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ABSTRACT

CD209, a c-type lectin expressed by dendritic cells (DCs) acts as a pathogen recognition receptor. A single nucleotide polymorphism (SNP) in the promoter region of *CD209* (-336 A/G; rs4804803) affects transcription and is associated with the severity of Tuberculosis and Dengue fever. As CD209 binds Hepatitis C Virus (HCV) glycoprotein-E2, we investigated this SNP in the context of chronic HCV infection. 131 Irish women who had received HCV contaminated Anti-D immunoglobulin and 79 healthy controls were genotyped. We found no association between rs4804803 and the risk of HCV chronicity. However, of those with chronic infection, possession of at least one *g*-allele was associated with more advanced liver disease, with significantly higher liver fibrosis scores and levels of alanine transaminase (ALT) observed. We conclude that rs4804803, a SNP in the *CD209* promoter contributes to the severity of liver disease in chronic HCV infection.

INTRODUCTION

HCV infection is a major global public health problem [1]. Approximately 70% of acute HCV infections result in chronic infection with the subsequent potential to develop a wide spectrum of clinical sequelae from asymptomatic chronic hepatitis to end-stage liver disease. The host's immune response to HCV is critical in determining whether resolution or persistent viraemia ensues after initial infection [2]. Viral impairment of Dendritic Cell (DC) function may be a major contributory factor to the establishment of chronic infection, though this remains controversial [3, 4].

DCs employ a wide variety of c-type lectin receptors to recognise pathogen associated carbohydrate (CHO) structures. CD209 (DC-SIGN, DC-specific ICAM-3 grabbing non-integrin), the most widely studied molecule of this family exhibits mannose type CHO specificity, and mediates the recognition of a plethora of diverse pathogens, including HCV, HIV, Dengue, *H. pylori* and *M. tuberculosis* by the innate immune system [5]. Capture of circulating HCV particles by CD209 expressed by DCs may facilitate virus infection of proximal hepatocytes and lymphocyte subpopulations and may be essential for the establishment of persistent infection [6]. However, the precise role this receptor plays in regulating the immune response to HCV infection or pathogenesis remains unclear.

A variant in the CD209 promoter region (rs4804803) that results in altered CD209 transcription is associated with the severity of Dengue fever in a Thai population [7], Tuberculosis in a sub-Saharan African population [8] and an increased risk of parental acquisition of HIV in an American-European population [9]. In this study, we investigated the association of rs4804803 with failure resolve HCV infection or the severity of liver disease in a cohort of Irish women who received HCV genotype 1b contaminated Anti-D immunoglobulin in 1977/1978.

PATIENTS & METHODS

Study Subjects

One hundred and thirty one patients who attend the Hepatitis C Clinic at St. Vincent's University Hospital, Dublin or the Mater Misericordiae Hospital, Dublin were enrolled in this study. All were infected with HCV genotype 1b after receiving contaminated anti-D immunoglobulin from a single source between May 1977 and November 1978 [10]. All patients consumed less than 14 alcohol units per week and had no other risk factors for liver disease. Informed written consent was obtained from each patient. The study received ethical approval from the Research and Ethics Committee at St. Vincent's University Hospital and conforms to the guidelines of the 1975 Declaration of Helsinki. All 131 patients tested positive for antibodies to HCV using a third-generation enzyme immunoassay (Abbott Diagnostics, Germany), confirmed with an immunoblot assay (RIBA-3) (Chiron Corp., Emeryville, CA). Seventy nine of these patients (60 %) remain chronically infected with the virus as determined by testing consistently positive for HCV RNA by a qualitative reverse transcriptase-polymerase chain reaction (RT-PCR), (Amplicor; Roche Diagnostic Systems, Nutley, NJ), over a 15 year follow- up period. Fifty two patients (40 %) achieved spontaneous (untreated) viral clearance and tested negative for HCV RNA by RT-PCR. None of the patients had received antiviral therapy at the time of entry to the study. Seventy-nine healthy volunteers were recruited from the general public. All of the completed a detailed health questionnaire and none of the volunteers reported risk factors for viral hepatitis.

Assessment of liver disease

Alanine aminotransferase (ALT) data was available for all patients from medical records. At the time of diagnosis in 1994, percutaneous liver biopsies were performed

on all HCV RNA positive patients, with repeat biopsies performed on a subset of patients at irregular time intervals. Liver fibrosis scores were available for all patients in the study and fibrosis and inflammation was assessed by a single experienced pathologist in the SVUH patient cohort and included in our analysis. Fibrosis was classified according to the following scale: 0 indicated no fibrosis, 1: periportal or portal fibrosis in a minority of tracts, 2: portal fibrosis in most portal tracts, 3: ocasional portal-portal septae, 4: occasional bridges, 5: marked fibrosis with early nodule formation, and 6: probable or definite cirrhosis. Inflammation was graded by light microscopic examination of the sections. The stage of fibrosis was assessed by Masson's trichrome staining and the Shikata orcein method. Inflammation was graded on a cumulative 18-point scale, with interface hepatitis graded from 0 to 4, confluent necrosis from 0 to 6, lobular inflammation from 0 to 4, and portal inflammation from 0 to 4.

Genomic DNA Extraction & SNP genotyping

Genomic DNA was extracted from whole blood (HCV patients) using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions or from saliva swabs (controls) using a salting out technique as described [11]. Genomic DNA concentration was assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and samples were diluted to 2 ng/ml in dH_20. Genotyping of the *CD209* variant rs4804803 was performed using a custom Taqman SNP Genotyping Assay (Applied Biosystems, Foster City, CA). The primer sequences used were as follows: 5'-ACTGTGTTACACCCCCTCCACTAG-3' (sense), 5'- AGGAAAGCCAGGAGGTCACA-3' (antisense). The sequences of the Taqman probes were, 5'-CTACCTGCCCACCC-3' and 5'-CTGCCTACCCTTGC-3'. The probes were labelled with the fluorescent dyes VIC and FAM, respectively.

Polymerase chain reactions (PCRs) were set up according to the manufacturer's instructions. Thermal cycling was performed on a fast optical 96-well reaction plate on the 7500 Fast Real-Time PCR system (Applied Biosystems) as follows: Initial denaturation and enzyme activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. The genotype of each sample was attributed by measuring the allele-specific fluorescence on the 7500 Fast Real-Time PCR system (Applied Biosystems).

Statistical Analysis

Allelic and genotypic frequencies were compared between the patient and control groups by the χ^2 test. Differences in ALT levels, liver fibrosis scores and liver inflammation scores between patients of differing genotypes were determined using a two-tailed Mann-Whitney U-test. In addition, we verified that Fibrosis scores (0 vs above 0) were associated with the SNP using Fisher's exact test on a two-by-two contingency table of counts, with columns corresponding to genotype and rows to fibrosis scores (e.g., equal to 0 vs. above 0). P values < 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism (Version 5) (GraphPad Software Inc., La Jolla, CA). Deviations from the Hardy-Weinberg equilibrium were calculated using a $\chi 2$ Hardy-Weinberg equilibrium test calculator for biallelic markers [12].

RESULTS

We investigated the association of the SNP rs4804803 in the promoter region of *CD209* with the resolution of HCV infection and the progression of liver disease. Genomic DNA was obtained from healthy Irish volunteers (n=79), women with chronic HCV infection (n=79) and women who had resolved infection (n=52) having received HCV contaminated Anti-D immunoglobulin and genotyped for the rs4804803 SNP. We found no difference in the frequency of the *a* or *g* alleles or *aa, ag* and *gg* genotypes between healthy volunteers, women with chronic HCV infection (HCV RNA positive) or women who had spontaneously cleared the virus (HCV RNA negative) (Table 1).

Women in this cohort, in general, have relatively mild liver disease [10, 13], however some have progressed to develop liver fibrosis or cirrhosis. We wished to examine if there was any difference in the progression of liver disease in women with the *aa* or *ag* and *gg* genotypes of the rs4804803 SNP in the *CD209* promoter. Clinical data including liver biopsy results were available for n=41 for the *aa* genotype and n=34 for the *ag* and *gg* genotype. Interestingly, patients with the *ag* or gg genotype had significantly worse liver fibrosis scores (Fig 1a), taking the most recently available fibrosis score of patients at both of the centres included in this study, and higher ALT levels (Fig 1b) than patients with the *aa* genotype. No significant differences in HCV viral loads were observed between the groups (median viral loads (range); *aa*, 2.532 x 10^6 I.U. /ml (0.29 x 10^6 – 4.94 x 10^7) and *ag/gg,* 4.035x10^6 (1.257 x 10^6 - 2.36 x 10^8); *P*=0.1519, Mann-Whitney U-test.

The majority of the patients in this cohort have mild liver inflammation (<6, based on 18-point HAI scale). We found no significant difference between the inflammation scores of HCV RNA positive women based either on recent biopsy data (Fig 1d) or

their 1994 baseline biopsy (Fig 1e). This data was from the SVUH cohort and was scored by a single experienced pathologist.

DISCUSSION

The underlying genetic reasons why some people resolve HCV infection and others fail to do so is still not fully understood. The Irish cohort is ideal to study genetic associations related to HCV infection as they are infected with virus from a single source and confounding factors are minimal. There is good evidence that certain human leucocyte antigen (HLA) class I and II alleles influence the outcome of HCV infection in this cohort of patients. HLA-A*03, HLA-B*27 and HLA-Cw*01 occurred more frequently in those who cleared the virus [14], while the HLA-A*11, Cw*04 haplotype was associated with persistent infection [15]. The HLA Class II allele DRB1*01 was associated with spontaneous viral clearance [16]. Variation in genes encoding for other immune mediators such as Killer cell Immunoglobulin-like receptors [17] and cytokines e.g. IL-10, IL-22 [18] and IL-28 [19] have a role in determining an individual's response to HCV infection.

Previous work has demonstrated that CD209 has a crucial role in determining the outcome of infection with Dengue virus, which like HCV is a member of the *Flaviviridae* family of viruses [7]. Among individuals with dengue, genotypes *gg* or *ag* strongly increased the risk of contracting dengue hemorrhagic fever verus dengue fever. Dengue haemorrhagic fever (DHF) is a potentially deadly complication that is characterized by high fever, often with enlargement of the liver, and in severe cases circulatory failure. In our patient cohort, though liver inflammation was generally mild, there was a tendency to higher inflammation scores (p=0.0569) in patients with the *ag* or *gg* phenotype, which along with higher levels of ALT levels and fibrosis scores suggests that the possession of at least one *g* allele may increase the risk for liver disease progression. The SNP rs4804803 was demonstrated to be in linkage

disequilibrium with 3 other intronic polymorphisms, in the Thai population [7], and these may also contribute to the observed phenotype.

Interestingly, the allele frequency of rs4804803 in the healthy Irish control population deviated from Hardy-Weinberg equilibrium (HWE), with an over representation of *aa* genotypes and a lower than expected frequency of *ag* genotypes observed. Higher prevalence of the *a* variant, which is associated with higher CD209 expression, in the healthy population may be associated with increased pathogen recognition and control of infection. For example the *a* allele may confer protection against TB, resulting in an increased frequency in European populations compared to Africans due to a longer history of TB exposure [20].

Pathogen interaction with CD209 influences the subsequent antigen-specific immune response [21]. Mannose-dependent binding of pathogens such as *M. tuberculosis* and HIV-1 to CD209 leads to Raf-1 dependent up-regulation of IL-10, IL-6 and IL-12 secretion [22]. In contrast, the fucose-dependent interaction of *H. pylori* or soluble *S. mansoni* egg antigen with CD209 induces IL-10 but suppresses IL-12 resulting thus inhibiting of Th1 responses. This difference in response is due to the effect of the ligand on the composition of the CD209 signalosome, effector proteins that activate Raf-1 are recruited in response to mannose based ligands, whereas fucose expressing pathogens actively dissociate the KSR1-CNK-Raf-1 complex [22]. HCV glycoprotein E2 has 11 N-linked glycosylation sites, the majority of oligosaccharides on E2 are high mannose structures, suggesting that the interaction of E2 with CD209 would lead to the induction of both IL-10 and IL-12 as is the case with HIV-1 and *M. tuberculosis*. Therefore, we hypothesise that the DCs of individuals who possess the *g* allele of rs4804803 and thus lower levels of CD209 do not respond to E2 as efficiently as individuals who are homozygous for the *a* allele.

In conclusion, we have evidence that a variant in the promoter of CD209 is associated with worse liver disease in a well defined cohort of patients who are homogenous for age, gender, race, mode of acquisition and HCV genotype. Overall, the risk of increased disease severity in HCV infection results from the complex multi-factorial virus-host interaction of which CD209 variability is one important factor.

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Table 1. Allelic and genotype frequencies of rs4804803 in a homogenous cohort of Irish patients who were exposed to HCV contaminated Anti-D immunoglobulin and healthy controls. No significant differences in genotype or allele frequency were observed between the groups (allele, P=0.3945; genotype, P=0.5239, χ^2 test).

	HCV RNA Positive		HCV RNA Negative		Healthy Controls		Total	
	n	**Frequency**	**n**	**Frequency**	**n**	**Frequency**	**n**	**Frequency**
a	118	0.75	80	0.77	128	0.81	326	0.78
g	40	0.25	24	0.23	30	0.19	94	0.22
Total	158	1	104	1	158	1	420	1
aa	45	0.57	32	0.62	55	0.70	132	0.63
ag	28	0.35	16	0.31	18	0.23	62	0.30
gg	6	0.08	4	0.07	6	0.07	16	0.08
Total	79	1	52	1	79	1	210	1

FIGURE LEGEND

Figure 1. Comparison of clinical parameters of liver disease of HCV PCR^{+} patients,with either the *aa* or *ag* genotype of rs4804803. Clinical data, including liver biopsy results were available for n=41 patients with the *aa* genotype and n=34 patients with the *ag* or *gg* genotype of rs4804803. Biopsy specimens were scored according to Ishak's grading and staging method, that is the modified histological activity index (HAI) system [23]. The most recent ALT results were obtained from medical records. (A) Most recent liver fibrosis scores (Median year, 2002; range 1994-2009) (B) 1994 fibrosis scores for SVUH patients and (C) and ALT levels (U/L) (D) Liver Biopsy Liver Inflammation Scores were compared between the two groups using a two-tailed Mann-Whitney *u*-test. *, $p < 0.05$ and **, $p < 0.01$. The central bar represents the mean and the error-bars the standard error of the mean.

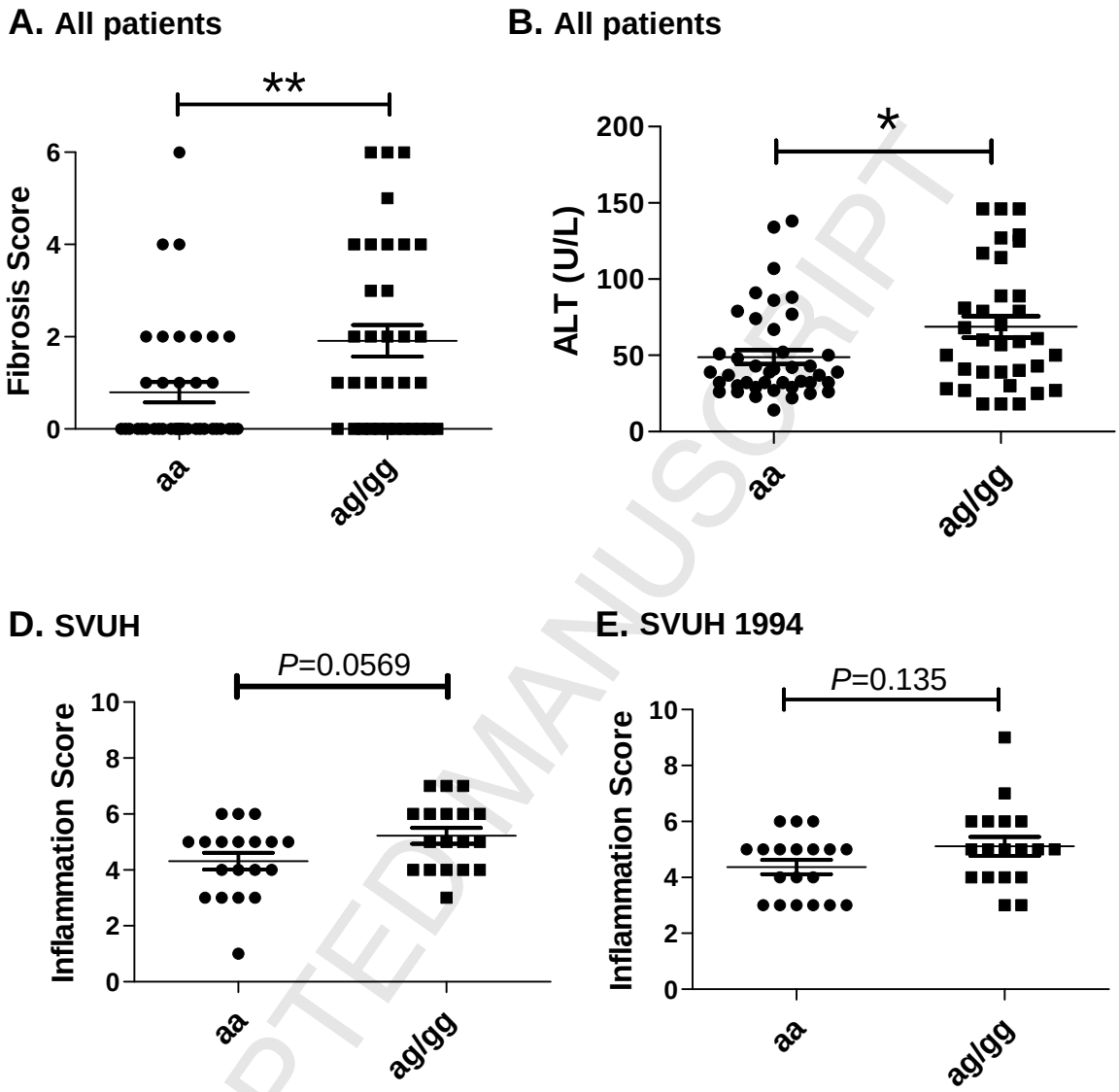

Figure 1