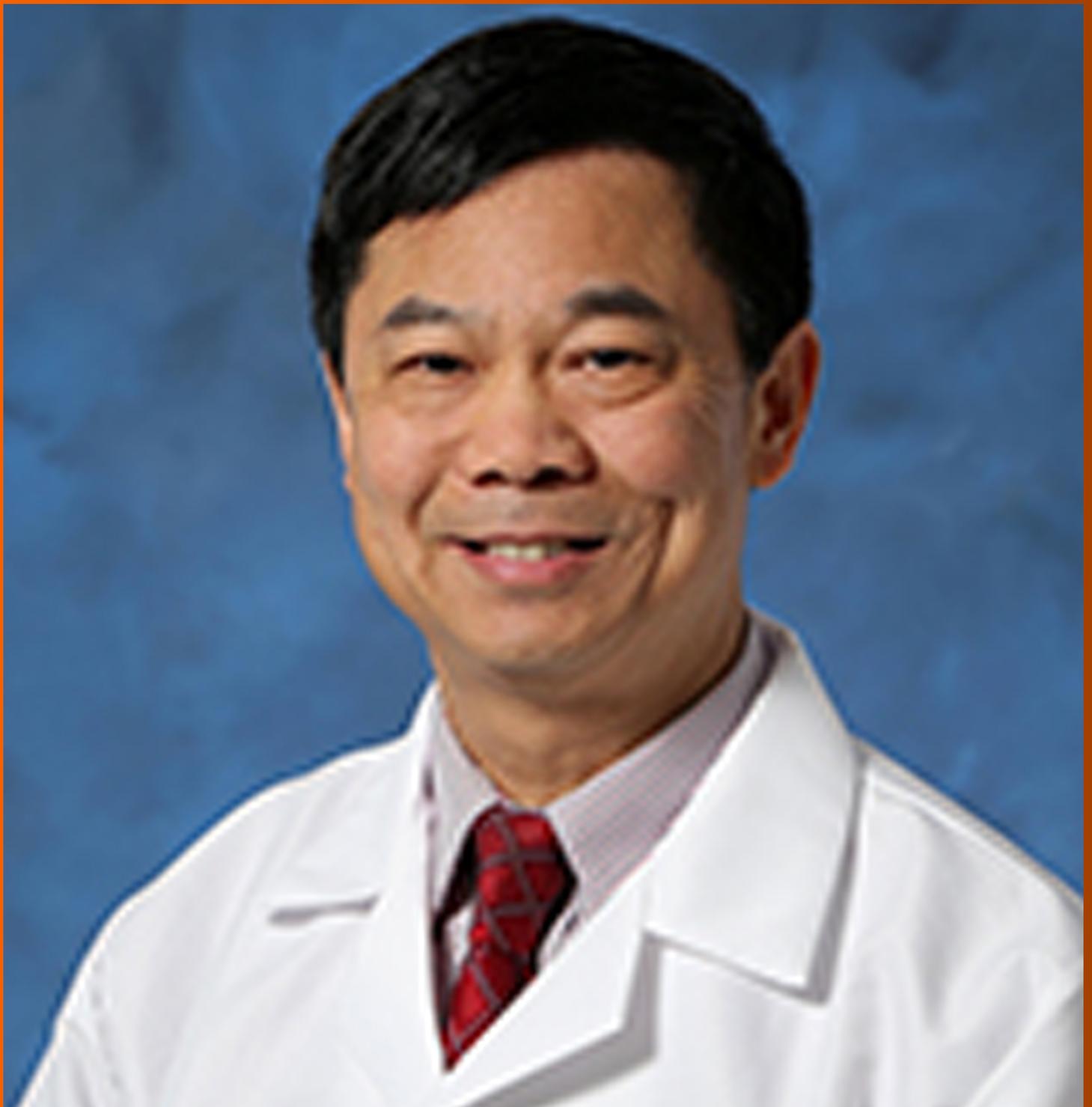


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Case Control Study

Comprehensive analysis of *HFE* gene in hereditary hemochromatosis and in diseases associated with acquired iron overload

Wagner Narciso de Campos, Juliana Doblas Massaro, Eduardo Luiz Rachid Caçado, Cláudia Emília Vieira Wiesel, Aguinaldo Luiz Simões, Andreza Correa Teixeira, Fernanda Fernandes de Souza, Celso Teixeira Mendes-Junior, Ana de Lourdes Candolo Martinelli, Eduardo Antônio Donadi

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Abstract**BACKGROUND**

Patients with hepatitis C virus (HCV) and hepatocellular carcinoma (HCC) may or not develop iron overload (IO), which is associated with worst prognosis, because can cause serious damage to organs. *HFE* gene controls the iron uptake from gut, particularly in patients with hereditary hemochromatosis (HH).

AIM

To identify associations between *HFE* coding region in patients exhibiting hereditary hemochromatosis and in diseases associated with acquired IO.

outpatients followed-up at gastroenterology clinics; Donadi EA mentor of the research, provided financial support, technical facilities and performed final review.

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statement: This study was approved by the local Ethics Research Committee, process number HCRP 4822/2011. The samples are deposited in the Bank of Samples of the Nucleus in Research in Immunogenetics (BAMPI), process number HCFMRP 3530/2007, under the coordination of Eduardo Antônio Donadi, and in the Bank of Samples HCFMRP 3416/2003, under the responsibility of Ana de Lourdes Candolo Martinelli.

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METHODS

We sequenced exons 2 to 5 and boundary introns of *HFE* gene, evaluating all polymorphic sites in patients presenting hereditary (hemochromatosis) or acquired iron overload HCV and HCC) and in healthy controls, using Sanger sequencing. We also determined the ensemble of extended haplotype in healthy control individuals, including several major histocompatibility complex loci, using sequence specific probes. Haplotype reconstruction was performed using the Arlequin and Phase softwares, and linkage disequilibrium (LD) between histocompatibility loci and *HFE* gene was performed using the Haploview software.

RESULTS

The *HFE**003 allele was overrepresented ($f = 71\%$) and *HFE**001 allele was underrepresented ($f = 14\%$) in HH patients compared to all groups. A strong linkage disequilibrium was observed among the *H63D-G*, *IVS2(+4)-C* and *C282Y-G* gene variants, particularly in HH; however, the mutation *IVS2(+4)T>C* was not directly associated with HH susceptibility. The *HFE**001/*HFE**002 genotype conferred susceptibility to HCC in HCV patients exhibiting IO ($P = 0.02$, OR = 14.14). Although *HFE* is telomeric to other histocompatibility genes, the *H63D-G/IVS2(+4)-C* ($P \leq 0.00001/P \leq 0.0057$) combination was in LD with *HLA-B*44* allele group in healthy controls. No LD was observed between *HFE* alleles and other major histocompatibility loci.

CONCLUSION

A differential *HFE* association was observed for HH and for diseases associated with acquired IO (HCV, HCC). Since *HFE* is very distant from other histocompatibility loci, only weak associations were observed with these alleles.

Key words: *HFE* gene; Hepatocellular carcinoma; Hepatitis C; Hemochromatosis hereditary; Alleles; Haplotypes

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Core tip: Patients with hepatitis C virus (HCV) and hepatocellular carcinoma (HCC) may or not develop iron overload (IO), which is associated with worst prognosis. The sequencing of the *HFE* gene permitted to assemble the previously described variation sites (*H63DC>G-*, *S65CA>T* and *C282YG>A*) associated with hereditary hemochromatosis into *HFE* haplotypes, under the standardized HLA nomenclature. A differential association of *HFE* alleles was observed for hereditary and acquired IO (HCV, HCC). In addition to the *HFE* gene, we also typed other major histocompatibility loci (*HLA-A/-B/-C/DRB1/-DQB1*, and *HLA-G 14bp INDEL* and *TNFA-d* microsatellites) in the healthy population to understand how the *HFE* gene variability is associated with these loci.

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INTRODUCTION

The *HFE* gene has seven exons and five introns, which code the α -heavy chain of the molecule. Exon 1 codes the signal peptide, exons 2-4 encode the $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains, exons 5 the transmembrane domains, and the 5' portion of exon 6 the cytoplasmic tail^[1]. Considering that *HFE* gene controls the iron uptake from gut, defects of the encoded molecule have been associated with iron overload (IO), particularly in hemochromatosis hereditary (HH). Major variation sites observed at exons 2 to 4 have been associated with HH, including the *H63DC>G* (exon 2),

S65CA>T (exon 2) and C282YG>A (exon 4) variants^[2]. However, not all HH patients exhibit these mutations^[1].

Besides HH, some acquired liver disorders have been associated with IO and fibrosis, including chronic hepatitis C virus (HCV), cirrhosis and hepatocellular carcinoma (HCC)^[3]. The C282Y-A allele is associated with high iron serum levels, increased hepatic iron content and advanced fibrosis in HCV patients. Increased frequency of the classical HFE mutations has also been reported for HCC patients^[4].

We sequenced exons 2 to 5 and boundary introns in HH patients, HCV patients presenting or not IO, and HCC patients exhibiting or not chronic HCV infection to associate with iron overload. We also evaluated the linkage disequilibrium (LD) between the HFE and HLA-A, HLA-B, HLA-C, HLA-DRB1 and DQB1 genes, as well as HLA-G 14bp INDEL and TNFa-d microsatellites to understand the association between HFE alleles and other major histocompatibility genes.

MATERIALS AND METHODS

This study was approved by the local Ethics Research Committee (Process HCRP-FMRP, USP n° 4822/2011), and informed consent was obtained from all participants.

Subjects

A total of 204 patients followed-up at Gastroenterology Units of University Hospitals of the University of São Paulo (USP) were studied: (1) 14 patients (9 men) aged 32-81 years (55.35 ± 15.16) exhibited HH, defined by high transferrin saturation ($\geq 45\%$) and liver IO in the absence of secondary causes; (2) 130 patients with HCV (93 men) aged 19-69 years (42.60 ± 10.98), exhibiting (71 patients, 57 men) or not IO (59 patients, 36 men) (HCV-IO⁺ and HCV-IO⁻, respectively) in the absence of chronic alcohol ingestion (> 60 g/d). All patients exhibited IgG antibody against recombinant HCV antigens by second-generation ELISA (Abbott, Chicago, IL) for at least 6 mo and positive serum HCV RNA (Roche Diagnostic Systems, Branchburg, NJ). Serum levels of liver enzymes, iron, ferritin, and transferrin saturation were also determined. Liver specimens were scored for necroinflammatory activity, as previously described by Desmet *et al.*^[4]. Iron deposits were assessed and scored on the basis of the amount and cellular/lobular location^[4,5]; and (3) 60 patients (43 men) aged 14-78 years (57 ± 14) exhibiting HCC, of whom 24 (18 men) presented IO and chronic hepatitis C (HCC HCV-IO⁺), and 36 (25 men) presented several underlying disorders including cryptogenic hepatitis, hepatitis B, non-alcoholic steatohepatitis and other comorbidities. Since there is no need for liver biopsy for HCC diagnosis, liver iron was not screened in these patients (HCC-IO[?]). The diagnosis of HCC was performed according to Bruix and Sherman^[6].

Iron overload was defined when iron deposits were detected in liver biopsy using Perl's iron staining^[7,8] and/or when serum transferrin saturation was higher than or equal to 45% with or without elevated ferritin. Patients presenting other types of congenital, virus or autoimmune liver disorders were excluded.

A total of 100 healthy unrelated blood donors (CTL), 80 men, and aged 20-52 years (33.31 ± 8.18) was also studied.

HFE typing

Exons 2 to 5 and boundary introns were evaluated using Sanger sequencing^[9] (Figure 1). HFE nucleotide variations were retrieved from the NCBI (NC_000006.12) and Ensembl (ENSG00000010704) databases. Primer sequences, amplification conditions and allele nomenclature were defined as previously reported^[10]. Sequencing was performed using an ABI 3500 sequencer (Applied Biosystems, Foster City, CA).

Major histocompatibility complex loci typing

HLA-A/-B/-C/-DRB1 and -DQB1 typing was performed using commercial kits (One-Lambda, Canoga Park, CA). HLA-G 14bp INDEL^[11] and TNFa-d microsatellites^[12] were typed as previously described. Haplotype inferences combining major histocompatibility genes were performed only for healthy controls.

Statistical analysis

Allelic and genotype frequencies (f), Hardy Weinberg Equilibrium (HWE), Fisher exact test, and linkage disequilibrium (LD) were performed using the GENEPOP v.4.2 and ARLEQUIN v.3.1 softwares. Image map of the pairwise LD parameters [Log of the Odds (LOD) and Linkage Disequilibrium Coefficient (D')] was generated using the HAPLOVIEW v.3.32 software.

Extended major histocompatibility alleles were reconstructed by means of the EM (ARLEQUIN) and PHASE v.2 algorithms. For all situations, P values ≤ 0.05 were

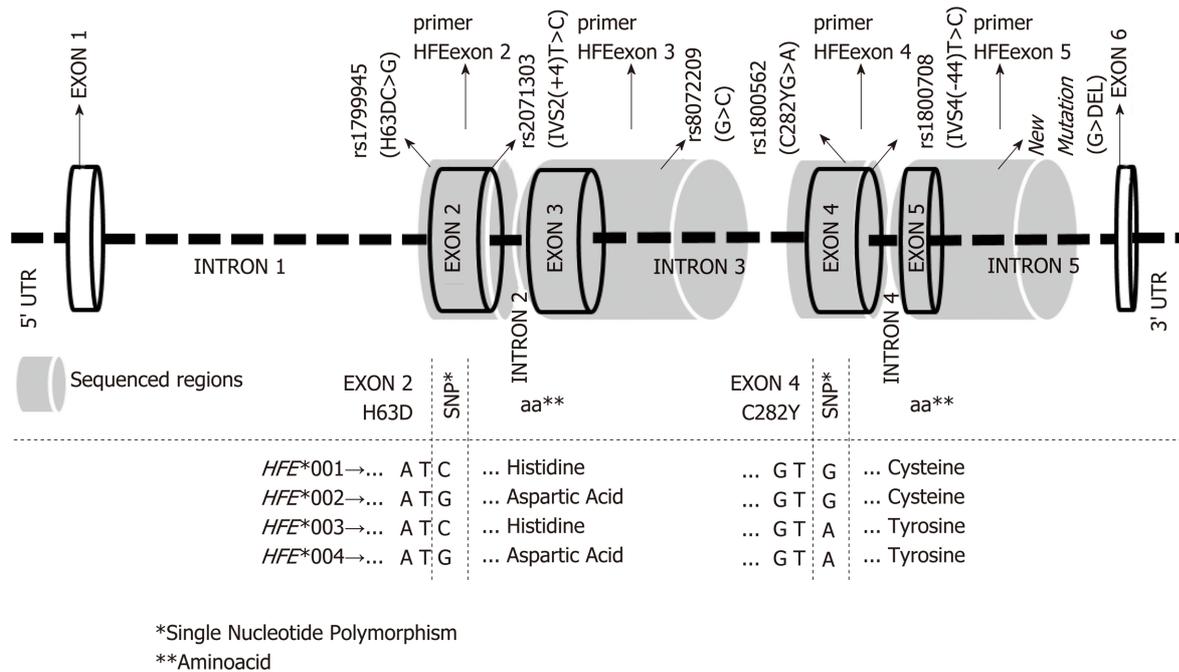


Figure 1 Structure of the *HFE* gene (ID# ENSG0000010704 - <http://www.ensembl.org>) at chromosome region 6p21.3, showing the reference number (rs) of variation sites (NCBI Data base - <http://www.ncbi.nlm.nih.gov/snp>), previously associated with iron disorders. Shaded grey areas indicate the sequenced gene regions and the respective pairs of primers, as previously described^[10]. The combination of these variation sites, translated into the official nomenclature for *HFE* alleles is also shown in the bottom chart; i.e., the combination of the triplet bases and respective encoded residues of the two most important mutations (H63DC>G and C282YG>A) that defined the four major *HFE* allele groups (mutated bases are shown in bold type). ¹Single nucleotide polymorphism; ²Aminoacid.

considered to be significant.

RESULTS

The results regarding *HFE* alleles are presented in two forms: (1) as previously reported in the literature, including the single nucleotide polymorphism (SNP) reference number (rs), the usual SNP names (H63DC>G, C282YG>A, IVS2(+4)T>C and IVS4(-44)T>C) and new variation sites (Table 1); and (2) as the newly described official *HFE* allele nomenclature (Table 2)^[10]. The location of the previously reported variation sites with respect to the nucleotide sequence that defined the new *HFE* nomenclature is illustrated in Figure 1.

HFE alleles and genotypes according to previously described variation sites

All population samples adhered to the HWE, except HCC patients (IO⁺) at the C282YG>A variation site ($P = 0.031$). Overall, patients and healthy controls shared the same most frequent alleles at each SNP, except when HH patients were compared to healthy controls, for whom the C282Y-A ($f = 0.714$) allele was the most frequently observed, significantly associated with susceptibility to HH ($P < 0.001$; OR = 53.06; 95% CI: 18.41-152.90). The C282Y-G allele was protective against HH ($P < 0.001$; OR = 0.01; 95% CI: 0.006-0.05). On the other hand, when the genotype frequencies were compared between HH patients and healthy controls several differences were observed. The IVS2(+4)-TT genotype was associated with susceptibility to HH ($P = 0.04$, OR = 3.91; 95% CI: 1.14-13.34). The C282Y-GG genotype was associated with protection against HH ($P < 0.001$; OR = 0.007; 95% CI: 0.0008-0.065), while the C282Y-AA genotype was associated with susceptibility to HH ($P < 0.001$; OR = 201.00; 95% CI: 10.44-3,871) (Table 1).

The most remarkable LD among these loci included: (1) H63DC>G and IVS2(+4)T>C in almost all groups analyzed separately and in the whole population; (2) IVS2(+4)T>C and IVS4(-44)T>C in most patient samples; and (3) IVS2(+4)T>C and C282YG>A in the HH population (Table S1). The Haploview software was used to analyze and visualize the patterns of linkage disequilibrium observed in these data and confirmed the strong LD between H63DC>G and IVS2(+4)T>C ($D' = 95$) and IVS2(+4)T>C and IVS4(-44)T>C ($D' = 90$), and a less strong linkage between IVS2(+4)T>C and C282YG>A ($D' = 77$) (Figure 2). Therefore, the most relevant SNPs

Table 1 The single nucleotide polymorphism reference number (rs), the usual single nucleotide polymorphism names (H63DC>G, C282YG>A, IVS2(+4)T>C and IVS4(-44)T>C) and new variation sites

SNPs	Allele/genotype	HH	HCV-IO ⁺	HCV-IO ⁻	HCC HCV-IO ⁺	HCC-IO [?]	CTL
H63DC>G (rs1799945) [+3511]	C	0.893	0.859	0.864	0.805	0.921	0.825
	G	0.107	0.141	0.136	0.195	0.079	0.175
	GG	0.000	0.042	0.000	0.024	0.000	0.030
	CG	0.214	0.197	0.271	0.341	0.158	0.290
	CC	0.786	0.761	0.729	0.634	0.842	0.680
	HW <i>P</i> value	1.000	1.000	0.580	1.000	1.000	1.000
IVS2(+4)T>C (rs2071303) [+3668]	T	0.857	0.641	0.669	0.585	0.684	0.610
	C	0.143	0.359	0.331	0.415	0.316	0.390
	TT	0.714 ¹	0.408	0.424	0.390	0.526	0.390
	TC	0.286	0.465	0.492	0.390	0.316	0.440
	CC	0.000	0.127	0.085	0.220	0.158	0.170
	HW <i>P</i> value	0.528	1.000	0.556	0.212	0.295	0.528
G>C (rs807209) [+5197]	G	0.000	0.007	0.017	0.000	0.053	0.035
	C	1.000	0.993	0.983	1.000	0.947	0.965
	GG	0.000	0.000	0.000	0.000	0.000	0.010
	GC	0.000	0.014	0.034	0.000	0.105	0.050
	CC	1.000	0.986	0.966	1.000	0.895	0.940
	HW <i>P</i> value	-	-	1.000	-	1.000	0.103
C282YG>A (rs1800562) [+5473]	G	0.286 ²	0.979	0.983	0.902	1.000	0.955
	A	0.714 ²	0.021	0.017	0.098	0.000	0.045
	GG	0.071 ²	0.958	0.966	0.854	1.000	0.910
	GA	0.429	0.042	0.034	0.098	0.000	0.090
	AA	0.500 ²	0.000	0.000	0.049	0.000	0.000
	HW <i>P</i> value	1.000	1.000	1.000	0.031	-	1.000
IVS4(-44)T>C (rs1800708) [+5635]	T	1.000	0.880	0.907	0.817	0.842	0.925
	C	0.000	0.120	0.093	0.183	0.158	0.075
	CC	0.000	0.014	0.000	0.024	0.000	0.000
	TC	0.000	0.211	0.186	0.317	0.316	0.150
	TT	1.000	0.775	0.814	0.659	0.684	0.850
	HW <i>P</i> value	1.000	1.000	1.000	1.000	1.000	1.000
New mutation (G>Del) at intron 5 [+5811]	G	1.000	1.000	1.000	1.000	1.000	0.995
	Del	0.000	0.000	0.000	0.000	0.000	0.005
	GG	1.000	1.000	1.000	1.000	1.000	0.990
	G Del	0.000	0.000	0.000	0.000	0.000	0.010
	Del Del	0.000	0.000	0.000	0.000	0.000	0.000
	HW <i>P</i> value	-	-	-	-	-	-

Frequency of single nucleotide polymorphism (SNP) alleles and genotypes observed at the *HFE* coding region (ordered from 5' to 3') in patients with hereditary hemochromatosis (HH), hepatitis C exhibiting (HCV-IO⁺) or not (HCV-IO⁻) iron overload, hepatocellular carcinoma and hepatitis C plus iron overload (HCC HCV-IO⁺), hepatocellular carcinoma caused by diverse etiologies other than HCV and without information regarding iron overload (HCC-IO[?]), and in healthy control individuals (CTL). The reference SNP numbers (rs) and the position SNP base [] are also shown for the previously described *HFE* variation sites and were assigned according to NCBI (<http://www.ncbi.nlm.nih.gov>) and Ensembl (<http://www.ensembl.org>) databases. Significant Fisher's exact test *P* values (≤ 0.05) and Hardy-Weinberg equilibrium adherence are shown in table.

¹HH x CTL: TT (*P* = 0.04; OR = 3.91; 95%CI: 1.14-13.34);

²HH x CTL: G (*P* < 0.001; OR = 0.01; 95%CI: 0.006-0.05), A (*P* < 0.001; OR = 53.06; 95%CI: 18.41-152.90), GG (*P* < 0.001; OR = 0.007; 95%CI: 0.0008-0.065) and AA (*P* < 0.001; OR = 201.00; 95%CI: 10.44-3.871). The most frequent allele/genotype different of healthy controls. In italics: alleles and genotypes that presented statistically significant values.

in LD with each other were H63DC>G, IVS2(+4)T>C, IVS4(-44)T>C and C282YG>A. Considering that: (1) H63DC>G and IVS2(+4)T>C were in LD in almost all analyses; (2) H63DC>G and C282YG>A presented LD only in HH patients; and (3) H63DC>G and C282YG>A polymorphic sites were frequently associated with susceptibility to HH in the literature, a third LD approach was performed, analyzing only HH and healthy control individuals to evaluate specifically-linked alleles and the strength of these associations. Accordingly, in both healthy controls and HH populations, a

Table 2 HFE coding region allele frequency in individuals exhibiting congenital or acquired iron overload and healthy control population

Allele	SNPs sequences	Population frequencies (f)															
		IO ⁻				IO ⁺				IO ²		Whole					
		1-2-3-4-5-6 ³		CTL	HCV-IO ⁻	HCV-IO ⁺	HCC HCV-IO ⁺	HCC HCV-IO ⁺	HH	TOTAL	HCC-IO ²	n = 304	%				
n =	%	n =	%	n =	%	n =	%	n =	%	n =	%	n =	%				
		100		59		71		24		28		109		36			
HFE*001 ⁴	C-T-C-G-T-G	107	0.54	74	0.63	86	0.61	23	0.48	4 ²	0.14	113	0.52	40	0.56	334	0.55
HFE*001:unofficial:02 ⁵	C-C-C-G-T-G	28	0.14	12	0.10	15	0.11	3	0.06	1	0.04	19	0.09	4	0.06	63	0.10
HFE*001:unofficial:03 ³	C-T-G-G-T-G	8	0.04	2	0.02	1	0.01	0	0.00	0	0.00	1	0.00	2	0.03	12	0.02
HFE*001:unofficial:04 ⁵	C-T-C-G-C-G	0	0.00	0	0.00	1	0.01	1	0.02	0	0.00	2	0.01	1	0.01	3	<0.01
HFE*001:unofficial:05 ⁵	C-C-C-G-C-G	13	0.07	11	0.09	16	0.11	7 ¹	0.15	0	0.00	23	0.11	12	0.17	59	0.10
HFE*001:unofficial:06 ⁵	C-C-G-G-T-Del	1	0.01	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	<0.01
HFE*002 ⁴	G-C-C-G-T-G	35	0.17	16	0.13	20	0.14	9 ¹	0.19	3	0.11	32	0.15	8	0.11	92	0.15
HFE*003 ⁴	C-T-C-A-T-G	8	0.04	3	0.02	3	0.02	3	0.06	20 ²	0.71	26	0.12	4	0.06	40	0.06
HFE*004 ⁴	G-C-C-A-T-G	0	0.00	0	0.00	0	0.00	1	0.02	0	0.00	1	0.00	0	0.00	1	<0.01
Number of alleles		200		118		142		48		28		218		72		608	

Patients with hereditary hemochromatosis (HH), patients with hepatitis C exhibiting (HCV-IO⁺) or not (HCV-IO⁻) iron overload, hepatocellular carcinoma (HCC) plus HCV-IO⁺, HCC caused by diverse etiologies other than HCV and without information regarding iron overload (HCC-IO²), and population healthy control individuals (CTL).

¹HCC HCV-IO⁺ x CTL: HFE*001:unofficial:06/HFE*002 ($P = 0.02$; OR = 14.14; 95%CI: 1.40-142.80);

²HH x CTL: HFE*001 ($P < 0.001$; OR = 0.14; 95%CI: 0.04-0.43), HFE*003 ($P < 0.001$; OR = 60.00; 95%CI: 20.31-177.20), HFE*001/HFE*003 ($P = 0.03$; OR = 7.20; 95%CI: 1.40-36.85), HFE*003/HFE*003 ($P < 0.001$; OR = 174.20; 95%CI: 8.92-400.00);

³ Order of base changes for each single nucleotide polymorphism (SNP) observed, encompassing H63DC>G; IVS2(+4)T>C; rs807209G>C; C282YG>A; IVS4(-44)T>C; new deletionG>DEL (5' to 3');

⁴ Alleles recognized by ImMunoGeneTics information system - IMGT;

⁵ Alleles in validation process.

LD of H63D-G and IVS2(+4)-C was detected as well as an absence of the recombinant H63D-G in linkage with the IVS2(+4)-T. Another relevant result was the linkage of both mutant H63D-G and IVS(+4)-C mutants with the C282Y-G ($D' = 1.000$ in both analyses, and $r^2 = 0.3000$ and 0.4267 , respectively) (Table S2).

HFE alleles and genotypes using the HFE nomenclature

The reconstruction of the meiotic phase generated nine alleles, included into four major allele groups (HFE*001 to *004), as standardized by IMGT^[10]. These allele groups encoded four distinct proteins (HFE*001 to *004) on the basis of polymorphic sites along the coding region, encompassing the H63DC>G (exon 2), IVS2(+4)T>C (intron 2), rs807209 (G>C intron 3), C282YG>A (exon 4) IVS4(-44)T>C (intron 4) and the new mutation (G>DEL at intron 5) (Table 2).

The HFE*001:01:01 was the most frequently observed allele in all studied populations (f varying from 48-63%), except in HH patients ($f = 14%$). In contrast, the HFE*003 allele was underrepresented in all studied populations (f varying from 2%-12%), except in HH patients ($f = 71%$). Therefore, the HFE*001, containing the H63D-C; IVS2(+4)-T; rs807209-C; C282Y-G; IVS4(-44)-T variation sites (from 5' to 3'), conferred protection against the development of HH ($P < 0.0001$, OR = 0.14) and the HFE*003 allele, containing the H63D-C; IVS2(+4)-T; rs807209-C; C282Y-A; IVS4(-44)-T (from 5' to 3'), conferred a high risk for HH development ($P < 0.0001$, OR = 60.00). The HFE*001/HFE*003 ($P = 0.03$, OR = 7.2) and HFE*003/HFE*003 ($P < 0.001$, OR = 174.20) genotypes, both containing the HFE*003 allele, were also overrepresented in HH patients. On the other hand, the HFE*001/HFE*002 genotype was associated with the development of HCC ($P = 0.02$, OR = 14.14) in patients exhibiting the underlying HCV infection and iron overload (HCC HCV-IO⁺).

Linkage disequilibrium between other major histocompatibility complex genes and HFE

The major histocompatibility complex (MHC) LD analysis was performed using two approaches: (1) considering HFE alleles (Table S3); and (2) considering separately the two HFE SNPs most frequently reported in association with HH (H63DC>G and

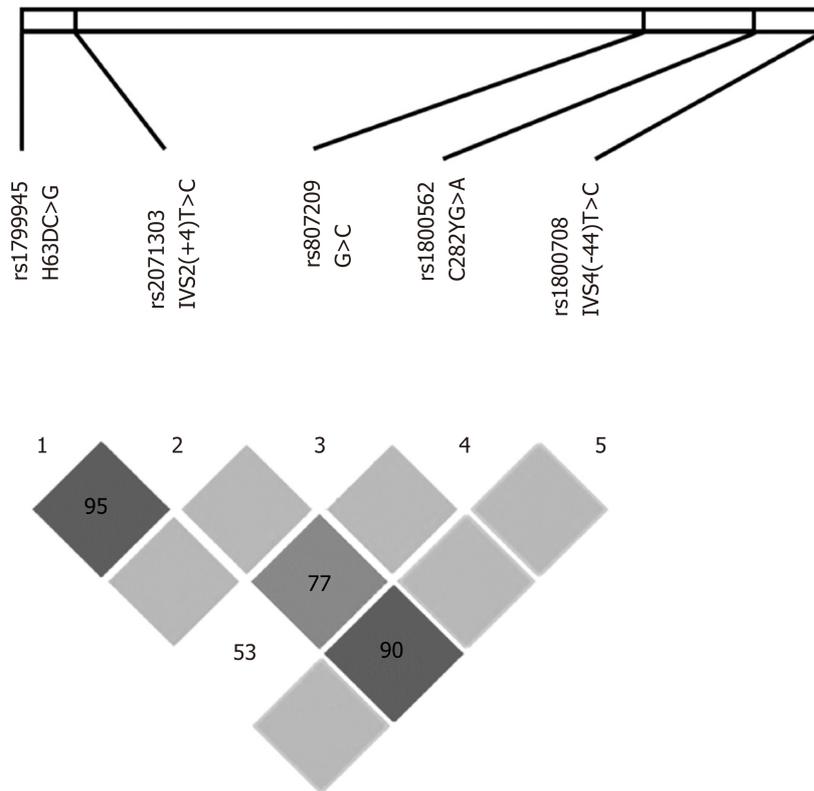


Figure 2 Linkage disequilibrium among single nucleotide polymorphisms observed along the coding region of the *HFE* gene. Areas in dark gray represent strong linkage disequilibrium (LD) ($LOD \geq 2$ and $D' = 1$), medium gray indicates moderate LD ($LOD \geq 2$, $D' < 1$), light gray indicates weak LD ($LOD < 2$, $D' = 1$); and white indicates no LD ($LOD < 2$, $D' < 1$). Values of D' different from 1.00 are represented as a percentage within the square. LOD: Log of the odds; D' : Linkage disequilibrium coefficient.

The major histocompatibility complex (MHC) LD analysis was performed using two approaches: (1) considering *HFE* alleles (Table S3); and (2) considering separately the two *HFE* SNPs most frequently reported in association with HH (H63DC>G and C282Y G>A) (Table 3). Considering the first approach, no LD was observed between *HFE* alleles and MHC alleles, except for H63DC>G and *HLA-B* locus ($P = 0.03$), showing a weak association between H63DC>G and *HLA-B*44* (Table 3). We also observed an absence of LD between the classical C282Y G>A SNP and *HLA-A*, *HLA-B*, *HLA-C*, 14bp *HLA-G*, *TNFA*-d microsatellites. Since the variation site IVS2(+4)T>C is located only 157bp downstream from the H63DC>G site and since these loci are in LD, the IVS2(+4)T>C would be a good candidate to be analyzed regarding the disequilibrium between *HFE* and *HLA-B* genes (Table 4). The weak *HLA-B* associations were confirmed.

DISCUSSION

Individual *HFE* gene variation sites

The frequency of the H63D-G allele in healthy controls varies from 7.9% to 17.5% in worldwide populations, exhibiting high frequencies in Netherlands and Iberian Peninsula (around 20%)^[13,14]. The frequency of the C282Y-A allele decreases from North (4%-10%) to South Europe (0%-3%)^[15], and in populations without a high European genetic ancestry, the frequency of this allele is negligible. The frequency of the C282Y-A allele in our healthy control series, as well as in other Southern Brazilian samples^[16-18], is closely similar to South European populations, indicating the European ancestry influenced on the Brazilian gene pool. The mutant S65C-T allele is observed at low frequency (0-1%) in European populations^[19-21], as well as in the Brazilian population^[22,23] (absent in our samples - data not shown).

Although the IVS2(+4)T>C SNP does not change protein sequence, it is in LD with H63DC>G, C282Y G>A and IVS2(+4)-T alleles. Considering that IVS2(+4)-T allele is increased in HH population, and considering that this allele is only 157bp distant from

Table 3 Linkage disequilibrium between HLA-B alleles and HFE coding region H63DC>G single nucleotide polymorphism alleles

HLA-B	Observed frequency			Expected frequency		Standardized value of disequilibrium(D')		Standardized value of correlation (r ²)	QUI ² value	P value of QUI ²
	C	G	C	G	C	G				
07	16	2	14.83	3.17	0.3683	-0.3683	0.0029	0.5728	0.4492	
08	12	1	10.71	2.29	0.5626	-0.5626	0.0047	0.9396	0.3324	
13	3	1	3.30	0.70	-0.0899	0.0899	0.0008	0.1547	0.6941	
14	10	0	8.24	1.76	1.0000	-1.0000	0.0113	2.2471	0.1339	
15	17	0	14.01	2.99	1.0000	-1.0000	0.0199	3.9669	0.0464	
18	10	0	8.24	1.76	1.0000	-1.0000	0.0113	2.2471	0.1339	
27	2	2	3.30	0.70	-0.3933	0.3933	0.0149	2.9586	0.0854	
35	17	4	17.31	3.69	-0.0177	0.0177	0.0002	0.0345	0.8526	
37	1	2	2.47	0.53	-0.5955	0.5955	0.0254	5.0617	0.0245	
38	3	0	2.47	0.53	1.0000	-1.0000	0.0033	0.6500	0.4201	
39	4	2	4.94	1.06	-0.1911	0.1911	0.0053	1.0582	0.3036	
40	3	0	2.47	0.53	1.0000	-1.0000	0.0033	0.6500	0.4201	
41	3	0	2.47	0.53	1.0000	-1.0000	0.0033	0.6500	0.4201	
42	1	0	0.82	0.18	1.0000	-1.0000	0.0011	0.2145	0.6433	
44	8	11	15.66	3.34	-0.4891	0.4891	0.1183 ¹	23.5443	< 0.0001	
45	6	0	4.94	1.06	1.0000	-1.0000	0.0066	1.3203	0.2505	
48	2	0	1.65	0.35	1.0000	-1.0000	0.0022	0.4312	0.5114	
49	8	0	6.59	1.41	1.0000	-1.0000	0.0089	1.7788	0.1823	
50	2	0	1.65	0.35	1.0000	-1.0000	0.0022	0.4312	0.5114	
51	9	5	11.54	2.46	-0.2199	0.2199	0.0172	3.4137	0.0647	
52	7	0	5.77	1.23	1.0000	-1.0000	0.0078	1.5484	0.2134	
53	5	0	4.12	0.88	1.0000	-1.0000	0.0055	1.0946	0.2955	
55	2	0	1.65	0.35	1.0000	-1.0000	0.0022	0.4312	0.5114	
56	0	1	0.82	0.18	-1.0000	1.0000	0.0237	4.7094	0.0300	
57	8	2	8.24	1.76	-0.0293	0.0293	0.0002	0.0423	0.8371	
58		2	4.94	1.06	-0.1911	0.1911	0.0053	1.0582	0.3036	
67	1	0	0.82	0.18	1.0000	-1.0000	0.0011	0.2145	0.6433	

¹The higher value of correlation. Identification of single nucleotide polymorphisms and most frequent allele according to NCBI (<http://www.ncbi.nlm.nih.gov>) and Ensembl (<http://www.ensembl.org>): rs1799945 (H63DC>G). Shaded cells are showing significant LD values.

the H63D-G allele, this association probably reflects a hitch-hiking effect, and possibly does not present biological significance in the susceptibility to HH. Indeed, de Lucas *et al*^[24] reported that HH patients presenting homozygosity for the C282Y-A allele did not exhibit the IVS2(+4)-C allele, indicating that the presence of the C282Y-A allele excludes the presence of IVS2(+4)-C allele in the same haplotype. Therefore, the sole analysis of the allelic frequency of the IVS2(+4)T>C SNP is not adequate to evaluate HH susceptibility, since the frequency of the C282Y-A allele is high in HH patients, and consequently, there is a high frequency of IVS2(+4)-T allele in the same sample (Table 1). The C282Y-A allele and the AA genotype have been associated with susceptibility to HH patients^[24,25], including the HH patients of this study and other Brazilian HH populations^[23]. Although the HH cohort is small, the mutated AA genotype appeared in high frequency in patients and was not observed in the healthy control group. The C282Y-G allele and the GG genotype have been associated with protection against HH development in various worldwide populations^[21]. The H63D-G allele and the GG genotype have been associated with HH in European and North American patients^[1,25]. However, these associations were not observed in ours nor in other HH Brazilian samples^[26].

The role of H63DC>G and C282YG>A variation sites in acquired IO disorders is

Table 4 Linkage disequilibrium between HLA-B alleles and HFE coding region IVS2(+4)T>C single nucleotide polymorphism alleles

HLA-B	Observed frequency			Expected frequency	Standardized value of disequilibrium(D')		Standardized value of correlation(r ²)	QUI ² value	P value of QUI ²
	T	C	T		T	C			
				HFE IVS2(+4)T>C					
				C	T	C			
07	13	5	10.98	7.02	0.2877	-0.2877	0.0052	1.0471	0.3062
08	8	5	7.93	5.07	0.0138	-0.0138	0.0000	0.0017	0.9672
13	4	0	2.44	1.56	1.0000	-1.0000	0.0130	2.6096	0.1062
14	4	6	6.10	3.90	-0.3443	0.3443	0.0098	1.9513	0.1624
15	11	6	10.37	6.63	0.0950	-0.0950	0.0005	0.1073	0.7433
18	8	2	6.10	3.90	0.4872	-0.4872	0.0080	1.5973	0.2063
27	1	3	2.44	1.56	-0.5902	0.5902	0.0111	2.2235	0.1359
35	17	4	12.81	8.19	0.5116	-0.5116	0.0196	3.9264	0.0475
37	0	3	1.83	1.17	-1.0000	1.0000	0.0238	4.7638	0.0291
38	2	1	1.83	1.17	0.1453	-0.1453	0.0002	0.0411	0.8393
39	1	5	3.66	2.34	-0.7268	0.7268	0.0256	5.1103	0.0238
40	3	0	1.83	1.17	1.0000	-1.0000	0.0097	1.9472	0.1629
41	2	1	1.83	1.17	0.1453	-0.1453	0.0002	0.0411	0.8393
42	1	0	0.61	0.39	1.0000	-1.0000	0.0032	0.6426	0.4228
44	6	13	11.59	7.41	-0.4823	0.4823	0.0382 ¹	7.6388	0.0057
45	3	3	3.66	2.34	-0.1803	0.1803	0.0016	0.3146	0.5749
48	2	0	1.22	0.78	1.0000	-1.0000	0.0065	1.2916	0.2558
49	8	0	4.88	3.12	1.0000	-1.0000	0.0266	5.3279	0.0210
50	2	0	1.22	0.78	1.0000	-1.0000	0.0065	1.2916	0.2558
51	10	4	8.54	5.46	0.2674	-0.2674	0.0034	0.6882	0.4068
52	3	4	4.27	2.73	-0.2974	0.2974	0.0050	1.0037	0.3164
53	5	0	3.05	1.95	1.0000	-1.0000	0.0164	3.2787	0.0702
55	0	2	1.22	0.78	-1.0000	1.0000	0.0158	3.1598	0.0755
56	1	0	0.61	0.39	1.0000	-1.0000	0.0032	0.6426	0.4228
57	4	6	6.10	3.90	-0.3443	0.3443	0.0098	1.9513	0.1624
58	1	5	3.66	2.34	-0.7268	0.7268	0.0256	5.1103	0.0238
67	1	0	0.61	0.39	1.0000	-1.0000	0.0032	0.6426	0.4228

¹The higher value of correlation. Identification of single nucleotide polymorphisms and most frequent allele according to NCBI (<http://www.ncbi.nlm.nih.gov>) and Ensembl (<http://www.ensembl.org>): rs2071303 (IVS2(+4)T>C). Shaded cells are showing significant LD values.

controversial. Apart from HH, no other association involving such polymorphisms was observed in the present study. A previous study evaluating chronic hepatitis C patients reported an association between HFE mutations (H63DC>G and C282YG>A) and elevated serum transferrin saturation, but not with liver iron deposits^[5]. On the other hand, some authors have observed an increased prevalence of C282YG>A mutation in hepatitis C patients from North England^[27], Austria^[28], and North America^[29]. These studies have shown an association between HFE mutations and higher serum iron indices and liver iron deposits, especially for C282Y homozygotes. In contrast, another study did not show association between HFE mutations and liver iron deposits^[30].

The association between the C282YG>A mutation and the HCC risk is also still controversial. HH is a condition characterized by hepatic IO, leading to higher cancer incidence^[31]. However, the role of moderate liver IO and of the carriage of HFE mutations on the HCC risk remains unclear. Some studies have shown higher prevalence of the C282YG>A mutation in patients with HCC compared with cirrhotic patients without HCC^[32], whereas other studies found no association between HFE and HCC^[33]. Additionally, another study reported an association between liver IO and C282YG>A with a higher risk of HCC in patients with alcoholic but not with HCV-related cirrhosis^[34].

HFE alleles and genotypes

The *HFE**001 allele was underrepresented, while the *HFE**003 was overrepresented in HH patients of this series. These findings corroborate the importance of the C282Y>A SNP on the susceptibility to HH, since only the *HFE**003 allele has an Adenine at this position (C282Y-A), which is the unique difference between both alleles. In addition, the *HFE**001/*HFE**003 and *HFE**003/*HFE**003 genotypes were also significantly associated with high risk for HH development. The homozygosis for the *HFE**003 allele group, which was not observed in the healthy control population, drastically increased the susceptibility to HH. Indeed, the *HFE**003 allele was present in 13 out of 14 patients and its presence in double doses was observed in 7 out of 14 HH patients.

In relation to acquired diseases exhibiting IO, the *HFE**001/*HFE**002 genotype was overrepresented in HCC patients exhibiting HCV infection and IO. When the *HFE* SNPs were analyzed separately, no significant differences were observed. Noteworthy, these results indicate that these populations are heterogeneous and in some circumstances represented small groups.

Extended MHC haplotypes encompassing the HFE SNPs and alleles

HH was initially associated with the *HLA-A3*, *HLA-A14* and *HLA-B14* antigens^[35]. Microsatellite evaluations pointed out a susceptibility locus for HH. This locus was initially named as *HLA-H*^[25], which is the same name of a pseudogene, located close to *HLA-A*, stressing the disequilibrium concept between *HLA-A/B* genes and the HH locus. Later, this HH locus was renamed *HFE* to put an end on this ambiguity^[36]. Considering the great distance between the *HFE* and *HLA-A*, *-B* and *-C* loci, strong LD between these genes are not expected; however, some studies reported LD between H63DC>G and C282Y>A SNPs with *HLA-A* and *HLA-B* alleles. Taking advantage of the fact that our healthy control population was typed for ten additional MHC loci, LD between *HFE* and all these loci was evaluated.

The pairwise test detected no disequilibrium between the *HFE* alleles and other MHC loci (Table S3), which is in agreement with the argument that the *HFE* gene is far from the other loci tested. When LD analyses were performed evaluating the H63DC>G and C282Y>A SNPs, a significant disequilibrium between the H63DC>G and *HLA-B* ($P = 0.03$) was observed, encompassing *HLA-B**15/H63D-C, *HLA-B**37/H63D-G, *HLA-B**44/H63D-G and *HLA-B**56/H63D-G alleles (Table 3), being stronger for *HLA-B**15/H63D-C and *HLA-B**56/H63D-G alleles ($D' = 1$). Since *HLA-B* locus is multiallelic, H63DC>G is biallelic, and H63D-G is rare, it is possible that not all H63DC>G/*HLA-B* haplotypes were represented in our CTL. In addition, the recombination coefficient, which indicates the power of the correlation between alleles, was weak for all these combinations, except for the *HLA-B**44/H63D-G ($r^2 = 0.11$) (Figure 3 and Table 3) which was much stronger than in the other combinations ($r^2 = 0.01-0.02$). Most likely, this *HLA-B**44/H63D-G disequilibrium has a historical origin.

Since the IVS2(+4)T>C SNP exhibited a significant LD with the H63DC>G SNP, as we discussed before, and considering that both SNPs are located at a relatively short distance, we further evaluated the LD between this SNP and *HLA-B*, which showed similar results: *HLA-B**35/IVS2(+4)-T; *HLA-B**37/IVS2(+4)-C; *HLA-B**44/IVS2(+4)-C; *HLA-B**49/IVS2(+4)-T and *HLA-B**58/IVS2(+4)-C. The analyses of LD between *HLA-B* alleles and IVS2(+4)T>C and H63DC>G showed that *HLA-B**37 and *B**44 exhibited weaker correlations in relation to H63DC>G ($r^2 = 0.02$ and 0.03 , respectively) (Table 4). This analysis resulted on the identification of the extended H63D-G/IVS2(+4)-C/*HLA-B**44 haplotype (Figure 3).

Regarding genetic studies in patients with IO, *HLA-B**44 and C282Y-A alleles are reported to be overrepresented in patients with HH^[1] or in patients with acquired diseases associated with IO^[37], however, without reaching significance. Since haplotypes containing *HLA-B**44 are common in Europe, West and North Africa, and in North-American Caucasians^[38], there is a high probability of overrepresentation of the H63D-G/*HLA-B**44 haplotype in these populations. Although the present study revealed that C282Y-A is not a part of this extended haplotype, the mentioned associations suggest an independent role of H63D-G and C282Y-A on HH susceptibility.

In conclusion, this study systematically reports variation sites along the *HFE* gene using *HFE* allelic official nomenclature, previously described by our group. The *HFE**003 was frequently observed in HH patients, whereas the *HFE**001 was frequently observed in healthy controls. The *HFE**001/*HFE**002 genotype was identified as a risk factor for HCC HCV patients exhibiting IO. Even if a strong LD has been observed among the H63D-G, IVS2(+4)-C and C282Y-G alleles, particularly in HH patients, the mutation IVS2(+4)T>C was not directly associated with HH susceptibility. Although the *HFE* gene is distant from other MHC genes, the *HFE*

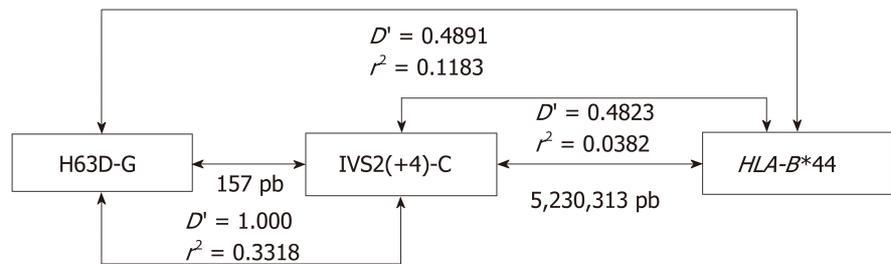


Figure 3 Linkage disequilibrium observed between two relevant *HFE* coding region [H63D>G and IVS2(+4)T>C] single nucleotide polymorphism alleles and *HLA-B* alleles.

H63D-G/IVS2(+4)-C alleles were in weak LD with the *HLA-B*44* allele.

ARTICLE HIGHLIGHTS

Research background

HFE gene controls the iron uptake from gut, and defects of the encoded molecule have been associated with iron overload (IO), particularly in hemochromatosis hereditary (HH), which can cause serious damage to the liver. Besides HH, patients with hepatitis C virus (HCV) and hepatocellular carcinoma (HCC) may or not develop IO.

Research motivation

The search for markers associated with IO may be very useful for the early diagnosis of these patients, which is essential for their survival.

Research objectives

The main objectives of this work is to identify associations between *HFE* coding region variable sites in patients exhibiting HH and in diseases associated with acquired IO.

Research methods

We sequenced exons 2 to 5 and boundary introns of the *HFE* gene to evaluate all polymorphic sites in patients presenting HH or acquired IO (HCV and HCC), and in healthy controls, using Sanger sequencing. We also determined the extended haplotype in healthy controls, including other major histocompatibility genes (*HLA-A/-B/-C/-DRB1/-DQB1* alleles, and *HLA-G* 14bp INDEL and *TNFA-d* microsatellites). Haplotype reconstruction was performed using the Arlequin and Phase softwares, and linkage disequilibrium (LD) between histocompatibility loci and *HFE* gene was performed using the Haploview software.

Research results

The *HFE*003* allele was overrepresented ($f = 71\%$) and *HFE*001* allele was underrepresented ($f = 14\%$) in HH patients compared to all groups. A strong LD was observed among the previously reported H63D-G, IVS2(+4)-C and C282Y-G gene variants, particularly in HH; however, the mutation IVS2(+4)T>C was not associated with HH susceptibility. The *HFE*001/HFE*002* genotype conferred susceptibility to HCC in HCV patients exhibiting IO ($P = 0.02$, OR = 14.14). Although *HFE* is telomeric to other histocompatibility genes, the H63D-G/IVS2(+4)-C ($P \leq 0.00001/P \leq 0.0057$) combination was in LD with *HLA-B*44* allele group in healthy controls.

Research conclusions

This study systematically evaluated variation sites along the *HFE* gene using the HLA official nomenclature, previously described by our group. The *HFE*003* allele that was overrepresented in HH patients encompasses major variation sites previously described in association with HH in several worldwide populations, in contrast with the *HFE*001* allele which does not present HH-associated variation sites and predominates among healthy controls. On the other hand, the *HFE*001/HFE*002* genotype was identified as a risk factor for HCC and HCV patients exhibiting IO. Although the *HFE* gene is distant from other histocompatibility genes, the *HFE* H63D-G/IVS2(+4)-C alleles were in weak LD with the *HLA-B*44* allele. Thus, a differential *HFE* association was observed for HH and for diseases associated with acquired IO (HCV, HCC).

Research perspectives

Besides the identification of markers associated with IO, which may permit an early detection of patients prone to develop iron deposits, the knowledge of the major gene associated with iron uptake may help on the understanding of the IO pathogenesis.

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