

Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR

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Interferon stimulates the expression of a number of genes encoding enzymes with antiviral activities, including myxovirus resistance-1 (MxA), 2-5-oligoadenylate synthetase 1 (OAS-1) and double-stranded RNA-dependent protein kinase (PKR). We examined whether polymorphisms in these genes influenced the outcome of hepatitis C virus (HCV) infection. We observed a lower frequency of the GG genotype at position –88 in the MxA gene promoter in self-limiting HCV infection (OR = 0.56; 95% CI: 0.35–0.8; P = 0.010) and in nonresponders to therapy (OR = 0.49; 95% CI: 0.25–0.95; P = 0.020). This genotype predominantly influenced the outcome of treatment in patients with viral genotype 1 (OR = 0.22 95% CI: 0.07–0.67; P = 0.002). A polymorphism in the 3'-untranslated region of the OAS-1 gene was associated with outcome of infection (GG genotype less frequent in self-limiting infection: OR = 0.43; 95% CI: 0.21–0.86; P = 0.010). A polymorphism at position –168 in the promoter region of the PKR gene was associated with self-limiting infection (CT genotype: OR = 2.75; 95% CI: 1.45–5.24; P = 0.002). Further associations were found with a CGG trinucleotide repeat in the 5' UTR region of the PKR gene. Polymorphisms in the interferon-induced genes, MxA, OAS-1 and PKR appear thus associated with HCV outcome.

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Introduction

The interferon system is a crucial component of the innate immune response to infectious agents. Type I interferons induce numerous proteins with antiviral action, including myxovirus resistance 1 (MxA), 2'-5'-oligoadenylate synthetase 1 (OAS-1) and double-stranded RNA(dsRNA)-dependent protein kinase (PKR).¹ MxA protein has selective activity against several viruses.^{2–4} However, the precise mechanism of antiviral action has not been elucidated. Studies of *in vivo* MxA levels in patients treated with interferon for hepatitis C virus (HCV) have shown greater levels in virological responders than nonresponders.⁵ Similarly, MxA mRNA levels have been reported to increase significantly after initiation of interferon therapy only in those who ultimately respond to treatment.⁶

Upon binding to dsRNA, OAS-1 catalyses the formation of 2'-5'-linked oligoadenylate and activates RNaseL, which breaks down viral and cellular RNA.^{7,8} PKR is also activated by dsRNA; this leads to the phosphorylation of its substrate, eIF2 α , which inhibits the guanosine nucleotide exchange factor, eIF2 β , and halts viral replication.^{9,10} PKR may act by shutting down protein

synthesis following infection of a cell and limit the transmission of virus to uninfected cells.¹¹ Interactions between the HCV and PKR are believed to be an important mechanism behind the resistance of HCV to interferon therapies.¹²

Individuals infected with HCV may exhibit a spectrum of possible outcomes, including the ability to clear the virus naturally (self-limiting infection). Unfortunately, the majority of individuals develop persistent infections. Interferon has been an important agent used in anti-HCV therapy, first as monotherapy^{13,14} and more recently, in combination with ribavirin.^{15,16} The addition of a polyethylene glycol moiety to interferon- α has also been developed, and may be administered alone or in combination with ribavirin.^{17,18}

During the first 3 months of therapy, HCV viral loads usually fall to undetectable levels in response to interferon- α . However, in a small proportion of patients (nonresponders) HCV viral loads persist at or near pretreatment levels. Among those patients with an initial response to treatment up to 50% will relapse after treatment is discontinued (relapsed responders), whereas the remainder will have a sustained response as determined by the absence of detectable viraemia 6 months after treatment has stopped.

It is known that interferon- α modulates the expression of over 100 genes of which the function is recognised for only a minority.¹⁹ It is unlikely that all these genes are

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involved in the resistance to all viral infections. We therefore used disease association studies to determine the relevance of MxA, OAS-1 and PKR to the outcome of HCV infection and response to interferon- α treatment.

Results

The characteristics of the patients from the present study are given in Table 1. As previously observed, female gender was found more frequently in subjects who had self-limiting HCV infection (OR = 2.01; 95% CI: 1.17–3.45; $P = 0.007$). Viral genotype 1 was found more frequently in those with nonresponse to interferon (OR = 2.81; 95% CI: 1.11–7.19; $P = 0.016$) when compared to sustained responders and initial responders.

Polymorphisms in the MxA, OAS-1 and PKR genes

Using automated sequencing, we identified the following single-nucleotide polymorphisms (SNPs): an A/G substitution at position -545 of the MxA gene, a C/T substitution at position -168 and a G/T substitution at position -180 of the PKR gene, and a CCG trinucleotide repeat in the promoter region of the PKR gene. The A/G substitution in the 3' untranslated region (3'-UTR) of OAS-1 at position 347 of GenBank Acc No. M11810 was identified through a BlastN search and confirmed by sequencing. Subsequent comparison of the -545 MxA (A/G) SNP and the 3'UTR-OAS-1(A/G) SNP with known polymorphisms in the NCBI database confirmed that these SNPs correspond to the previously described polymorphisms rs462903 and rs2660, respectively. The -88 MxA polymorphism has been taken from the literature²⁰ and the CCG repeat in the 5'UTR of the PKR gene has been subsequently published.^{21,22} In the whole group, all polymorphic sites approximated to Hardy-Weinberg equilibrium.

MxA

The SNP at position -88 with respect to the transcription initiation site was associated with the natural outcome of

HCV infection as well as with response to interferon therapy (Table 2). The TT genotype was found more frequently in patients who had self-limiting infection (OR = 3.30; 95% CI: 0.80–14.15; $P = 0.050$) together with the GT genotype (OR = 1.59; 95% CI: 0.98–2.59; $P = 0.050$) indicating a gene dosage effect. The GT genotype was also found more frequently in patients with an initial response (OR = 2.06; 95% CI: 1.05–4.07; $P = 0.023$ and those with sustained response (OR = 1.80; 95% CI 0.85–3.82; $P = 0.096$) to interferon compared to nonresponders. Carriage of the T allele was associated with self-limiting infection (OR 1.80, 95% CI: 1.12–2.88; $P = 0.010$) and with initial response to interferon (OR 2.06; 95% CI: 1.05–4.07; $P = 0.023$).

Stratification by viral genotype revealed that the -88 MxA polymorphism predominantly influenced the response to interferon in patients infected with viral genotype 1 (OR = 0.22; 95% CI: 0.07–0.67; $P = 0.002$), rather than those infected with other genotypes (OR = 1.75; 95% CI: 0.31–9.79; $P = 0.359$). Stratification of the results by gender made no difference to the observed associations.

The SNP at position -545 was not found to be associated with the outcome of HCV infection or treatment response.

OAS-1

The SNP in the 3'UTR of the OAS-1 gene showed evidence of association with self-limiting HCV infection, but not with the response to interferon therapy (Table 3). The GG genotype at this locus was found in 9.0% of patients with self-limiting infection compared to 18.8% of patients with persistent infection (OR = 0.43; 95% CI: 0.21–0.86; $P = 0.010$). Stratification by gender suggests that the predominant effect of 3'UTR-OAS-1 SNP with respect to self-limiting infection is within females: OR = 0.37; 95% CI: 0.11–1.19; $P = 0.0648$, rather than males: OR = 0.76; 95% CI: 0.22–2.43; $P = 0.611$. Stratification by viral genotype did not affect the observed association.

Table 1 Baseline characteristics of the Caucasian patients included in the present study

	Self-limiting infection N=94 (%)	Persistent infection N=177 (%)	Sustained responder N=93 (%)	Relapsed responder N=12 (%)	Nonresponder N=61 (%)
<i>Gender</i>					
Female	55 (58.5)	73 (41.2)	39 (41.9)	4 (33.3)	24 (39.4)
Male	39 (41.5)	104 (58.8)	54 (58.1)	8 (66.7)	37 (60.6)
<i>Viral genotype</i>					
Genotype-1	5 (5.3)	70 (39.5)	31 (33.3)	4 (33.3)	33 (54.1)
Non-1	4 (4.2)	44 (24.8)	29 (31.2)	3 (25.0)	11 (18.0)
Unknown	85 (90.4)	63 (35.6)	33 (35.5)	5 (41.7)	17 (27.9)
<i>Source of infection</i>					
IVDU	16 (17.0)	32 (18.1)	21 (22.6)	2 (16.7)	7 (11.5)
Blood products	19 (20.2)	59 (33.3)	30 (32.3)	4 (33.3)	23 (37.7)
Misc	3 (3.2)	7 (3.9)	4 (4.3)	5 (41.7)	2 (3.3)
Unknown	56 (59.6)	79 (44.6)	38 (40.8)	1 (8.3)	29 (47.5)
<i>Average age at infection</i> (\pm St Dev)	26.8 \pm 14.6	32.6 \pm 14.7	32.2 \pm 13.2	29.4 \pm 14.9	33.6 \pm 16.4

IVDU=intravenous drug user; StDev=standard deviation.

Table 2 Distributions of genotype frequencies for *MxA* polymorphisms and the outcome of hepatitis C virus infection among Caucasian patients. (a) Distributions with respect to self-limiting *vs* persistent infections. (b) Distributions with respect to initial response to interferon monotherapy. (c) Distributions among sustained responders and nonresponders to interferon monotherapy

(a)						
<i>Locus</i>	<i>Variant</i>	<i>Self-limiting N (%)</i>	<i>Persistent N (%)</i>	<i>OR</i>	<i>95% CI</i>	<i>P-value</i>
<i>MxA</i>	–88					
	GG	88 (65.2)	222 (77.1)	0.56	0.35–0.89	0.010
	GT	41 (30.4)	62 (21.5)	1.59	0.98–2.59	0.048
	TT	6 (4.4)	4 (1.4)	3.30	0.80–14.19	0.050*
<i>MxA</i>	–545					
	AA	14 (17.9)	42 (27.3)	0.58	0.54–3.27	0.120
	AG	48 (61.5)	87 (56.5)	1.23	0.68–2.23	0.462
	GG	16 (20.5)	25 (16.2)	1.33	0.63–2.82	0.420
(b)						
<i>Locus</i>	<i>Variant</i>	<i>Initial response N (%)</i>	<i>Nonresponse N (%)</i>	<i>OR</i>	<i>95% CI</i>	<i>P-value</i>
<i>MxA</i>	–88					
	GG	108 (73.5)	97 (85.1)	0.49	0.25–0.95	0.020
	GT	39 (26.5)	17 (14.9)	2.06	1.05–4.07	0.023
	TT	0 (0.0)	0 (0.0)	—	—	—
<i>MxA</i>	–545					
	AA	23 (30.3)	16 (21.6)	1.57	0.71–3.52	0.230
	AG	40 (52.6)	46 (62.2)	0.68	0.33–1.36	0.238
	GG	13 (17.1)	12 (16.2)	1.07	0.42–2.73	0.880
(c)						
<i>Locus</i>	<i>Variant</i>	<i>SR N (%)</i>	<i>NR N (%)</i>	<i>OR</i>	<i>95% CI</i>	<i>P-value</i>
<i>MxA</i>	–88					
	GG	73 (76.0)	97 (85.1)	0.56	0.26–1.18	0.100
	GT	23 (24.0)	17 (14.9)	1.80	0.85–3.82	0.096
	TT	0 (0.0)	0 (0.0)	—	—	—
<i>MxA</i>	–545					
	AA	22 (30.1)	16 (21.6)	1.56	0.7–3.53	0.238
	AG	39 (53.4)	46 (62.2)	0.70	0.34–1.42	0.283
	GG	12 (16.4)	12 (16.2)	0.46	0.17–1.26	0.092

OR=odd's ratio; 95% CI=95% confidence interval.

*Fisher's exact *P*-value.

PKR

Polymorphisms in the PKR gene appear to influence both the natural outcome and the response to interferon therapy in HCV infection (Table 4). Heterozygotes for the SNP at position –168 relative to the transcription initiation site were more likely to have self-limiting HCV infection (OR = 2.75; 95% CI: 1.45–5.24; *P* = 0.002), but this genotype had no significant influence on the outcome of treatment. The SNP at position –180 had no significant influence on the outcome of HCV infection or treatment.

Stratification of the association between the heterozygous genotype at position –168 in the PKR gene with self-limiting infection by gender suggested that the predominant effect of this variant is among females: OR = 2.98; 95% CI: 1.26–7.11; *P* = 0.006, rather than males: OR = 1.81; 95% CI: 0.66–5.00; *P* = 0.201).

As trinucleotide repeats may influence gene transcription, we categorised alleles as short (<9 repeats) or long (≥9 repeats). Individuals with two short alleles were

found more frequently among subjects who had self-limiting infection (OR = 5.3; 95% CI: 0.3055–92.75; *P* = 0.201) as were individuals with one short allele (OR = 2.03; 95% CI: 1.01–4.07; *P* = 0.030). Conversely, patients with two long alleles were found more frequently in the initial responder group (OR 2.29; 95% CI: 1.05–5.07; *P* = 0.023) and sustained responder group (OR = 3.29; 95% CI: 1.10–10.52; *P* = 0.017).

Stratification of the heterozygous short/long genotype of the CGG repeat by gender did not show a discernable difference between the two groups (*P* > 0.10 for both). In contrast, stratification of the long/long homozygous genotype and response to interferon by gender yielded significant associations for males: OR = 2.70; 95% CI: 1.02–7.1; *P* = 0.026 for initial response and OR = 5.90; 95% CI: 1.7–22.7; *P* = 0.001 for sustained response. Similar significant associations were not seen among females: OR = 2.30; 95% CI: 0.52–12.00; *P* = 0.182 for initial response and OR = 3.50; 95% CI: 0.39–80.00; *P* = 0.213 for sustained response. Stratification by viral genotype

Table 3 Distributions of genotype frequencies in *OAS-1* gene polymorphisms and the outcome of hepatitis C virus infection among Caucasian patients. (a) Distributions with respect to self-limiting *vs* persistent infections. (b) Distributions with respect to initial response to interferon monotherapy. (c) Distributions among sustained responders and nonresponders to interferon monotherapy

(a)						
Locus	Variant	Self-limiting N (%)	Persistent N (%)	OR	95% CI	P-value
<i>OAS-1</i>	3'-UTR					
	AA	45 (36.9)	185 (35.9)	1.05	0.68–1.61	0.830
	AG	66 (54.1)	234 (45.3)	1.42	0.94–2.15	0.082
	GG	11 (9.0)	97 (18.8)	0.43	0.21–0.86	0.010
(b)						
Locus	Variant	Initial response N (%)	Nonresponse N (%)	OR	95% CI	P-value
	AA	97 (36.1)	51 (34.7)	1.06	0.68–1.65	0.780
	AG	117 (43.5)	72 (49)	0.80	0.52–1.22	0.283
	GG	55 (20.4)	24 (16.3)	1.32	0.75–2.31	0.310
(c)						
Locus	Variant	SR N (%)	NR N (%)	OR	95% CI	P-value
	AA	51 (35.7)	51 (34.7)	1.04	0.63–1.74	0.860
	AG	60 (41.9)	72 (49.0)	0.75	0.46–1.23	0.223
	GG	32 (22.4)	24 (16.3)	1.48	0.79–2.77	0.190

See Table 2.

did not produce any significant relation for initial or sustained response ($P > 0.190$ for all markers in PKR). The relatively low frequency of many of these variants precluded simultaneous adjustment of both gender and viral genotype as potential confounders through the use of multiple logistic regression.

Discussion

Our observations suggest that polymorphisms in the interferon-induced genes *MxA*, *OAS-1* and *PKR* may be involved in determining the outcome of HCV infection. With respect to the *MxA* gene, we found that both the TT and the GT genotypes at the -88 locus were associated with self-limiting infection indicating that the T allele conferred a protective effect with respect to viral clearance. Furthermore, the GT genotype was found more frequently in individuals with an initial response or sustained response to interferon therapy. This observation is consistent with a previous report in a Japanese population in which the GG genotype was associated with nonresponse to interferon.^{20,23} Interestingly, the allele frequency of the T allele is much lower in our Caucasian population than in the Japanese population (14.5% in Caucasians and 29.0% in Japanese). The apparent effect of the T-allele is supported by *in vitro* functional work, suggesting that this variant has higher transcriptional activity than the G allele when stimulated with interferon- α .²³ This is because the polymorphism at position -88 lies within a sequence element similar to an interferon sensitivity response element (ISRE), and the T-allele increases this similarity.²⁰ Consequently, patients with the GG genotype at position -88 may produce a suboptimal *MxA* response when given interferon- α . Clinical studies have also suggested that those who

respond to interferon- α treatment express increased amounts of *MxA* mRNA during treatment.⁶

Hijkata *et al*²³ describe a second SNP at position -123 from the transcription start site, which is in close linkage disequilibrium with the SNP at position -88 , and was therefore ignored in our analysis.

We observed an association between the 3'UTR *OAS-1* GG genotype with persistent infection, but not with response to therapy. This observation may be explained by studies indicating that *OAS-1* levels do not change with the administration of interferon- α or that levels may become elevated, before quickly diminishing again.^{24,25} While levels of *OAS-1* protein expression have been reported to be increased in persistent HCV infection, administration of exogenous interferon- α does not appear to have a modulatory effect on its expression.^{24,26–28} It is quite possible that the natural induction of interferon- α by an infectious agent such as HCV is different from the administration of exogenous interferon- α , or that persistent induction of *OAS-1* through persistent HCV infection reduces sensitivity to exogenously supplied interferon. Thus, it is possible that *OAS-1* plays a greater role in mediating self-limiting *vs* persistent HCV infections, rather than when viral persistence is established.

The length of the alleles in the PKR promoter trinucleotide repeat polymorphism influenced the outcome of infection. Patients who possessed one short allele were approximately two-fold more likely to clear HCV spontaneously, whereas those with two short alleles were estimated to have an 18-fold increased chance of self-limiting infection although, because of the low allele frequency, this fails to reach statistical significance. Variation in the number of repeats in this PKR microsatellite may alter binding capabilities of transcription factors and affect PKR expression during

Table 4 Distributions of genotype frequencies in *PKR* gene polymorphisms and the outcome of hepatitis C virus infection among Caucasian patients. (a) Distributions with respect to self-limiting *vs* persistent infections. (b) Distributions with respect to initial response to interferon monotherapy. (c) Distributions among sustained responders and nonresponders to interferon monotherapy

(a)						
Locus	Variant	Self-limiting N (%)	Persistent N (%)	OR	95% CI	P-value
PKR	-180					
	GG	2 (4.7)	8 (10.5)	0.41	0.06–2.27	0.226
	GT	17 (39.5)	20 (26.3)	1.83	0.77–4.38	0.134
PKR	-168					
	CC	6 (9.8)	39 (19.2)	0.46	0.16–1.21	0.090
	CT	37 (60.7)	77 (37.9)	2.75	1.45–5.24	0.002
PKR	TT	18 (29.5)	87 (42.9)	0.56	0.29–1.08	0.060
	CGG					
	Long/long	43 (70.5)	180 (78.9)	0.64	0.32–1.26	0.162
	Short/long	18 (29.5)	39 (17.1)	2.03	1.01–4.07	0.030
	Short/short	0 (0.0)	9 (3.9)	5.3	0.30–92.75	0.201
(b)						
Locus	Variant	Initial response N (%)	Nonresponse N (%)	OR	95% CI	P-value
PKR	-180					
	GG	2 (6.9)	6 (14.6)	0.43	0.06–2.68	0.320
	GT	9 (31.0)	10 (24.4)	1.40	0.43–4.58	0.538
PKR	-168					
	CC	19 (17.0)	18 (22.2)	0.72	0.33–1.56	0.360
	CT	43 (38.4)	27 (33.3)	1.25	0.66–2.37	0.471
PKR	TT	50 (44.6)	36 (44.4)	1.01	0.54–1.87	0.978
	CGG					
	Long/long	76 (85.4)	74 (71.8)	2.29	1.05–5.07	0.023
	Short/long	12 (13.5)	22 (21.4)	0.57	0.25–1.32	0.154
	Short/short	1 (1.1)	7 (6.8)	0.16	0.01–1.30	0.050
(c)						
Locus	Variant	SR N (%)	NR N (%)	OR	95% CI	P-value
PKR	-180					
	GG	2 (7.4)	6 (14.6)	0.47	0.06–2.91	0.370
	GT	9 (33.3)	10 (24.4)	1.55	0.47–5.16	0.421
PKR	-168					
	CC	12 (15.2)	18 (22.2)	0.63	0.26–1.51	0.250
	CT	31 (39.2)	27 (33.3)	1.29	0.64–2.59	0.437
PKR	TT	36 (45.6)	36 (44.4)	1.05	0.54–2.05	0.890
	CGG					
	Long/long	42 (89.4)	74 (71.8)	3.29	1.10–10.52	0.017
	Short/long	5 (10.6)	22 (21.4)	0.44	0.13–1.34	0.113
	Short/short	0 (0.0)	7 (6.8)	0.14	0.008–2.42	0.11

See Table 2.

interferon therapy. Expansion of the number of trinucleotide repeats within the promoter region has been correlated with transcriptional silencing as shown for the *FMR1* gene and Fragile x syndrome.²⁹ While the number of repeats in the *FMR1* gene expands from 10–50 to 52–2000, we only found a maximum number of 10 repeats in the 5'UTR of the *PKR* gene, with nine repeats being the most common number of repeats and a low number (1–8) of repeats being found less frequently.

Our observed differences in the association between the long/long repeat and response to therapy but lack of association between this genotype and self-limiting infection may be due to, at least in part, differential

effects from endogenously derived interferon- α (self-limiting infection) and exogenous interferon- α (administered as anti-viral therapy) or differences in the level of interferon- α between natural infections and therapies. While *PKR* expression occurs at low levels under most physiological conditions, its expression is greatly enhanced within a few hours of interferon- α treatment.¹¹ The large dose of interferon- α (3 million IU) given during interferon therapy may strongly increase *PKR* expression, and the increased quantity of *PKR* may facilitate enhanced clearance of HCV.

Our study showed that the SNP at position -168 in the *PKR* gene may also be important in the outcome of HCV.

Table 5 Sequences of primers used in the present study

<i>Gene</i>	<i>Region</i>	<i>Oligo Name</i>	<i>Oligo Sequence (5'-3')</i>	<i>Annealing positions and reference sequence (Genbank accession number)</i>
<i>MxA</i>	Promoter -88	Forward	TGAAGACCCCCAATTACCAA	269-287 of X55639
	Promoter -88	Reverse	CTCTCGTTCGCCTCTTTCAC	619-600 of X55639
	Promoter -545	Forward	GGCCTGGCCTGACAACCTAT	29666-29684 of AL773577
	Promoter -545	Reverse	CATCCAAGCCTGCACGTAT	30068-30050 of AL773577
<i>OAS-1</i>	Exon 8	Reverse A/ G-allele specific primers	CTCACTGAGGAGCTTTGTCT	323-343 of M11810
	Exon 8	Forward common primer	CTCACTGAGGAGCTTTGTCC CAGGTGGGACTCTTGATCCAG	366-348 of M11810
<i>PKR</i>	Promoter region including exon 1	PPKR3S	AGGGTTCCTGGCCGTGCAGG	442-461 of HSU51035; 441-460 according to reference. ²¹
	Promoter region including exon 1	PPKR4A	CCGCCTCCCTCGGCTGC	751-734 of HSU51035; 441-460 according to Kuhen and Samuel ²¹
	Promoter region	PPKR-168F	GACTAGGCCAGCGGAGAAC	
	Promoter region	PPKR-168R	GCTTCGGGAGAGCTGGTT	
	Exon 4	PKR4S	ATATTCTCTTTGTAATCAGG	2-21 of U5635
	Exon 4	PKR4A	AAAAATGGCAATCACTCACC	160-141 of U5635
	Exon 6	PKR6S	CCTTCTATGATTTCCTAG	1-21 of U5637
	Exon 6	PKR6A	ATCCAAAGGCAATACGTACC	167-147 of U5637
	Exon 11	PKR11S	ACAGTGTTTTATCTTTAAGG	2-21 of U50642
	Exon 11	PKR11A	GTAAACATTTACTACTACTCG	162-141 of U50642
	Exon 12	PKR12S	CCCTGTTCCCTTTAACTAGG	2-21 of U50643
	Exon 12	PKR12A	CTCAGGATCATAATCACTGC	159-140 of U50643
	Exon 13	PKR13S	CTGTGAATTTTATACCCAGG	1-21 of U50644
	Exon 13	PKR13A	GTATTACTTTTTCCACTTACC	221-201 of U50644
	Exon 17	PKR 17S	GACTTCACTGTCATTGCAG	1-20 of U50648
Exon 17	PKR 17A	GTGTCATTGCACTCCAGCCT	401-420 of U50648	

However, in this case it appears that heterozygosity confers an advantage for spontaneous elimination of the virus. Such observations have been reported initially for haemoglobinopathies in falciparum malaria³⁰ and more recently for MHC class II genes for hepatitis B virus infection.³¹

Although a number of concurrent hypotheses have been tested in this study, we have avoided the application of a Bonferroni correction. A number of statistical experts have addressed this issue and it is now generally accepted that the Bonferroni correction is too conservative and does not replace the need for a credible hypothesis and replication of the results in independent cohorts.³²

The present study highlights the important role of host genetic factors, in particular variation in the interferon-induced *MxA*, *OAS-1* and *PKR* genes in the modulation of the outcome of HCV. In addition, it identifies important polymorphisms that may be further examined for potential use as genetic markers of HCV or other infectious disease pathogenesis.

Patients and methods

Patients

We studied 638 Caucasian subjects enrolled in the HENCORE (Hepatitis C European Network for Collaborative Research) study; the details of this study have

been described before.³³ Briefly, this is a multicentre European study of HCV that enrolled individuals from nine centres between October 1995 and June 2001, and the cohort includes individuals with both self-limiting and persistent HCV infections. A portion of these individuals have also been treated with interferon- α monotherapy.

Patients were recruited randomly or sequentially in each centre in order to minimise 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 have no evidence of viraemia on at least two occasions and who have persistently normal liver transaminase levels; (2) persistent HCV infection, individuals who have evidence of viraemia for at least 6 months; (3) sustained treatment response (SR), patients with persistent HCV infection treated with interferon- α alone who had normal liver transaminases and no evidence of viraemia 6 months after the end of treatment; (4) response-relapse (RL), patients with persistent HCV infection treated with interferon- 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) nonresponse (NR) to treatment, patients with persistent HCV infection treated with interferon- α who never lost viraemia during treatment. Individuals considered "initial responders" to interferon

were those who experienced a loss of HCV viraemia during the first 12 weeks of treatment, regardless of whether they ultimately achieved sustained response (SR+RL).

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, IL, USA). 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.

HCV genotyping

Since the patients included in this study were treated with interferon- α monotherapy, which was administered for the same duration of time irrespective of viral genotype, the individuals in the cohort were not tested for viral genotype. We retrospectively viral genotyped 192 individuals using stored sera. HCV genotyping was conducted using the Inno LiPA HCV Kit (Innogenetics, Gent, Belgium). Genotypes were dichotomised into genotype-1 and non-1 to facilitate categorical analyses.

Screening for novel polymorphisms

Novel polymorphisms were sought in the promoter region and the functional domains of the *PKR* and *OAS-1* genes, along with the promoter region of *MxA* by direct sequencing of these regions. Sequencing was performed using Big-Dye Terminator Chemistry (Applied Biosystems, Warrington, UK) and an ABI377 automated DNA sequencer. Sequences were aligned and analysed using Sequence Navigator Software (Applied Biosystems, Warrington, UK).

Genotyping of allelic variants

Primers used for sequencing and genotyping can be found in Table 5.

OAS-1. An A-G SNP at position 84 bp in the untranslated 3' end of exon 8 of OAS-1 (position 347 of GenBank Acc. no. M11810) was genotyped using allele-specific real-time PCR, which is a modified method according to Germer *et al.*³⁴ Each reaction comprised 0.2 μ M each of the primers specific for exon 8 (Table 1); 2.5 U of Stoffel Gold Polymerase (David Birch, RMS); 1 \times Stoffel Gold buffer (10 mM Tris-HCl, 10 mM KCl at pH 8.0); an additional 30 mM KCl for a final concentration of 40 mM; 2 mM MgCl₂; 50 μ M each dATP, dCTP and dGTP; 25 mM TTP; 75 mM dUTP; 2 U of UNG (PE), 0.2 \times SybrGreen I (Roche Molecular Probes); 2 μ M ROX (Roche Molecular Probes); 5% DMSO; and 2.5% Glycerol. Kinetic PCR reactions were performed on a GeneAmp 5700 Sequence detection System (PE Applied Biosystems). An initial incubation step of 2 min at 50°C (to allow UNG-mediated elimination of carryover PCR product contamination), and an enzyme heat activation step of 12 min at 95°C were followed by 40 two-step amplification cycles of 20 s at 95°C for denaturation and 20 s for 58°C for annealing and extension, and a final 5 min extension at 72°C. All PCR

reactions were performed with 5–50 ng genomic DNA in a total volume of 20 μ l.

MxA. The biallelic G/T polymorphism in the promoter region of *MxA* at position -88 from the transcription start site²⁰ was genotyped by restriction fragment length polymorphism (RFLP) using the enzyme *HhaI* (New England Biolabs) to digest the PCR fragment of 351 bp. Amplification was carried out in a volume of 20 μ l, containing 10–100 ng DNA, 2.5 mM MgCl₂, 500 nM of each primer, 500 μ M dNTP's, 1 \times PCR buffer (Qiagen), 1 U *Taq* DNA polymerase (Qiagen), 0.16 μ l *TaqStart* Antibody (BD Clontech). The cycling conditions in an Applied Biosystems 2400 or 9700 machine were: denaturation at 94°C for 5 min, subsequently 35 cycles of denaturation at 94°C for 30 s; annealing at 58°C for 30 s; and extension at 72°C for 1 min. This was followed by a final extension step at 72°C for 7 min. For the *HhaI* restriction digest 8 μ l of the PCR product were digested for at least 4 h in a volume of 20 μ l with 5 U of *HhaI* according to the manufacturer's specifications. Digested PCR product 10 μ l were run out on 2% agarose gels and analysed. In the presence of the G allele, the 351 bp long product is cut into fragments of 261, 51, 23 and 16 bp, and in the presence of the T allele into fragments of 312, 23 and 16 bp. The SNP (G/A) at position -545 from the transcription start site was genotyped using *BsaI* (New England Biolabs) to differentiate between the alleles. A 402 bp fragment was amplified at under the same conditions as described for the SNP at position -88. Restriction digestion was carried out using 8 μ l of the PCR product in 20 μ l volume in the presence of 2 U *BsaI*. The presence of the A-allele was indicated by the absence of the *BsaI* cutting site and consequently by the presence of a 402 bp fragment, whereas the G allele resulted in the creation of a 287 and a 115 bp fragment.

PKR

Point mutations in the promoter region of PKR were genotyped by sequence analysis as described earlier, using the primers PPKR3S and PPKR4A (Table 5). In addition, RFLP with the enzyme *SgrAI* was used to genotype the SNP at position -168 from the transcription start of the PKR gene. A 282 bp fragment was amplified using the forward primer PPKR-168F and the reverse primer PPKR 168R (Table 1). In the presence of the T allele, a second *SgrAI* site was introduced, which leads to the separation of 19, 97 and 166 bp fragments after digestion and gel separation, whereas in the presence of the T allele the *SgrAI* digest only gives rise to two 116 and 166 bp fragments.

A short tandem repeat (CGG) in exon 1 of the 5'UTR of PKR was genotyped on the automated ABI377 sequencer (Perkin-Elmer Applied Biosystems). PCR products were obtained using a FAM labelled sense primer (primer PPKR3S, see Table 1) and allowed size discrimination of the fluorescently labelled products. The products were diluted 1:5 in water and 1 μ l of the diluted product was added to 4 μ l of formamide, TAMRA 350 size marker and BlueDextran/EDTA buffer (ratio 3:1:1). The samples were heated to 94°C for 5 min and then cooled on ice immediately. A volume of 2 μ l of this solution was loaded onto a denaturing

4.25% polyacrylamide gel and run on an ABI 377 genotyping system. Allele discrimination was performed using Genescan and Genotyper software (Applied Biosystems).

Statistical analyses

Standard univariable analyses were conducted using contingency tables. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated along with maximum likelihood and Fisher's exact *P*-values, as appropriate.

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