

Transforming growth factor- β and toll-like receptor-4 polymorphisms are not associated with fibrosis in haemochromatosis

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Abstract

AIM: To investigate the role of genetic polymorphisms in the progression of hepatic fibrosis in hereditary haemochromatosis.

METHODS: A cohort of 245 well-characterised C282Y homozygous patients with haemochromatosis was studied, with all subjects having liver biopsy data and DNA available for testing. This study assessed the association of eight single nucleotide polymorphisms (SNPs) in a total of six genes including toll-like receptor 4 (TLR4), transforming growth factor-beta (TGF- β), oxoguanine DNA glycosylase, monocyte chemoattractant protein 1, chemokine C-C motif receptor 2 and interleukin-10 with liver disease severity. Genotyping was performed using high resolution melt analysis and sequencing. The results were analysed in relation to the stage of hepatic fibrosis in multivariate analysis incorporating other cofactors including alcohol consumption and hepatic iron concentration.

RESULTS: There were significant associations between the cofactors of male gender ($P = 0.0001$), increasing age ($P = 0.006$), alcohol consumption ($P = 0.0001$), steatosis ($P = 0.03$), hepatic iron concentration ($P < 0.0001$) and the presence of hepatic fibrosis. Of the candidate gene polymorphisms studied, none showed a significant association with hepatic fibrosis in univariate or multivariate analysis incorporating cofactors. We also specifically studied patients with hepatic iron loading above threshold levels for cirrhosis and compared the genetic polymorphisms between those with no fibrosis vs cirrhosis however there was no significant effect from any of the candidate genes studied. Importantly, in this large, well characterised cohort of patients there was no association between SNPs for TGF- β or TLR4 and the presence of fibrosis, cirrhosis or increasing fibrosis stage in multivariate analysis.

CONCLUSION: In our large, well characterised group

of haemochromatosis subjects we did not demonstrate any relationship between candidate gene polymorphisms and hepatic fibrosis or cirrhosis.

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Key words: Haemochromatosis; Genetic polymorphism; Liver fibrosis; Toll-like receptor 4; Interleukin 10; Monocyte chemoattractant protein 1; Chemokine (C-C motif) ligand 2; Transforming growth factor beta; 8-oxoguanine DNA glycosylase

Core tip: This study does not support the previously proposed role of mutations in both toll-like receptor 4, transforming growth factor-beta in the progression of hepatic fibrosis associated with hereditary haemochromatosis.

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INTRODUCTION

It is generally believed that genetic factors may influence the progression of hepatic fibrosis in chronic liver disease of differing aetiologies. Many case-control studies have been performed in an attempt to elucidate these genetic influences, however, results have been inconsistent. Possible explanations for this include relatively small sample sizes and the difficulties in controlling for factors such as the duration of hepatic insult (*e.g.*, in chronic hepatitis C virus infection and disease co-morbidities (*e.g.*, alcohol)^[1]. In hereditary haemochromatosis iron accumulation begins in early adulthood in males and despite similarity in the age of onset, there is a highly variable disease progression both in iron loading and in hepatic fibrosis progression^[2-6]. It is likely that genetic factors play a role in influencing both iron accumulation and the development of cirrhosis^[7]. The aim of this study was to explore potential genetic polymorphisms involved in hepatic disease progression in haemochromatosis with particular attention to candidate molecules associated with the processes of hepatic fibrogenesis. This study was conducted using a well-characterised cohort of patients with HFE-associated hereditary haemochromatosis with known fibrosis stage and quantitative hepatic iron loading.

Candidate genes for analysis were chosen based either on their existing association between gene mutations and fibrogenesis in other disease aetiologies, or their demonstrated role in hepatic stellate cell biology and hepatic injury/fibrosis. Candidate genes included:

(1) molecules associated with hepatic inflammation including monocyte chemoattractant protein 1 (MCP-1), the MCP-1 receptor, chemokine C-C motif receptor 2 and interleukin-10 (IL10); and (2) mediators of hepatic injury/inflammation/fibrosis including transforming growth factor-beta (TGF- β), toll-like receptor 4 (TLR4) and human 8-oxoguanine DNA glycosylase (hOGG1).

MCP-1, also known as chemokine (C-C motif) ligand 2, is a cytokine belonging to the CC chemokine family which acts as a potent inducer of monocyte, macrophage and hepatic stellate cell migration^[8-11] and is involved in the early stages of hepatic inflammation and fibrogenesis^[9]. Several clinical studies have shown an association between single nucleotide polymorphisms (SNPs) in the *MCP-1* gene and fibrosis in various organs including liver, kidney, and skin^[12-14] although others have shown conflicting results. The MCP-1 receptor, CCR2, mediates much of chemokine response of MCP-1^[15]. While the precise role of CCR2 in human liver disease is relatively unknown, studies investigating CCR2 variants in alcoholic liver disease or liver carcinoma have produced varying results^[16,17]. IL10 acts as an anti-inflammatory cytokine^[18] in different forms of human chronic liver disease, regulating inflammatory and fibrogenic responses (reviewed in^[19]). In haemochromatosis, hepatic IL10 mRNA expression is decreased^[20].

TGF- β has been described as a “master switch” in hepatic fibrosis due to its central role in the activation of hepatic stellate cells and the production of fibrillar collagen *via* Smad^[21]. SNPs in the *TGF- β* gene have previously been studied in a relatively small cohort of subjects with haemochromatosis where results suggested that the presence of the proline substitution (C) may accelerate hepatic fibrosis^[22]. TLRs are a group of receptors involved in both the recognition of pathogens and in mediating non-infectious and ischaemic causes of liver injury^[23,24]. TLR4 has been shown to be associated with signalling leading to hepatic fibrosis, with hepatic stellate cells being the main direct mediator promoting fibrogenesis *via* TGF- β signalling^[25]. Studies designed to assess the role of *TLR4* polymorphisms in the susceptibility to inflammatory or infectious diseases have provided conflicting results^[26,27]. In haemochromatosis, however, one study has shown that a *TLR4* polymorphism was associated with clinical disease without any notable effect on iron loading^[28], although again this was conducted in a relatively small cohort of patients. hOGG1 is an enzyme responsible for repairing the 8-oxo-7,8-dihydroguanine 8 lesion of DNA subjected to oxidative stress. Although oxidative stress is one of the common mechanisms for hepatocyte injury and an inflammatory cascade, particularly in iron loading, to our knowledge no studies have considered the role of *OGG1* gene polymorphisms in the progression of liver damage.

Few studies have assessed the contribution of polymorphisms in genes associated with hepatic injury and fibrosis in the phenotypic disease expression in haemochromatosis. Those that have were limited by both

cohort size and the lack of well characterised patients with liver biopsy-proven fibrosis staging and quantitative hepatic iron loading. This study represents one of the largest cohorts of patients with haemochromatosis to be assessed for the role of SNPs associated with hepatic disease expression.

MATERIALS AND METHODS

Ethics statement

All subjects in this study provided written informed consent and the study was approved by the Human Research Ethics Committees of the Royal Brisbane and Women's Hospital (RBWH) and the Queensland Institute of Medical Research (QIMR), Brisbane, Australia. Written informed consent was witnessed and documented in each patient hospital file, a procedure approved by both ethics committees.

Study subjects

The subjects in this study were derived from the Haemochromatosis Database at the QIMR. This is a cohort recruited over approximately 30 years from clinical review at the RBWH. The database lists more than 2400 patients of whom 722 are C282Y homozygous. The database includes clinical and laboratory data, with DNA available from a subset of these patients.

Inclusion criteria for selection for this study were: (1) genetic testing confirming C282Y homozygosity; (2) patients had previously undergone a liver biopsy for clinical indications and information was available with respect to iron loading and fibrosis stage; and (3) patients had previously provided blood for the extraction of DNA from peripheral white cells or were available to do so prospectively.

Patients were excluded from this study if aged less than 16 years at the time of liver biopsy as iron loading at this age may indicate the presence of other mutations in iron homeostatic genes. Patients with viral hepatitis were excluded. Excessive alcohol consumption was not an exclusion criterion however this information was included in the data collection.

Control subjects were obtained from the QIMR DNA bank and represented a selection of healthy subjects who had previously provided peripheral blood for the extraction of DNA. Those with European ethnicity were preferentially utilised for this study in order to provide a genetically comparable group for the study subjects. Allele frequencies were also compared to subjects in the International HapMap project selected from United States residents with Northern and Western European ancestry^[29].

Rationale for candidate gene analysis

While this cohort of haemochromatosis patients is one of the largest and most well characterised studied to date, investigation using genome wide association in a cohort of this size was not considered viable, thus we

used a candidate gene approach.

MCP-1 and CCR2: Human MCP-1 production is regulated in part by a region 1.8 to 2.7 kb upstream of the transcriptional start site and a polymorphism at position -2518 (G/A rs1024611) affects the transcriptional activity^[30]. Individuals with a G allele at this site (G/A or G/G) produce more MCP-1 from monocytes in response to stimulation with interleukin-1 β ^[30]. A variant in the CCR2 gene Val64Ile (A/G rs1799864) has been shown to be associated with a delay in progression in human immunodeficiency virus^[31] and other studies have shown a possible role for this polymorphism in inflammatory conditions such as sarcoidosis and atherosclerosis^[32,33]. Thus, the MCP1-2518 and CCR2-190 SNPs were chosen for study.

IL10: The promoter region of the *IL10* gene has several polymorphisms at positions -1082 (G/A: rs1800896), -819 (C/T: rs1800871) and -592(C/A: rs1800872) with only three haplotypes found in Caucasian populations: GCC, ACC and ATA^[34]. Heritability factors are thought to account for some of the variability in IL10 production although environmental influences are also important^[35,36]. The role of specific SNPs in differential IL10 production is controversial^[37-40]. The GCC promoter haplotype may have greater transcriptional activity compared to the ATA and ACC haplotypes^[35,41] and this would be consistent with other studies showing decreased IL10 production in those with the -1082A genotype^[34,39,42]. Most studies considering the relationship between *IL10* promoter polymorphisms and liver fibrosis in HCV infected patients have failed to show statistically meaningful effects although many of these have suffered from small sample sizes (reviewed in^[43]). Therefore, although it seems attractive to consider IL10 as a mediator of hepatic inflammation and fibrosis, promoter polymorphisms in this gene have not been unambiguously linked with either cytokine production or hepatic fibrosis. For this study, IL10-1082 and IL10-592 SNPs were selected for investigation.

TGF- β : A single nucleotide polymorphism in the *TGF- β* gene at position -915, codon 25(G/C: rs1800471) results in an amino acid substitution of proline for arginine. Leukocytes from those homozygous for the arginine (G) molecule at this site appear to produce more TGF- β in response to stimuli suggesting this is the "high producing" genotype^[44]. This report has been challenged by other groups who have suggested that there may be important differences between total TGF- β secretion and bioavailable forms^[45,46]. The TGF β -915 SNP was used in this evaluation of disease expression susceptibility in haemochromatosis.

TLR4: Two common missense mutations in the *TLR4* gene have been suggested to have functional significance with defective signalling resulting. Aspartic acid substi-

Table 1 Gene specific primers for polymerase chain reaction amplification and dissociation analysis

Gene	Forward primer	Reverse primer
MCP1-2518	TTICTTGACAGAGCAGAAGTGGGAG	TGCTGGCTGAGTGTTCACATAGG
CCR2 190	ATACCAACGAGAGCGGTGAAGAAG	AAAGCAGATCAGAGATGGCCAGG
IL10-592	AAAGGAGCTGGAACACATCCTGT	AAAGTTCCAAGCAGCCCTTCCAT
IL10-1082	TCCAAGACAACACTACTAAGGCTTC	GCTGGATAGGAGGTCCCTTACTTT
TGF- β	CTACCGCTGCTGTGGCTACTGGT	TCACCAGTCCATGTCGATAGTCT
TLR4-299	CCGATTAGCATACTTAGACTACTACCTC	CCTTCAATAGTCACACTCACCAGG
TLR4-399	GCTTGAGTTTCAAAGGTTGCTGTCTC	GCCCAAGAAGTTTGAACTCATGGTAA
hOGG1	ACCCTCTACAGGTGCTGTTCAGT	CCTTTGGAACCCTTTCTGCGCTTT

MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: Interleukin-10; TGF- β : Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

Table 2 Polymerase chain reaction profiles for candidate gene high resolution melt analysis

Gene	Annealing temperature	Extension temperature	Melt analysis range
MCP-1	58 °C	72 °C	74 to 84 °C
CCR-2	58 °C	72 °C	74 to 84 °C
IL10-592	57 °C	72 °C	75 to 85 °C
IL10-1082	59 °C	72 °C	70 to 81 °C
TGF- β	65 °C (20 s)	72 °C (20 s)	79 to 89 °C
TLR4 299	57 °C	68 °C	66 to 78 °C
TLR4 399	58 °C	70 °C	69 to 81 °C
hOGG1	57 °C	68 °C	78 to 88 °C

MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: Interleukin-10; TGF- β : Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

tuted for glycine at amino acid position 299 (Asp299Gly) (A/G: rs4986790) and 399 (Thr399Ile) (C/T: rs4986791) are said to induce hypo-responsiveness to lipopolysaccharide although this has not been supported by all investigations^[47-49]. The CC variant of the Thr399Ile polymorphism was one SNP included in a panel of tests demonstrating discrimination of patients with advanced fibrosis in chronic hepatitis C^[50]. Functional studies have shown that the polymorphisms in TLR4 which confer protection from hepatic fibrosis are associated with a reduced threshold for HSC apoptosis and attenuation of fibrogenic responses stimulated by MCP-1, BAMBI and IL6^[51]. Both TLR4-299 and TLR4-399 SNPs were assessed in the present study.

OGG1: It can be shown on paraffin sections of diseased liver that there is increased staining of 8-oxodG consistent with oxidative DNA damage to hepatocytes and this does not appear to be specific to any particular type of liver disease^[52]. A polymorphism exists in the human *OGG1* gene leading to Ser326Cys conversion and affecting the function of this glycosylase due to changes in localization and phosphorylation (C/G: rs1052133)^[53]. There is some epidemiological evidence to suggest that certain tumours may have an increased prevalence in those with the variant of *OGG1*^[54]. Functional studies

have shown that individuals with the GG genotype in Ser326Cys have a reduced capacity for repair of DNA damage compared to wild types or heterozygous subjects^[55]. Thus *OGG1*-326 was included for evaluation in this study.

SNP mutation analysis, real-time PCR and sequencing

Patients provided blood samples, often at the time of therapeutic venesection to allow DNA extraction from buffy coats using a high salt extraction method. DNA was utilised in all PCR experiments at a concentration of 25 ng/ μ L. Primers were designed using Genbank to obtain genetic sequences adjacent to the area of interest and using specific software (Primer Quest) to optimise the temperature difference for denaturing and minimise secondary structures (Table 1). Specificity was tested using specific software (Blast). Primers were purchased from Sigma-Aldrich Pty Ltd (NSW, Australia).

High resolution melt (HRM) analysis (Corbett Life Science Rotor-GeneTM 6000 HRM) was used to evaluate nucleotide sequences based on the dissociation profile of the fragment of DNA containing the polymorphism when amplification of the template occurs in the presence of specific dye. Sensimix HRMTM (Quantace, London) was used in PCR reactions with the following volumes for 1 reaction to give a total reaction volume of 25 μ L. Each gene was amplified and analysed under optimised conditions (Table 2).

From initial HRM analysis, subjects with variable dissociation characteristics were selected for sequencing in order to confirm the presence of the suspected gene polymorphism. Once identified, a sample from these subjects was used in each experiment to provide a positive control. Any sample that could not be genotyped with HRM analysis was subjected to sequencing. Each patient sample was tested in duplicate. All experiments contained negative control samples (H₂O) to confirm the absence of contamination by PCR product. All patient and control sample identities were number coded for inclusion in experiments.

Sequencing was performed with ABI BigDye Version 3.1 according to specific instructions and using primers designed to incorporate the area of interest.

Table 3 Patient characteristics grouped according to the presence or absence of hepatic fibrosis (univariate analysis) *n* (%)

Parameter	No fibrosis (<i>n</i> = 136)	Fibrosis present (<i>n</i> = 109)	<i>P</i> value
Male gender	72 (53)	89 (82)	0.0001
Age at biopsy (yr) (mean ± SD)	39.7 (14.9)	44.3 (12.4)	0.006
Alcohol (g/d) (median: range)	5 (0-120)	20 (0-200)	0.0001
Steatosis present ¹	36 (37)	42 (53)	0.03
Serum ferritin (µg/L) (median: range)	531 (33-3000)	2134 (155-6000)	0.0001
HIC (µmol/g <i>dw</i>) (median: range)	124 (20-537)	218 (43-847)	< 0.0001
Iron grade			
0	1 (1)	0	
1	5 (4)	0	
2	27 (20)	7 (6)	
3	63 (46)	25 (23)	
4	40 (29)	77 (71)	< 0.0001

¹Steatosis data available for 176 subjects (72% of total cohort).

Statistical analysis

Normally distributed data were summarized by mean and standard deviation and differences tested by Student's *t* test. Non-parametric data were summarized by median and range and tested by Mann-Whitney or Kruskal-Wallis test. Categorical variables were summarized by frequencies and tested by Pearson χ^2 or Fisher's Exact test. Ordinal multivariate logistic regression analysis was performed using increasing hepatic fibrosis grade as the outcome variable incorporating predictor variables including age, gender, alcohol consumption and the genetic polymorphism in question. Results are presented as OR with 95%CI. *P* values of < 0.05 or less were considered significant. Polymorphisms were grouped in both possible ways for testing but reporting has been limited to that commonly described in the literature. Grouping for the outcome variable of hepatic fibrosis was done in several ways including "no fibrosis (F0) *vs* any hepatic fibrosis (F1-4)", "minimal fibrosis (F0-2) *vs* advanced fibrosis (F3-4)" and as an ordered logistic regression analysis with increasing hepatic fibrosis grade as the outcome variable. In order to consider those patients with the greatest difference in clinical outcome the analysis was repeated incorporating only those patients with heaviest iron loading (iron grade 3 and 4) and comparing those with no fibrosis (F0) against those with advanced fibrosis (F3/F4). Stata/IC software (version 10.1; Stata-Corp LP, College Station, TX) was used for statistical analysis. Deviation from Hardy-Weinberg equilibrium was tested for each gene using an on-line calculation tool (<http://www.oege.org/software/hwe-mr-calc.shtml>).

RESULTS

Of the 245 C282Y homozygous patients included in this study, 161 (66%) were male. The majority of biopsies performed in these patients (82%) were done so prior

Table 4 Allele frequency in candidate genes assessed in patient and control groups by high resolution melt analysis: Compared to published data for Caucasian ethnicity populations

Gene	Patient group	Control group	Published results ¹
<i>MCP1</i>	A 0.729	A 0.736	A 0.695
	G 0.271	G 0.264	G 0.305
<i>CCR2</i>	G 0.892	G 0.976	G 0.892
	A 0.108	A 0.024	A 0.108
<i>TGFβ</i>	G 0.918	G 0.892	G 0.887
	C 0.082	C 0.108	C 0.113
<i>hOGG1</i>	C 0.695	C 0.694	C 0.776
	G 0.305	G 0.306	G 0.224
<i>IL10-1082</i>	G 0.539	G 0.500	G 0.531
	A 0.461	A 0.500	A 0.469
<i>IL10-592</i>	C 0.794	C 0.794	C 0.792
	A 0.206	A 0.206	A 0.208
<i>TLR4 299</i>	A 0.934	A 0.930	A 0.967
	G 0.066	G 0.070	G 0.033
<i>TLR4 399</i>	C 0.934	C 0.931	C 0.955
	T 0.066	T 0.069	T 0.045

¹NCBI dbSNP (HapMap CEU-Utah residents with Northern and Western European ancestry from the CEPH collection). *MCP-1*: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

to 1996 when the *HFE* gene was cloned and therefore many patients are likely to have been biopsied for diagnostic purposes. Fibrosis stages were as follows; F0: 136 (56%), F1: 26 (11%), F2: 23 (9%), F3: 13 (5%), F4: 47 (19%). The demographic, laboratory and histological characteristics of the patients with and without hepatic fibrosis are summarized in Table 3. Of those with the grade of steatosis reported, 56% had no steatosis present and 12% had grade 2 or 3 steatosis. Male gender, age, alcohol consumption, steatosis and iron indices from serum and liver sections all showed significant associations with the presence of hepatic fibrosis, as has been previously demonstrated^[56,57].

Allele frequencies for the genes of interest were assessed in patient and control populations and these results compared to published data for Caucasian groups (Table 4). There was a significant difference in allele frequencies for the *CCR2* gene polymorphism when comparing the haemochromatosis and control populations (*P* = 0.001). This appeared to be due to an unexpectedly low number of heterozygous and A allele homozygous control subjects. When the patient population was compared to data published from the International HapMap Project there was no significant difference (*P* = 0.985). For all other genes there were no significant differences in allele frequencies between patient and control populations.

No patient or control subject was identified to have the uncommon homozygous polymorphism (Pro/Pro or C/C) in the *TGF-β* gene but as this is present in low frequency in the population this is not an unexpected finding. For the analyses of this polymorphism, Arg/Arg (G allele homozygosity) was compared to Arg/Pro (G/C).

Table 5 Genetic polymorphisms in Hereditary Haemochromatosis patients grouped according the presence or absence of advanced fibrosis and subjected to univariate analysis *n* (%)

Gene	No/minimal fibrosis (F0-2)	Advanced fibrosis (F3-4)	<i>P</i> value
<i>MCP1</i>			
AA	99 (53.5)	28 (46.7)	0.546
AG	76 (41.1)	27 (45.0)	
GG	10 (5.4)	5 (8.3)	
<i>CCR2</i>			
GG	142 (78.0)	50 (84.8)	0.401
AG	37 (20.3)	9 (15.3)	
AA	3 (1.7)	0 (0)	
<i>TGFβ</i>			
GG	154 (83.2)	51 (85)	0.749
GC	31 (16.8)	9 (15)	
<i>hOGG1</i>			
CC	99 (53.8)	26 (43.3)	0.352
CG	64 (34.8)	25 (41.7)	
GG	21 (11.4)	9 (15)	
<i>IL10-1082</i>			
GG	50 (27.1)	17 (28.3)	0.806
GA	97 (52.4)	33 (55.0)	
AA	38 (20.5)	10 (16.7)	
<i>IL10-592</i>			
CC	120 (64.9)	37 (61.7)	0.173
AC	58 (31.3)	17 (28.3)	
AA	7 (3.8)	6 (10.0)	
<i>TLR4 299</i>			
AA	159 (86.4)	54 (91.5)	0.305
AG	24 (13.0)	4 (6.8)	
GG	1 (0.6)	1 (1.7)	
<i>TLR4 399</i>			
CC	161 (87.0)	53 (89.8)	0.502
CT	23 (12.4)	5 (8.5)	
TT	1 (0.5)	1 (1.7)	

MCP-1: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

All genes showed no deviation from Hardy-Weinberg equilibrium in the haemochromatosis patient population except the *OGG1* polymorphism ($P = 0.03$). This may relate to selective pressure in this group. The control population showed no deviation from Hardy-Weinberg equilibrium.

No significant associations were present between the polymorphisms of any of the candidate genes and fibrosis stage when the patients were grouped into those with minimal or no fibrosis (F0-2) and compared with those having severe fibrosis (F3-4) (Table 5). Each candidate gene was assessed in ordered logistic regression analysis incorporating increasing fibrosis stage as outcome before and after adjustment for age, gender, iron loading and alcohol consumption and in each analysis, there was no statistically significant effect from the SNP of interest on fibrosis stage (Table 6). Analyses were repeated incorporating data on steatosis grade however this produced no statistically significant effect. Iron loading, alcohol consumption, male gender and age remained important in multivariate analyses as previously reported^[2,4-6,58-62].

Table 6 Multivariate ordered logistic regression analysis determining role of genetic polymorphisms in increasing hepatic fibrosis stage

Gene	OR	95%CI	<i>P</i> value	Adjusted <i>P</i> value ¹
<i>MCP1</i>	1.11	0.69-1.80	0.660	0.681
<i>CCR2</i>	0.71	0.36-1.32	0.284	0.432
<i>TGFβ</i>	1.03	0.54-1.95	0.936	0.740
<i>hOGG1</i>	0.94	0.44-1.98	0.864	0.588
<i>IL10-1082</i>	1.10	0.65-1.88	0.720	0.897
<i>IL10-592</i>	0.82	0.50-1.35	0.441	0.639
<i>TLR4 299</i>	0.73	0.35-1.53	0.403	0.745
<i>TLR4 399</i>	0.87	0.42-1.81	0.706	0.990

¹Adjusted for age at biopsy; gender; iron grade and alcohol consumption. *MCP-1*: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

After considering only those patients with heaviest grades of iron loading (iron grade 3 and 4) there was no significant association with hepatic fibrosis (F0 vs F3/F4) and any of the genetic polymorphisms studied when assessed in univariate or multivariate analysis (after adjustment for age, gender and alcohol consumption) (Table 7). A threshold hepatic iron concentration for cirrhosis of 236 μmol/g dry weight has previously been identified in this haemochromatotic patient cohort^[59]; this cut off was used to isolate those with the greatest risk of hepatic fibrosis and subjects with no fibrosis (F0) were again compared to those with advanced disease (F3/F4) (Table 8). Age and alcohol consumption were important in disease progression in this group but no difference was seen for any of the genetic polymorphisms studied. Gender was not a significant risk factor in this cohort with very heavy iron stores.

DISCUSSION

Studies investigating the clinical penetrance of haemochromatosis have consistently identified the severity of iron loading, male gender and alcohol consumption as being crucial factors in determining the risk of liver fibrosis (reviewed in^[7]). It is clear that steatosis accelerates the hepatic injury^[57] and that diabetes is a risk factor for advanced fibrosis^[56]. Despite these known risk factors, family studies have suggested a clustering of phenotypes that may indicate a role for genetic disease modifiers quite separate to those influencing iron loading. Relatively few studies have explored this area in haemochromatosis subjects, particularly when compared to a large body of literature that exists with respect to viral hepatitis.

Many candidate gene studies have suffered from methodological flaws which increase the risk of misleading results. Perhaps the most common scenario is a small subject group which allows the finding of a false positive result due to chance. Although haemochromatosis is relatively common in terms of genetic diseases, its expression remains uncommon in the general popula-

Table 7 Genetic polymorphism frequencies in patients with heavy iron loading (Grade 3 and 4) grouped according to no fibrosis (F0) vs advanced fibrosis (F3/F4)

Gene	No fibrosis (F0) (n = 103)		Advanced fibrosis (F3/4) (n = 60)		P value	Adjusted P value ¹
MCP1	AA 53/103	AG/GG	AA 28/60	AG/GG 32/60	0.555	0.433
	51.50%	50/103 48.5%	46.70%	53.3%		
CCR2	GG 81/100	AG/AA	GG 50/59	AG/AA 9/59	0.549	0.277
	81%	19/100 19%	84.70%	15.3%		
TGF β	GG 86/103	CG/CC 17/103	GG 51/60	CG/CC 9/60	0.800	0.968
	83.50%	16.5%	85%	15%		
hOGG1	CC 54/102	CG/GG 48/102	CC 26/60	CG/GG 34/60	0.238	0.790
	52.90%	47.1%	43.30%	56.7%		
IL10-1082	AA/AG 75/103	GG 28/103	AA/AG 43/60	GG 17/60	0.874	0.998
	72.8%	27.20%	71.80%	28.30%		
IL10-592	AA/AC 34/103	CC 66/103	AA/AC 23/60	CC 37/60	0.492	0.659
	33.0%	67.0%	38.3%	61.7%		
TLR4 299	AA 87/102	AG/GG 15/102	AA 54/59	AG/GG 5/59	0.248	0.848
	85.3%	14.7%	91.5%	8.5%		
TLR4 399	CC 89/103	CT/TT 14/103	CC 53/59	CT/TT 6/59	0.524	0.985
	86.4%	13.6%	89.8%	10.2%		

¹Adjusted for age:gender:alcohol consumption. MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: Interleukin-10; TGF- β : Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

Table 8 Logistic regression analysis performed in subjects with HIC > 236 $\mu\text{mol/g dw}$ and comparing outcome of F0 vs F3/4 (univariate analysis) (n = 47)

Factor	OR	95%CI	P value
Age	1.07	1.00-1.15	0.034
Alcohol	1.03	1.01-1.05	0.011
Female gender	0.27	0.05-1.55	0.140
MCP1	1.20	0.37-3.87	0.76
CCR2	0.74	0.13-4.12	0.73
TGF β	0.58	0.09-3.52	0.553
hOGG1	1.50	0.47-4.77	0.492
IL10-1082	1.89	0.48-7.44	0.363
IL10-592	0.98	0.30-3.21	0.980
TLR4 299	1.17	0.77-7.79	0.868
TLR4 399	1.24	0.19-8.19	0.824

MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: interleukin-10; TGF- β : Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

tion. Added to this is the fact that many patients have no indication, or wish to undergo a liver biopsy, it is clear that establishing a sizable cohort of subjects for study is difficult. Our group of 245 C282Y homozygous patients not only had liver biopsy data available but had also provided blood samples for extraction and storage of DNA. This is likely to represent one of the largest groups in the international literature and represents a recruitment period spanning decades, including the pre-HFE era when liver biopsies were used for diagnosis of the disease. This allowed inclusion of patients with early and late stage disease thus avoiding a recruitment bias. In order to allow conclusions regarding the role of genetic polymorphisms, data are also needed about other factors. We have included information from this group relating to iron grade, age, alcohol and gender and used this information to perform multivariate analyses con-

sidering gene-environment interactions.

We selected biologically plausible genes for analysis of functionally significant polymorphisms based on known mechanisms of hepatic injury and liver fibrosis and considering the results of previous studies. This approach has been used to describe the role of SNPs in many polygenic diseases although it does risk returning a null result. Genome wide association studies have since become an alternative approach which removes any need for a mechanistic approach to gene selection however these studies require very large patient cohorts in order to describe associations with a small effect on disease.

We did not find a role for the single nucleotide polymorphisms studied in genes coding MCP-1, TGF- β , IL10, OGG1, TLR4 or CCR2 when considered in univariate analysis with fibrosis stage or when incorporated in multivariate analysis including gender, iron loading, alcohol consumption and age. Genotyping was performed using high resolution melt analysis with confirmation of grouping controls with sequencing. Data were considered in several different analyses with the outcome variables of liver fibrosis grouped as being either present or absent, minimal or advanced and finally as an ordered logistic regression approach (F0-F4). Likewise, the predictor variables (genetic polymorphisms) were considered in all possible combinations but none proved to have a statistically significant association with the outcome variable. We particularly examined those patients where there is greatest variability in liver fibrosis despite significant iron loading. We used the previously published HIC threshold for cirrhosis in this cohort, *i.e.*, HIC > 236 $\mu\text{mol/g dry weight}$ ^[59], and compared the groups with no fibrosis (F0) against those with advanced fibrosis (F3/F4) who had this significant level of hepatic iron deposition. We hypothesized that these patients are most likely to have other factors accounting for disease progression; however, it was still evident that there was no significant

effect seen in any of the genes tested. In this subgroup analysis of patients with significant iron loading, it is apparent that alcohol consumption and age remain the important determinants of liver disease and this is consistent with previous studies. Although our overall study size is large, it must be noted that this subgroup with very heavy iron loading is much smaller and it is possible that this accounts for the lack of association.

Our results are not consistent with previous studies which have been performed in smaller cohorts. A European study reported that the TLR4 Asp299Gly gene polymorphism modulated phenotypic expression of haemochromatosis and described the effect on both liver histology and on an amalgamated clinical expression including liver disease, arthropathy, joint disease, cardiomyopathy and endocrine disease^[28]. The grouping of such diverse types of clinical expression is unusual when considering candidate gene testing and one may wonder whether this is biologically plausible. This study included 99 patients but of these, only 52 had histology available and 29 of these had liver iron quantification. Although allele frequencies were similar to our cohort, we did not replicate the SNP association with liver disease in a group almost five times larger and suspect that the European results may represent a type 1 error.

Similarly, a previous investigation into the role of TGF- β mutations in 149 biopsied haemochromatosis patients concluded that those with the proline substitution at codon 25 were more likely to be grouped into an outcome variable of cirrhosis (F4) *vs* all other stages (F0-3). This grouping could be considered somewhat arbitrary and it would be interesting to know the genotype frequencies across other fibrosis stages. A recent meta-analysis considering the role of TGF- β polymorphisms in liver disease (mainly viral hepatitis) concluded a lack of effect upon fibrosis progression which is in keeping with our results^[63].

There are substantial difficulties in studying disorders with polygenic and environmental interactions. Future research directions may involve non-targeted analysis of either whole genomes or exomes but this is likely to require greater numbers of subjects who have been well characterised in terms of liver disease and co-factors. Collaborations between research institutions would allow more patients to be involved in such studies but newer non-invasive tests of liver fibrosis such as transient elastography will allow assessment for liver disease in almost all patients and capture a much greater proportion of C282Y homozygous subjects in such studies.

In conclusion, the role of chemokines, chemokine receptors, oxidative stress and inflammatory mediators in fibrogenesis in haemochromatosis is established; however, the influence of genetic polymorphisms in the molecules studied here is less clear. In contrast to other published associations, in our large, well-characterised group of C282Y homozygous subjects we did not demonstrate any relationship between *MCP1*, *CCR2*, *TGF β* , *IL10*, *OGG1* and *TLR4* single nucleotide polymorphisms and

hepatic fibrosis or cirrhosis. Future studies utilising techniques such as exome sequencing may provide a better approach to identify genetic polymorphisms associated with hepatic fibrosis progression in haemochromatosis.

COMMENTS

Background

Hereditary haemochromatosis can lead to liver fibrosis and cirrhosis however not all patients with iron loading develop this complication. It is thought that genetic polymorphisms influence this process however previously reported studies may have had methodological flaws.

Research frontiers

Factors associated with an increased risk of hepatic fibrogenesis have been the subject of investigation and many of the clinical cofactors are now established. Genetic factors have proven more difficult to determine although international collaborations investigating this area are ongoing. This is one of the largest cohorts of C282Y homozygous patients studied in this field.

Innovations and breakthroughs

The cohort studied in this paper is large and carefully characterised which allows us to accurately test the relationship between genetic polymorphisms, cofactors for liver injury and fibrosis stage. Authors have tested polymorphisms in molecules related to inflammation and hepatic fibrogenesis and found no significant relationship which is in contrast to previous papers. Their work suggests that further well designed studies are needed to determine what genetic factors influence fibrosis in iron loading.

Applications

By understanding the molecular differences between patients who develop progressive liver disease and those who don't, they may eventually be able to develop therapeutic targets to modify disease development. Patients could also expect more personalised prognostic information and this may allow better informed treatment decisions.

Terminology

Hereditary haemochromatosis due to homozygosity in the C282Y substitution in *HFE* is a genetic disorder seen in those of Northern European ancestry. It is one of the most common genetic disorders in this population and can lead to heavy iron loading in the liver and liver scarring (cirrhosis). The molecules studied in this paper are thought to be involved in mediating inflammation, signalling to fibrosis-producing cells or repair of oxidative stress within the liver.

Peer review

The manuscript reports the lack of association between hepatic fibrosis risk and polymorphisms in the genes encoding toll-like receptor 4, transforming growth factor-beta, in a relatively large cohort of hemochromatosis patients. These results are not consistent with previous findings, which were obtained with error prone smaller cohorts of patients. The methodology employed here is appropriate and the paper is well written. The discussion puts the negative findings into context. The conclusions will be of interest to researchers and clinicians in the field of gastroenterology.

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