Case Control Study

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

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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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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 Haplovew 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)/+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 ≤ 0.0001/ P ≤ 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 worsen 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.

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 α1, α2 and α3 domains, exons 5 the transmembrane domains, and the 5' portion of exon 6 the cytoplasmic tail. 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 \textit{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 \textit{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 \textit{HFE} alleles and other major histocompatibility genes.

\section*{MATERIALS AND METHODS}

This study was approved by the local Ethics Research Committee (Process HCRP-FMRP, USP nº 4822/2011), and informed consent was obtained from all participants.

\subsection*{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 (≥ 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[3-5]; 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 co-morbidities. 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.

\subsection*{HFE typing}

Exons 2 to 5 and boundary introns were evaluated using Sanger sequencing[9] (Figure 1). \textit{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).

\subsection*{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.

\subsection*{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# ENSG00000010704 - 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). *Single nucleotide polymorphism; **Aminoacid.

**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). 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 (IC) 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: 1.14-13.54). 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’ = 27) (Figure 2). Therefore, the most relevant SNPs in LD with each other were H63DC>G, IVS2(+4)T>C, IVS4(-44)T>C and C282YG>A.
presented statistically significant values. The most frequent allele/genotype different of healthy controls. In italics: alleles and genotypes that

$$P < 0.001; \text{OR} = 2$$

$$P < 0.007; 95\% CI: 0.0008-0.065)$$ and

Fisher’s exact test variation sites and were assigned according to NCBI (http://www.ncbi.nlm.nih.gov) and Ensembl (http://www.ensembl.org) databases. Significant

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 remarkable LD between the H63DC>G and IVS2(+4)T>C was observed ($D' = 1.000$ in

**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-I0⁺ | HCC HCV-I0⁺ | HCC-I0⁺ | 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 P 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              | 0.714¹         | 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 P value                | 0.528           | 1.000          | 0.556   | 0.212       | 0.295   | 0.258         |
| 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 P value                | -               | -              | 1.000   | -           | 1.000   | 0.103         |
| C282YG>A (rs1800562) [+5473] | G               | 0.286¹         | 0.979   | 0.983       | 0.902   | 1.000         | 0.955         |
|                           | A               | 0.714¹         | 0.021   | 0.017       | 0.098   | 0.000         | 0.045         |
|                           | GG              | 0.071¹         | 0.958   | 0.966       | 0.854   | 1.000         | 0.910         |
|                           | GA              | 0.429          | 0.042   | 0.034       | 0.098   | 0.000         | 0.090         |
|                           | AA              | 0.500¹         | 0.000   | 0.000       | 0.049   | 0.000         | 0.000         |
| HW P 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 P 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         | 0.000         |
|                           | 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 P 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-I0⁺) or not (HCV-I0⁻) iron overload, hepatocellular carcinoma and hepatitis C plus iron overload (HCC HCV-I0⁻), hepatocellular carcinoma caused by diverse etiologies other than HCV and without information regarding iron overload (HCC-I0⁻), 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 ($\leq 0.05$) and Hardy-Weinberg equilibrium adherence are shown in table.

¹HH x CTL: TT ($P < 0.04; \text{OR} = 3.91; 95\% \text{CI: 1.14-13.34}$).
²HH x CTL: G ($P < 0.001; \text{OR} = 0.01; 95\% \text{CI: 0.006-0.05}$).
³A ($P < 0.001; \text{OR} = 53.06; 95\% \text{CI: 18.41-152.90}$), GG ($P < 0.001; \text{OR} = 0.007; 95\% \text{CI: 0.0008-0.065}$) and AA ($P < 0.001; \text{OR} = 201.00; 95\% \text{CI: 10.44-3.871}$). The most frequent allele/genotype different of healthy controls. In italics: alleles and genotypes that presented statistically significant values.
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-IO-), and population healthy control individuals (CTL).

 Alleles in validation process.

°Alleles recognized by ImMunoGeneTics information system – IMGT; 1Order 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 (intron 4) and new deletionG>DEL (5' to 3'); 2HH x CTL: 1HCC HCV-IO+ x CTL: 5Alleles 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 IVS2(+4)-C mutants with the C282Y-G (H63DC>G and IVS2(+4)-T). Another relevant result was the linkage of both mutant H63D-G and IVS2(+4)-C mutants with the C282Y-G (exon 4) IVS4(-44)T>C (intron 4) and the new mutation (G>DEL at intron 5) (Table 2).

### 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) | Whole |
|-----------------|----------------|-----------------------------|-------|
|                 | 1-2-3-4-5-6    | 1HCC: HCV-IO+; HCV-IO-; HH; TOTAL; HCC-IO- |       |
|                 |                | CTL | HCV-IO | HCC HCV-IO | HH | TOTAL | HCC-IO |       |
|                 |                | n  | %    | n  | %    | n  | %    | n  | %    | n  | %    |       |       |
| HFE*001        | C-T-C-G-T-G    | 107 | 0.54 | 74 | 0.63 | 63 | 0.61 | 23 | 0.48 | 47 | 0.14 | 113 | 0.52 |
| HFE*001:unofficial:02 | C-C-C-G-T-G    | 28  | 0.14 | 12 | 0.10 | 15 | 0.11 | 3  | 0.06 | 1  | 0.04 | 19  | 0.09 |
| HFE*001:unofficial:03 | C-T-G-G-T-G | 8   | 0.04 | 2   | 0.02 | 1  | 0.01 | 0  | 0.00 | 0  | 0.00 | 1   | 0.00 |
| HFE*001:unofficial:04 | C-T-C-G-C-G | 0   | 0.00 | 0   | 0.00 | 1  | 0.01 | 1  | 0.02 | 0  | 0.00 | 2   | 0.01 |
| HFE*001:unofficial:05 | C-C-C-G-C-G | 13  | 0.07 | 11  | 0.09 | 16 | 0.11 | 7  | 0.15 | 0  | 0.00 | 23  | 0.12 |
| HFE*001:unofficial:06 | C-C-G-T-G-Del | 1   | 0.01 | 0   | 0.00 | 0  | 0.00 | 0  | 0.00 | 0  | 0.00 | 1   | <0.01 |
| HFE*002        | G-C-C-G-T-G    | 35  | 0.17 | 16  | 0.13 | 20 | 0.14 | 9  | 0.19 | 3  | 0.11 | 32  | 0.15 |
| HFE*003        | C-T-C-A-T-G    | 8   | 0.04 | 3   | 0.02 | 2  | 0.02 | 3  | 0.06 | 20 | 0.71 | 26  | 0.12 |
| HFE*004        | G-C-C-A-T-G    | 0   | 0.00 | 0   | 0.00 | 0  | 0.00 | 1  | 0.02 | 0  | 0.00 | 1   | 0.00 |
| Number of alleles |                | 200 | 118  | 142 | 48  | 28  | 208 | 74  | 208 |

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-IO-), and population healthy control individuals (CTL).

**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®. 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; rs807209G>C; C282Y-G>A; IVS4(-44)T>C; new deletionG>DEL (5’ to 3’); Alleles recognized by ImMunoGeneTics information system – IMGT; Alleles in validation process.

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 = 7.20; 95%CI: 1.40-44.80) and the HFE*002 allele was underrepresented in all studied populations (f = 71%). Therefore, the HFE*001/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
**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, C282YG>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 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...
Table 3 Linkage disequilibrium between HLA-B alleles and HFE coding region H63DC>G single nucleotide polymorphism alleles

| HLA-B | HFE H63DC>G | Observed frequency | Expected frequency | Standardized value of disequilibrium(D') | Standardized value of correlation(r²) | QU1 value | P value of QU1
|-------|-------------|--------------------|--------------------|------------------------------------------|--------------------------------------|-----------|--------------
| 07    | C G         | C G                | C G                |                                          |                                      | 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 0.3056|
| 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    | 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 1        | 4.94 1.06          | -0.1911 0.1911     |                                          |                                      | 0.0053    | 1.0582 0.3056|
| 67    | 1 0        | 0.82 0.18          | 1.0000 -1.0000     |                                          |                                      | 0.0011    | 0.2145 0.6433|

The 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.

et al.[28] reported that HH patients presenting homozygosis 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)>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[21-23], 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[24]. The H63D-G allele and the GG genotype have been associated with HH in European and North American patients[24-25]. However, these associations were not observed in ours nor in other HH Brazilian samples[23].

The role of H63DC>G and C282YG>A variation sites in acquired IO disorders is 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[41]. 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...
Table 4  Linkage disequilibrium between HLA-B alleles and HFE coding region IVS2(+4)T>C single nucleotide polymorphism alleles

| HLA-B | HFE IVS2(+4)T>C |
|-------|-----------------|
|       | Observed frequency | Expected frequency | Standardized value of disequilibrium(D') | Standardized value of correlation(r) | QUI^1 value | P value of QUI^2 |
|       | T   | C   | T   | C   | T   | C   | T   | C   | T   | 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 | 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 | 0.0138 | 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://wwwensembl.org; rs2071305 (IVS2(+4)T>C). Shaded cells are showing significant LD values.

HH is a condition characterized by hepatic iron overload, leading to higher cancer risk. The association between the C282YG>A mutation and the HCC risk is still controversial. HH is a condition characterized by hepatic iron overload, leading to higher cancer risk. The association between the C282YG>A mutation and the HCC risk is still controversial. HH is a condition characterized by hepatic iron overload, leading to higher cancer risk. The association between the C282YG>A mutation and the HCC risk is still controversial. HH is a condition characterized by hepatic iron overload, leading to higher cancer risk. The association between the C282YG>A mutation and the HCC risk is still controversial. HH is a condition characterized by hepatic iron overload, leading to higher cancer risk. The association between the C282YG>A mutation and the HCC risk is still controversial.
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[39]. Microsatellite evaluations pointed out a susceptibility locus for HH. This locus was initially named as HLA-HF[36], 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[39]. Considering the great distance between the HFE and HLA-A, -B and -C loci, strong LD between these genes is not expected; however, some studies reported LD between H63DC>G and C282YG>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 C282YG>A SNPs, a significant disequilibrium between the H63DC>G and HLA-B (P = 0.03) was observed, encompassing HLA-B*15/H63DC-C, HLA-B*37/H63DC-C, HLA-B*44/H63DC-G and HLA-B*56/H63DC-G alleles (Table 3), being stronger for HLA-B*15/H63DC-C and HLA-B*56/H63DC-G alleles (D’ = 1). Since HLA-B locus is multiallelic, H63DC>G is biallelic, and H63DC-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/H63DC-G (r2 = 0.11) (Figure 3 and Table 3) which was much stronger than in the other combinations (r2 = 0.01-0.02). Most likely, this HLA-B*44/H63DC-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 (r2 = 0.02 and 0.03, respectively) (Table 4). This analysis resulted on the identification of the extended H63DC-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[4] or in patients with acquired diseases associated with IO[39], however, without reaching significance. Since haplotypes containing HLA-B*44 are common in Europe, West and North Africa, and in North-American Caucasians[40], there is a high probability of overrepresentation of the H63DC-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 H63DC-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 H63DC-G, IVS2(+4)-C and C282Y-G alleles, particularly in HH patients, the mutation IVS2(+4)>C was not directly associated with HH susceptibility. Although the HFE gene is distant from other MHC genes, the HFE H63DC-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* 14 bp INDEL and *TNFα*-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 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 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 knowledgement of the major gene associated with iron uptake may help on the understanding of the IO pathogenesis.

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