes; however, the significant association of these SNPs with disease progression in hepatitis C indicate that they may lie in LD with other

Table 3 Distribution of LDLR haplotypes

	Exon 2 C → T at 39	Exon 8 A → G at 11429	Exon 10 G → A at 13397	Exon 12 T → C at 16730	Exon 13 C → T at 20001	Intron 15 A → G at 26857	3'UTR G → A at 31125	No.	%
A	C	A	G	T	T	G	G	24	13.19
B	C	A	G	T	C	G	G	22	12.09
C	C	A	G	C	T	G	G	21	11.54
D	C	A	A	C	T	G	A	16	8.79
E	C	A	G	C	C	A	G	14	7.69
F	C	A	G	T	C	G	A	10	5.49
G	T	A	G	T	C	G	G	10	5.49
Others (<5%)	–	–	–	–	–	–	–	65	35.73
Total	–	–	–	–	–	–	–	182	100.00

Table 4 lists haplotypes of <5% frequency obtained from 91 individuals for which genotyping data for all SNPs analysed was available. A total of 25 haplotypes was identified in this patient cohort (data not shown). A common pattern for haplotypes appearing with a frequency of >5% was seen with the haplotype C-A-G-(T/C)-(T/C)-G-G comprising a collective frequency of 64.28%.

polymorphisms within this locus. We have thus assessed the LD across the LDLR locus including data available for all SNPs under investigation from 91 individuals and covering a region of approximately 32 kb. The two promoter SNPs at positions -175 and -124 sites proved to be monomorphic in our population and were thus excluded from all further analyses. We demonstrate a complex pattern of LD across the region and strong pairwise LD ($D' > 0.5$, $P < 0.001$) was observed between the SNPs in exons 2 and 10; exon 2 and intron 15, exons 10 and 13; exons 12 and 13 and intron 15 and the SNP in the 3'UTR, respectively. This shows that the three polymorphisms we found to be associated with disease progression are in LD with other SNPs within the gene. Considering that we have covered the whole length of the LDLR locus it seems that there is no single polymorphism exerting a major effect upon disease. It is unlikely that there is another SNP with a major effect upon hepatitis C infection that would not be in LD with the SNPs studied here. Since the polymorphism in exon 8 is a codon change and it shows the strongest association with severity of fibrosis, this is the most likely SNP to have a causative effect upon disease progression. Our data analysis was limited by the availability of information on potentially confounding factors such as age, viral genotype, regime of anti-viral treatment and steatosis. Polymorphisms in the LDLR gene may theoretically affect steatosis, which is fatty deposition in the liver, and an effect of steatosis on progression of severe liver disease has been proposed, but remains unclear. In the absence of a scoring system for steatosis it was unmanageable to assess the possible interaction between LDLR genotypes, steatosis and persistent HCV infection in our cohort. However, possible influences of sex and ethnicity were corrected for. In particular LDLR genotype frequencies have been shown to vary substantially in different populations, indicating genetic heterogeneity even within Europe.^{31–33}

The SNP in exon 2 at position 39 leads to an amino acid change from alanine to serine. To our knowledge this polymorphism has not been shown to have an involvement with any disease to date. The codon change in exon 8 has been associated with raised levels of total plasma cholesterol and LDL-cholesterol and apoB in men.³⁴ Vector expression experiments have shown that there was no difference in the production of LDL receptors or its activity from vectors carrying the alanine or threonine sequence, although a minor influence on receptor activity was not excluded. The polymorphism in exon 10 does not lead to an amino acid change. However, it has been shown that the G allele of this SNP cosegregated with elevated LDL cholesterol without exception in a Japanese pedigree.³⁵ The silent change in exon 12 has been shown to be associated with altered concentrations of total and LDL cholesterol in FH studies.^{31,33} Another study showed that this SNP was associated with obesity overall, but not with plasma lipid levels.¹¹ The C → T changes in exon 13 and in intron 15 have both been associated with disease pathogenesis in FH, although some reports are inconsistent.^{32,36,37} Finally, the G → A transition in 3'UTR is located 56 bp downstream of the termination codon; its potential function is unclear.

The LDLR has been proposed as the viral receptor for HCV and other flaviviridae. This hypothesis has been based on the following evidence: *in vitro* studies showed that HCV-RNA-carrying material bound to lipoproteins

and immunoglobulins, and HCV binding to human fibroblasts could be initiated with purified LDL.⁴ Since LDL is internalized by LDLR it was presumed that HCV in turn could be internalized by LDLR-mediated endocytosis. It has been shown that the endocytosis of viral particles is associated with LDLR activity, based upon the correlation of cells positive for HCV and for LDLR.³⁸ Furthermore, continued stimulation of LDLR expression enhanced virus replication *in vitro*.³⁹ The serum concentration of beta-lipoproteins is thought to influence HCV concentration, as the former could inhibit the binding of the virus to its proposed receptor, the LDLR. Indeed, it was demonstrated that beta-lipoproteins negatively affected the level of HCV-Ag.⁴⁰ Studies using HCV pseudotype viruses support the hypothesis that LDLR, CD81 and heparin are involved in HCV cell entry.⁴¹ Another study identified a LDLR-related molecule from *Caenorhabditis elegans* that was capable of binding to a complex of E1-E2 HCV envelope protein.⁴² Additionally, a soluble form of the LDLR has been described as a potent antiviral protein against vesicular stomatitis virus.⁴³ It contains only the ligand binding domain of the LDLR, is interferon-induced, secreted extracellularly and appears to interfere with viral replication. It was proposed that the production of soluble variants by host cells may be employed in order to control virus infections. Overall it remains uncertain as to whether virus uptake can be mediated directly by the LDLR, but lipoprotein bound to HCV may function as a ligand. It thus seems the LDLR or its homologues are involved in endocytosis of HCV, however, the exact mechanisms underlying this process are to date not fully understood. Therefore, polymorphisms in the LDLR could influence the activity of the receptor and thus may affect the level of cellular entry for HCV. This in turn may influence the ability of the virus to establish persistent infection, to evade interferon therapy and to induce liver fibrosis.

There is also compelling evidence for the involvement of components of the lipoprotein system in innate immunity, the primary defense against microorganisms such as HCV. Lipoproteins may be involved in the redistribution of nutrients, neutralisation, binding or lysis of viruses and the binding of endotoxins and other biological agents.⁴⁴ Indeed, it has been shown that high lipoprotein levels reduce cytokine responses,⁴⁵ increase macrophage chemotaxis⁴⁶ and impair antiviral T cell immunity in hypercholesterolemic LDLR deficient mice.⁴⁷ It is hence plausible that genetic variation in the LDLR gene could affect the immune responses in HCV infection.

A further molecule that has been proposed as a receptor mediating the uptake of HCV into hepatocytes is the cell membrane protein CD81.⁵ In particular the large extracellular loop (LEL), but not the small extracellular loop, of the CD81 protein has been shown to be critical for the interaction with HCV E2 envelope protein.^{15,48} We sequenced all exons of the CD81 gene in order to identify novel polymorphisms that may affect the protein binding affinity, in particular the region spanning exons 5 to 7, which encodes the LEL. However, we did not find any SNPs within the coding region of this locus in the 35 HCV patients sequenced. We thus showed that there is no genetic variation in this region that could affect the binding of virus particles and therefore susceptibility to HCV infection. This does not rule out the mediation of

viral uptake through CD81 as such, or the role of polymorphisms in other regulatory regions of the gene upon this process. However, no polymorphisms were found in CD81 cDNA sequence from a Japanese cohort consisting of 24 individuals, and although sequence variation was detected in the 3' and 5' non-coding regions, it was considered unlikely that CD81 would influence the progression of chronic hepatitis.⁴⁹ It thus seems doubtful that CD81 is the only cell surface receptor for HCV and indeed, it has been shown that CD81 has a poor capacity to mediate virus entry into hepatocytes.⁵⁰

In summary, it appears that variants in the LDLR gene, which influence the pathogenesis of FH and related diseases, may contribute to variation in severity of fibrosis, inflammation and response to treatment in hepatitis C. There is a complex interaction between IFN- α and LDLR activity, which could play an important role in these processes. LDLR activity appears to be increased by IL-1,⁵¹ which is downregulated by its receptor antagonist, IL-1RA.⁵² Circulating IL-1RA in turn has been shown to be induced by IFN treatment.^{53,54} IFN- α may therefore indirectly suppress LDLR activity by increasing secretion of IL-1 receptor antagonist production and thus decreasing IL-1 receptor-mediated stimulation by IL-1.³ Taking our findings into account we thus hypothesise that LDLR polymorphisms may have an influence upon disease progression rather than viral clearance, ie, our findings support a role in immune response modulation rather than the LDLR functioning as entry receptor for HCV as such. However, it is important to note the limitation of our data analysis with respect to confounding factors other than sex and ethnicity. Overall, our data add to the increasing body of evidence of the involvement of host genetic factors in infectious disease and hepatitis C infection in particular. Similar studies should be conducted in other population in order to confirm, or otherwise, these findings.

Materials and methods

Patients

Participants for this study were recruited at several large hepatology clinics in Greece, Spain, Germany, Sweden, France, Italy and the UK between October 1995 and February 2001. Patients were categorized on the basis of viral serology, liver histology and response to treatment. Exclusion criteria included co-infection with hepatitis B virus or HIV. All patients included were positive for HCV antibody (ELISA2 assay, Abbot Laboratories Ltd, Maidenhead, UK) and had no evidence of liver disease due to other causes. Patients classified as having a persistent infection were HCV antibody positive and PCR positive for >6 months post presentation or likely time of infection. Patients with a self-limiting infection showed normal concentrations of liver aminotransferase enzymes; they were HCV antibody positive and PCR negative at presentation on at least one occasion >3 months later.

The response to combined interferon- α and Ribavirin or interferon- α therapy alone was evaluated in patients who had received treatment for >3 months. SRS were defined as individuals with normal concentrations of liver aminotransferases and no detectable viraemia >6 months after the course of drug administration. Non-responders (NR) were defined as patients with continu-

ous viraemia >3 months into therapy. Relapsers (RL) were those patients with non-detectable viraemia, using the Roche Amplicor test (Roche Diagnostics, Lewes, UK) with a lower end sensitivity of 200 genomes per ml, during the course of treatment and detectable viraemia following the end of the drug administration.

Overall inflammation was scored according to the modified histological activity index (HAI-Ishak) for liver histology.⁵⁵ Patients were divided into two groups, those with a necro-inflammatory score of <5 (mild) or >5 (severe), respectively. The severity of fibrosis was also assessed by liver histology, scoring according to the Ishak's system. Patients with persistent infection were divided into those with a fibrosis score of <2 (mild) or >3 (severe) at the time of presentation. Further details of the patient criteria are reported by Thursz *et al*.¹⁷

Genotyping

Sample genotypes were analyzed by the ligation detection reaction (LDR).⁵⁶ Briefly, PCR products containing the SNP of interest were generated under standard conditions in a 15 μ l reaction containing 1 \times reaction buffer, 1–2 mM MgCl₂, 0.32 mM dNTP (all reagents Perkin Elmer Applied Biosystems, Warrington, UK), 0.12 μ M of forward (F) and reverse (R) primer respectively, 1 unit Taq polymerase, 50 ng DNA and dH₂O. Cycling parameters were 94°C for 14 min followed by 35 cycles of 94°C for 30 sec, the specified annealing temperature (see below) for 30 sec and 72°C for 30 sec, followed by a final step of 72°C for 5 min. For PCR primer sequences, PCR annealing temperatures and PCR product sizes refer to Table 4. PCR reactions were treated with 1.5 μ l of 1 mg/ml proteinase K (in 50 mM EDTA) in one cycle of 37°C for 15 min, 55°C for 10 min and 90°C for 10 min to destroy any remaining DNA polymerase activity prior to LDR amplification.

For genotyping by the LDR method, three oligonucleotide probes are required for each SNP. These comprise two allele specific probes labeled with a different fluorescent tag at the 5' end and the allele specific base at the 3' end as well as a phosphorylated unlabelled common probe. The phosphorylation is carried out in a 100 μ l reaction containing 2 μ M of the common probe, 250 μ M ATP, 1 \times Polynucleotide kinase buffer and 1 μ l Polynucleotide kinase (all reagents Promega) and incubated at 37°C for 45 min. To this mix 100 μ l of proteinase K (0.2 mg/ml in TE, pH 8.0) were added and the reaction incubated at 37°C for a further 30 min prior to increasing the incubation temperature to 90°C for 10 min. All probes were designed using the TM calc function in the Bench Mate programme under nearest neighbour conditions (<http://biochem.roche.com/benchmate>). If required the length of probes was adjusted by adding poly A's, these were not complementary to the sequence and did not affect the TM. Each 15 μ l LDR reaction typically contained 1 \times Taq Ligase Buffer (New England Biolabs Ltd, Hitchin, UK), 0.2 μ M of phosphorylated common probe, 0.067 μ M of each of the allelic probes, 1 μ l of PCR product, 10 units of Taq DNA Ligase (New England Biolabs) and dH₂O. LDR reactions for up to five SNPs could be pooled in one reaction. The LDR reactions were cycled as follows: 95°C for 1 min followed by 15 cycles at 95°C for 15 sec and the appropriate annealing temperature for 4 min. To prevent any further ligase activity samples were treated with 3 μ l of 100 mM EDTA. Further details

Table 4 Table 4 SNP positions, PCR and LDR oligonucleotide sequences and cycling parameters

SNP location Base change at position AA change (restriction enzyme)	PCR primer sequences	PCR annealing temperature and product size	LDR probe sequences	LDR annealing temperature and fragment size
Promoter T → G at -175	SchF: GAGGCAGAGAGACAATGGC SchR: CACGACCTGTGTCTAGTCTAGTGGAAACCC	58°C 439 bp	P1: AAATCAAAAGTGCCTGCCCTGCCT (FAM) P2: TTACAAAAGTGCCTGCCCT GCCTG (HEX) P3: ACCTTTCGAAGGACTGGAGTGGGAATCAGTAAAAATATAA	72°C 65 bp
Promoter T → C at -124	SchF: GAGGCAGAGAGACAATGGC SchR: CACGACCTGTGTCTAGTCTAGTGGAAACCC	58°C 439 bp	P1: GGAATCAGAGCTTCACRGGTTAAAAAGCT (FAM) P2: GGAATCAGAGCTTCACRGGTTAAAAAGCC (HEX) P3: GATGTACATCGGCCCTTCGAAACTCCAAAAAATAA	72°C 68 bp
Exon 2 C → T at 39 Ala → Ser (SmaI)	FH112: TTGAGAGACCTTTTCTCTTTTCC FH132: GCATATCATGCCCAAAGGGG	57°C 183 bp	P1: aCTCTCTCTCAGTGGGGCAGATGC (FAM) P2: aCTCTCTCTCAGTGGGGCAGATGT (TET) P3: GAAAGAAAACGAGTTCACAGTCCAAAGCAGGaa	64°C 56 bp
Exon 8 A → G at 11430 Ala → Thr (StuI)	F:GGAAGAGCCTCCCAACCAAGC R:GATGAAACTCCCAACCACTGCT	57°C bp	P1: CCAGCTGACCCCAACAGAAA (FAM) P2: CCAGCTGACCCCAACAGAA (TET) P3: GCCTGCAAGGCTGTGGTGAGCA	66°C bp
Exon 10 G → A at 13397 (HpaII)	FH10: AGATGAGGGCTCCTGTGCGATGCC FH29: GCCCTGGTATCCGCAACAGAGACA	57°C 202 bp	P1: ACTTCTCTATGACACCTGCATCAGCAGG (FAM) P2: TCTCTTCTATGACACCTGCATCAGCAGA (TET) P3: GACATCCAGGCCCTCCGACCGG	64°C 49 bp
Exon 12 T → C at 16730 (HindII)	FH13: GCACGTGACCTCTCCTTATCCACTT FH32: CACCTAAGTGCTTCGATCTCGTACG	57°C 211 bp	P1: aTTCACCTCACTCAAGCATCGATGTCAAC (FAM) P2: CTTCACTCACTCAAGCATCGATGTCAAT (TET) P3: GGGGGCAACCCGGAAGACCATCTTG	64°C 54 bp
Exon 13 Val → Gly (AvaII)	FH124: GTCATCTTCTGTGCTGCTG FH33: GTTCCACAAGGAGGTTTCAAGGTT	57°C 218 bp	P1: TGA AAAACCTACTGTCCCAAGAGGATATGGTC (FAM) P2: TGA AAAACCTACTGTCCCAAGAGGATATGGTT (TET) P3: CTCCTCCACAACCTCACCCAGCCAAAGA	64°C 58 bp
Intron 15 A → G at 26857 (PvuII)	F: TGTGGGGTCCGAAGGTG R: CACCTCAGCCTCCCAAAGT	57°C 323 bp	P2: GATTACAGGTGCCCAACCCGCA (FAM) P1: aATTACAGGTGCCCAACCCGCA (TET) P3: GCTGGCTAATTTTGTATTTTAGTAGAGACAGGGTTTCAaa	64°C 65 bp
3'UTR G → A at 31125 (MspI/HaeIII)	F: TCCGCTGTTACCAATTGTGGCAC R: ATAAAACAAAGCTCTGGCAGGCAAT	57°C 173 bp	P1: AAAAGAAACCTTCTCGAGACCTCGCCG (FAM) P2: AAAAGAAACCTTCTCGAGACCTCGCCA (TET) P3: GCCTTGTTTTATTCAAAAGACAGAGAACCAAGCC	66°C 62 bp

The accession number for the LDLR promoter and gene sequences used for primer and probe design were M93189 and AF217403, respectively. The SNP positions are given in relation to these sequences. Non-specific poly A's are shown in lower case. The restriction enzyme for RFLP typing as previously published (<http://www.ucl.ac.uk/fh/polypage.html>) is shown in parentheses. Primer sequences were obtained from <http://www.ucl.ac.uk/fh/primers.html> (labelled FH) or designed in our laboratory. Primers for the amplification of the LDLR promoter region, were kindly provided by CL Scholtz (University of Stellenbosch, South Africa).

regarding the LDR reactions can be found in Table 4. Finally, 1 µl of each LDR amplification product was run on an ABI prism 373 sequencer (Perkin Elmer Applied Biosystems, Warrington, UK) and sample genotypes analysed using GENESCAN and Genotyper software programmes (Perkin Elmer Applied Biosystems).

Statistical analysis

The distribution of genotype and allele frequencies was analyzed using standard 3×2 or 2×2 χ^2 tests or Fisher's exact tests applying SPSS 9.0 for Windows or the Statcalc programme (Epi2000, Centre for Disease Control and Prevention, Atlanta, Georgia, USA; <http://www.cdc.gov/epiinfo/index.htm>). A value of $P < 0.05$ was considered as significant. The OR was calculated to indicate the risk associated for individual alleles with 95% confidence intervals (CI). The potentially confounding effects of ethnic group and sex were corrected for by logistic regression.

Linkage disequilibrium analysis

LD analysis was performed using GOLD software (graphical overview of LD; <http://www.well.ox.ac.uk/asthma/GOLD/>⁵⁷). Significant LD was defined here as a D' value of >0.5 and a P -value of <0.05 .

Haplotype analysis

Haplotype analysis was carried out using the PHASE software programme (version 0.21, <http://www.stats.ox.ac.uk/mathgen/software.html>⁵⁸).

Sequencing analysis

Thirty-five patients were screened for polymorphism in the coding region of the CD81 gene. PCR primers and conditions were as follows: Exon 4: sense CAG ATC GCC AAG GAT GTG AAG C; antisense CTC GAA GAT CAT GAT CAC AGC G (Conditions: 94°C, 5 min, 1 cycle; 94°C, 30 sec, 55°C 30 sec, 72°C, 1 min, 35 cycles; 72°C, 7 min, 1 cycle). Exon 5: sense ATC GCC AAG GAT GTG AAG CAG TTC TAT; antisense CAG GAT TCA TCC AGG ACG (Conditions: 94°C, 5 min, 1 cycle; 94°C, 1 min, 50°C 30 sec, 72°C 1 min, 35 cycles; 72°C, 4 min, 1 cycle). Exon 6: sense TGG ATG CAT TCT GCA GTG GGG AG; antisense TGT GAC CTG CCC ACC CCT AGG A (Conditions: 94°C 5 min, 60°C 1 min, 72°C 30 sec, 1 cycle; 94°C 45 sec, 60°C 45 sec, 72°C 30 sec, 35 cycles; 72°C, 4 min, 1 cycle). Exon 7: sense ATT ACT GCG TGA CAA CGG GAA; antisense TAC ACG GAG CTG TTC CGG ATG CCA (Conditions: as for Exon 6). Exon 8: sense AGT CTG TGG GCA CTC TCT GC, antisense GCA TGC CTG ATG TTC CTT C (Conditions as for exon 4). Twenty µl PCR and sequencing reactions were run under standard conditions. Sequencing was performed using an AB1377 sequencer and each exon was sequenced in both directions.

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