Haptoglobin type neither influences iron accumulation in normal subjects nor predicts clinical presentation in HFE C282Y haemochromatosis: phenotype and genotype analysis

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Summary. In the UK, 90% of patients with hereditary haemochromatosis (HH) are homozygous for HFE C282Y, as are one in 150 people in the general population. However, only a minority of these will develop clinical haemochromatosis. Iron loss modifies iron accumulation but so may other genetic factors. Haptoglobin (Hp) exists as three major types (Hp 1-1, Hp 2-1 or Hp 2-2) and binds free plasma haemoglobin. In men, Hp 2-2 has been shown to be associated with increased macrophage iron accumulation and serum ferritin concentration. Furthermore, the frequency of Hp 2-2 was shown to be increased in patients with HH. We determined Hp types by phenotyping and genotyping 265 blood donor control subjects and 173 subjects who were homozygous for HFE C282Y. The latter group included 66 blood donors lacking clinical features suggestive of haemochromatosis and without a known family history, and 68 patients presenting clinically with haemochromatosis. Hp 2-2 frequencies did not differ in control subjects and C282Y homozygotes. Hp 2-2 was not a risk factor for disease development in HH. To investigate the relationship between iron accumulation and haptoglobin type, we determined transferrin saturation and serum ferritin concentration in 192 male, first-time blood donors aged 20–40 years who lacked both HFE C282Y and H63D. Transferrin saturation and serum ferritin concentrations did not vary with Hp type.

Keywords: haptoglobin, iron, haemochromatosis.

Hereditary haemochromatosis (HH) is an autosomal recessive disorder in which the patient has iron accumulation as a result of increased dietary absorption. In 1996, the haemochromatosis gene (HFE) was located on the short arm of chromosome 6 about 5 Mb telomeric to the human leucocyte antigen (HLA) Class I region (Feder et al, 1996). It codes for an HLA Class I protein, and 90% of chromosomes from patients with HH were found to have a single mutation of this gene (C282Y). In the UK, over 90% of patients are homozygous for this mutation (The UK Haemochromatosis Consortium, 1997) as are about one in 150 people in the general population (Jackson et al, 2001).

The replacement of cysteine by tyrosine at position 282 in the HFE gene (C282Y) causes the loss of a disulphide bridge essential for the protein’s ability to bind to β2-microglobulin (Feder et al, 1996). Unlike the wild-type protein, the mutant protein is not expressed at the cell surface. Ordinarily, the HFE protein binds to the transferrin receptor to inhibit the binding of iron-loaded transferrin, thereby regulating the amount of iron imported into cells. How this causes the enhanced iron absorption in haemochromatosis remains a matter for debate (Roy & Andrews, 2001; Townsend & Drakesmith, 2002).

Once diagnosed, the excess iron is readily removable by regular venesection and, if treatment is started before complications have arisen, life expectancy is not reduced (Niederau et al, 1996). The frequency of the mutation, the availability of a genetic test and the effective treatment by phlebotomy have led to pressure to implement population screening (Burke et al, 1998). However, the clinical significance of HFE mutations remains uncertain.

Iron accumulation is influenced by blood loss and diet but studies of twins (Whitfield et al, 2000) and several strains of HFE knock-out mice suggest that genetic factors other than HFE are also important (Levy, J.E. et al, 2000; Fleming et al, 2001). There is, therefore, considerable interest in other polymorphic genes that may modify iron accumulation in HH.

Haptoglobin (OMIM no. 140100) is an α₂ plasma glycoprotein (see review by Langlois & Delanghe, 1996). It is produced mainly by hepatocytes and released into the plasma where it remains for up to 10 d. It binds with high
there were no differences in the frequency of Hp 2-2 between subjects homozygous for HFE C282Y and control subjects lacking the HFE mutations (Beutler et al., 2002a). Here we have determined the haptoglobin types of a control group of blood donors (not homozygous for HFE C282Y), male first-time blood donors aged 20–40 years who lacked HFE C282Y and H63D mutations, and subjects homozygous for HFE C282Y (who were patients presenting with clinical haemochromatosis and subjects discovered by genetic testing). We have compared the transferrin saturation and serum ferritin concentrations between haptoglobin types within each group. As only frozen whole blood was available for testing in many cases, we developed a simple method to determine haptoglobin genotypes.

MATERIALS AND METHODS

Sample selection. Blood samples were available from 10 500 consenting blood donors from South Wales who had participated in a research-based genetic screening programme (Jackson et al., 2001). The control group consisted of 265 randomly selected samples, none of which were homozygous for C282Y. In addition, 192 samples were tested from male, first-time blood donors aged 20–40 years who lacked C282Y and H63D mutations of the HFE gene. Samples were available from 173 subjects homozygous for C282Y. These included 66 of the 72 blood donors found to be homozygous for HFE C282Y (Jackson et al., 2001), 68 unrelated patients presenting clinically with haemochromatosis in South Wales (McCune et al., 2002) and 39 individuals discovered through family screening of both groups. When available, serum was used to determine Hp phenotypes but for many blood donors only frozen whole blood was available for testing and these were tested by genotyping. The blood donor study and the study of families of C282Y homozygous individuals received approval from the local ethical committee.

Measurement of transferrin saturation and serum ferritin concentration. Serum iron concentration, unsaturated iron binding capacity, transferrin saturation and serum ferritin concentrations were available for all the blood donors...
(Jackson et al., 2001). For the patients, assays were carried out in several hospitals in South Wales, and the values reported were obtained at presentation.

**HFE genotyping.** HFE genotypes of all blood donor samples included in this study were determined by polymerase chain reaction (PCR) using the method of Guttridge et al. (1998). For patients, HFE genotypes were determined by heteroduplex analysis (Jackson et al., 1997; Worwood et al., 1999).

**Haptoglobin genotyping.** Frozen whole blood (500 μl) was added to an equal volume of cell lysis buffer (Tris-HCl 10 mmol/l pH 8.0, sucrose 11% w/v, MgCl\(_2\) 5 mmol/l, Triton X-100 1% w/v). After vortexing briefly and incubating at room temperature (RT) for 2 min, the nuclei were harvested by centrifugation (Heraeus microfuge 6000 r.p.m., 2 min, RT), resuspended by briefly vortexing in cell lysis buffer (500 μl) and centrifuged again. The nuclei were gently resuspended in 300 μl nuclei lysis buffer [Tris-HCl pH 8.0 10 mmol/l, EDTA 10 mmol/l, sodium citrate 10 mmol/l, sodium dodecyl sulphate (SDS) 1% w/v], NaCl (6 mol/l, 100 μl) and then chloroform (500 μl) were added. The mixture was inverted gently until a uniform emulsion formed and this was centrifuged at 6000 r.p.m. for 5 min at RT. A portion of the upper aqueous layer (300 μl) was decanted into absolute ethanol (600 μl) and was then mixed by gentle inversion until the high-molecular-weight DNA had precipitated as a small, fibrous ball. The DNA was transferred to sterile water (25 μl) and dissolved at 4°C for 3 h or at RT for 1 h.

The Hp\(^2\) cDNA (GenBank Entry: NT_010494, Homo sapiens chromosome 16) starts at position 1152337 (preceding exon 1) and ends at position 1146076 (the last amino acid of exon 5). The Hp\(^2\) allele runs in the 3′ to 5′ direction on the reverse complement strand of chromosome 16 (Fig. 1). Using this diagram, we estimated the approximate sizes of the bands produced by the primers annealing at exons 2 and 5. As Hp\(^2\) is completely documented, we were confident of our prediction of a 4.37-kb band. However, for Hp\(^1\) only the cDNA sequence was available. We estimated the size of the predicted band by eliminating any combination of exon 3 (i and ii) and 4 (i and ii) and their introns in turn, all of which gave an approximate size of 3.0 kb.

Primers Hp-F (5′ CTGCTCTGGGACAGCTTTTGCAGTGG 3′) and Hp-R (5′ TGTTGACATTTTAAAATTTGGCATTTTGCAGTGG 3′) exploit the size difference due to the extra two exons of the Hp\(^2\) allele. Primer design took into consideration the haptoglobin-related gene (HPR), which is highly homologous with the haptoglobin gene. Long primers were designed with 3′ ends that were mismatched against HPR.

Approximately 250 ng of high-molecular-weight DNA was amplified in a 15-μl reaction mix consisting of 1 × PCR Buffer II and 2.25 mmol/l MgCl\(_2\) (GeneAmp\(^{®}\); Perkin Elmer), 0.5 mmol/l dNTPs (GeneAmp\(^{®}\); Applied Biosystems, Warrington, UK), 0.6 μmol/l of primers F and R (Oswel DNA Service, Southampton, UK) and 1 unit of AmpliTaq DNA Polymerase (Applied Biosystems).

After a ‘hot start’ at 92°C for 2 min, there were 28 cycles of denaturing at 92°C for 30 s, annealing at 62°C for 30 s and extension at 68°C for 10 min with a 20-s increment in the extension step of each cycle. PCR product (4 μl) was added to 1.5 μl loading buffer, containing bromophenol blue, and loaded on a 0.8% (w/v) agarose gel (8 cm × 10 cm × 1 cm) in 0.5 × TBE (Tris-borate-EDTA) buffer pH 8.0 containing ethidium bromide for electrophoresis at 60 V for 3 h.

**Haptoglobin phenotyping.** A red cell lysate containing free haemoglobin was prepared by adding 500 μl of whole blood to 500 μl of water and centrifuging (Heraeus microfuge 13 000 r.p.m., 5 min). The supernatant was removed, added to 500 μl of water and re-centrifuged. The supernatant was removed, and 1.5 μl was added to 15 μl of serum and left for 10 min to enable the haptoglobin–haemoglobin complexes to form. The 15 μl sample was added to 1.5 μl loading dye and loaded onto a 2-mm (16 cm × 16 cm) 5% polyacrylamide gel (37:5:1 polyacrylamide gel: Appligene; Graffenstade, France) for electrophoresis in 1 × Tris-borate-EDTA (TBE) buffer, pH 8.0, at 200 V for 3 h. The gel was removed and incubated for 1 h with a solution of 15 ml 1× TBE in which was dissolved one tablet of 3.3′-diaminobenzidine (Sigma, Poole, UK) and 15 μl hydrogen peroxide (30%; Sigma), in order to detect haem.

**Analysis of results.** The distributions of haptoglobin frequencies in groups of subjects were compared using the chi-squared test. Transferrin saturations are given as mean ± standard deviation (SD), and mean values were compared using the two-sample t-test assuming unequal variance. Serum ferritin concentrations were not normally distributed so the median and the ranges are given. Median values were compared using the Mann–Whitney U-test. A probability of <0.05 was taken to indicate a significant difference.

**RESULTS**

**Genotyping**

We developed a new method of haptoglobin genotyping using frozen whole blood and a single PCR that exploits the 1.7 Kb size difference between the product of the Hp\(^1\) and Hp\(^2\) alleles. The PCR method was validated by testing 86 samples that had been previously typed using polyacrylamide gel electrophoresis (PAGE). There was 100% agreement. Figure 2 shows the PCR products for six subjects. The product present for samples 1 and 2 is approximately 4.7 kb in size while that for samples 5 and 6 is approximately 3.0 kb. The larger product was less intense, as it was less favourably amplified during the long-distance PCR.

The Hp0 type has a gene deletion from the Hp promoter to the 5′ end of HpRβ (Koda et al., 1998) and would be misinterpreted as a failure of the PCR in the very rare case of a homozygous deletion. Sometimes patients with hypo-haptoglobinemia may be compound heterozygotes (Hp\(^2\)/Hp0) (Koda et al., 1998). These would be considered to be Hp 2-2 type after PCR. However, the frequency of Hpo is very low in Europeans (Langlois & Delanghe, 1996). Furthermore, the observed haptoglobin frequencies did not differ from the expected Hardy–Weinberg distribution calculated by assuming that there were two genotypes, Hp\(^1\)
and Hp\(^2\). The haptoglobin frequencies in control subjects are those expected for a Northern European population (Langlois & Delanghe, 1996).

**Phenotyping**

Haptoglobin phenotypes were easily distinguishable by the number and position of the bands stained (data not shown). The Hp 1-1 phenotype has a single band that rapidly migrates towards the anode. The Hp 2-2 phenotype has a series of slower bands, while the Hp 2-1 phenotype has a mixture of both types. The Hp 1-1 band has a similar mobility to that of free haemoglobin (which must always be present to ensure staining of all haptoglobin bands). Subjects carrying Hp0 may have no haptoglobin, very low plasma concentrations so only the free haemoglobin will electrophorese on the gel and be stained. Subjects with Hp0 may, therefore, be reported as Hp 1-1 but the frequency of Hp0 is very low in European populations (see above).

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**Table I.** Haptoglobin types in blood donors and patients with haemochromatosis.

| Group                                      | No of subjects | Female/male | Hp 1-1 | Hp 2-1 | Hp 2-2 |
|--------------------------------------------|----------------|-------------|--------|--------|--------|
| Blood donor control subjects               | 265            | 140/125     | 43 (16.2%) | 124 (46.8%) | 98 (37%) |
| Male first-time blood donors                | 192            | N/A         | 27 (14.1%) | 87 (45.3%) | 78 (40.6%) |
| All homozygous for C282Y*                  | 173            | 76/97       | 26 (15%) | 76 (43.9%) | 71 (41%) |
| Blood donors homozygous for C282Y†         | 66             | 39/27       | 14 (21.2%) | 32 (48.5%) | 20 (30.3%) |
| Patients homozygous for C282Y†             | 68             | 22/46       | 8 (11.8%) | 34 (50%) | 26 (38.2%) |

*Including the two groups marked †and relatives (see text).
for C282Y in the control blood donor group (data not shown).

**Transferrin saturation, serum ferritin concentrations and haptoglobin type**

Table II shows the mean transferrin saturation and the median serum ferritin concentration for the various groups of blood donors, according to haptoglobin type and sex. Information about iron status was available for almost all control subjects and for the blood donors homozygous for C282Y. There were no differences in iron status for each haptoglobin type within the groups of subjects, either male or female.

We also examined the variation of transferrin saturation and serum ferritin concentration in male, first-time blood donors aged 20–40 years who lacked both the C282Y and H63D mutations of the *HFE* gene. There were no significant differences in either transferrin saturation or serum ferritin concentration between the haptoglobin types. For the patients presenting clinically with haemochromatosis, who were homozygous for C282Y, transferrin saturation and/or serum ferritin concentration was available for 44 of the 68 patients (data not shown). All had a raised transferrin saturation (> 50%) and serum ferritin concentrations (> 200 μg/l in women or 300 μg/l in men). There were no significant differences with Hp type.

**DISCUSSION**

It was necessary to develop a method to determine haptoglobin types by genotyping, because for many of the blood donors only frozen whole blood remained available for testing. Recently, a method of genotyping based on similar principles has been described (Koch *et al*, 2002). The authors confirmed their results using alternative PCR protocols. Both genotyping and PAGE are suitable methods for healthy subjects of European origin where the frequency of Hp0 is very low.

A number of large population surveys have now been completed and show that most men homozygous for *HFE* C282Y and about 50% of women have a raised transferrin saturation (Adams *et al*, 2000; Asberg *et al*, 2001; Beutler *et al*, 2002b; Deugnier *et al*, 2002; Phatak *et al*, 2002). However, the morbidity associated with homozygosity for *HFE* C282Y appears to be low. In California (Beutler *et al*, 2002b) and Norway (Asberg *et al*, 2001), the morbidity was similar to control subjects although, in Brittany, male homozygotes had a higher incidence of chronic fatigue and distal arthralgias (Deugnier *et al*, 2002). These findings suggest a low penetrance for homozygosity for C282Y for both significant iron overload and disease. This was confirmed by a survey of hereditary haemochromatosis as a clinical condition carried out in South Wales. Only 1.2% of adult C282Y homozygotes had received a confirmed diagnosis (McCune *et al*, 2002). Restricting the study to men over 45 years old, the figure rose to 2.8%. For this reason, there has been considerable interest in identifying other genetic factors which may enhance iron accumulation and morbidity.

In healthy men, Hp 2–2 was found to be associated with macrophage iron accumulation when compared with men with Hp 1–1 or Hp 1–2 (Langlois *et al*, 2000). Differences in turnover and haemoglobin binding may explain these changes in iron storage for Hp 2–2. The Hp 1–1 phenotype binds free haemoglobin more efficiently than either Hp 2–1 or Hp 2–2 (Langlois & Delanghe, 1996). It also has

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**Table II.** Transferrin saturation (TS) and serum ferritin (sFn) concentration according to Hp type in blood donors.

| Group                                           | Sex | Hp type | n   | TS (%) mean ± SD | sFn (μg/l) median (range) |
|-------------------------------------------------|-----|---------|-----|------------------|--------------------------|
| Randomly selected control subjects              | Male| 1-1     | 24  | 31 ± 11          | 104 (34–183)            |
|                                                |     | 2-1     | 61  | 31 ± 15          | 96 (6–299)              |
|                                                |     | 2-2     | 40  | 31 ± 11          | 87 (24–207)             |
|                                                | Female| 1-1     | 19  | 25 ± 12          | 57 (9–116)              |
|                                                |     | 2-1     | 63  | 24 ± 11          | 37 (7–301)              |
|                                                |     | 2-2     | 58  | 25 ± 11          | 41 (9–137)              |
| C282Y homozygotes                               | Male| 1-1     | 5   | 56 ± 15          | 141 (51–374)            |
|                                                |     | 2-1     | 14  | 63 ± 16          | 221 (14–650)            |
|                                                |     | 2-2     | 8   | 69 ± 23          | 135 (85–410)            |
|                                                | Female| 1-1     | 9   | 56 ± 25          | 58 (11–238)             |
|                                                |     | 2-1     | 18  | 49 ± 20          | 68 (13–418)             |
|                                                |     | 2-2     | 12  | 50 ± 19          | 96 (8–195)              |
| First-time donors aged 20–40 years lacking HFE C282Y and H63D | Male| 1-1     | 27  | 28 ± 10          | 112 (25–193)            |
|                                                |     | 2-1     | 87  | 30 ± 11          | 100 (34–263)            |
|                                                |     | 2-2     | 78  | 29 ± 9           | 108 (26–223)            |

The numbers tested are samples for which transferrin saturation or serum ferritin concentrations were available.
unrestricted access to tissues, possibly because of its smaller size and consequent ability to migrate across the endothelial cell barrier. These advantages may contribute to a more effective clearance of free haemoglobin from the plasma than is achieved by the other two phenotypes. Moreover Hp 2-2 complexed with haemoglobin exhibits a higher affinity for the CD163 receptor than the other phenotypes, which would suggest a preferential delivery of haemoglobin complex to the macrophage (Kristiansen et al. 2001). Plasma vitamin C concentrations were lowest for the Hp 2-2 phenotype (Langlois et al. 1997). These authors suggested that this might be a consequence of the weaker binding of haemoglobin by Hp 2-2 and the weaker antioxidative capacity.

The above findings provide a basis for differences in the handling of haemoglobin iron in the plasma by the major haptoglobin types. However, our studies of healthy normal men (aged 20–40 years) who had not donated blood and lacked the common HFE mutations demonstrated no differences in either transferrin saturation or serum ferritin concentration between the Hp types, and confirm the recent reports of Kasvosve et al. (2002) and Beutler et al. (2002a). Beutler et al. (2002a) studied only 60 HFE wild-type men, mean age 57 ± 12 years. In this group, the iron status may have been influenced by chronic disease (Beutler et al. 2002a).

There have been numerous reports of associations between Hp phenotypes and diseases (Langlois & Delanghe, 1996) but none indicated that possession of a particular Hp type is a risk factor for arthritis or diabetes. However, the Hp 1-1 phenotype has been associated with protection against vascular complications in both type I (Levy, A.P. et al. 2000) and type II (Nakhoul et al., 2001) diabetes. Zhao and Zhang (1993) found that Hp1-1 was over-represented in 107 patients with cirrhosis of the liver compared with 552 normal adults (relative risk 3.3, P = 2 × 10⁻⁸). Zipprich et al. (1986) found an increased frequency of Hp1-1 in 100 German patients with chronic, non-alcoholic liver disease but not in 90 patients with cirrhosis when compared with 1726 control subjects. There appear to be no clear associations between Hp type and risk of the major clinical manifestations of HH.

Beutler et al. (2002a) suggested that conflicting conclusions about the frequency of Hp 2-2 in haemochromatosis might reflect differences in ascertainment. However in our study, we did not find significant differences in frequency between cases ‘discovered’ by genetic testing to be homozygous for HFE C282Y, and the patients presenting clinically with signs and symptoms of iron overload who were homozygous for HFE C282Y. Possession of Hp 2-2 does not appear to be a risk factor for iron accumulation in normal subjects or for the development of disease in subjects homozygous for HFE C282Y.

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