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Analysis of genes implicated in iron regulation in individuals presenting with primary iron overload

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Abstract Extensive investigation into the molecular basis of iron overload disorders has provided new insights into the complexity of iron metabolism and related cellular pathways. The possible involvement of genes affecting iron homeostasis, including *HFE*, *SLC40A1*, *HAMP* and *CYBRD1*, was investigated in individuals who were referred for confirmation or exclusion of a diagnosis of haemochromatosis, but who tested negative or were heterozygous for the causative *HFE* mutation, C282Y. Denaturing high performance liquid chromatography analysis of these genes revealed a unique spectrum of mutations in the South African study population, including 67 unrelated patients and 70 population-matched controls. Two novel *CYBRD1* gene mutations, R226H and IVS1-4C→G, were identified in 11% of South African Caucasian patient referrals. We identified a novel D270V

mutation in the *SLC40A1* gene in a Black South African female with iron overload. These mutations were absent in the control population. In Africans with iron overload not related to the *HFE* gene, the possible involvement of the *SLC40A1* and *CYBRD1* genes was demonstrated for the first time. This study confirms the genetic heterogeneity of haemochromatosis and highlights the significance of *CYBRD1* mutations in relation to iron overload.

Nucleotide sequence data reported are available in the Genbank database under the accession numbers AJ604512, AJ609539, AJ616848, AJ616847, AJ609540, AJ715523, AJ715524 and AJ715525.

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Introduction

Hereditary haemochromatosis (HH) is generally an autosomal recessive condition and is characterised by iron overload, primarily in parenchymal cells (Bothwell et al. 1995). Iron accumulation usually results in tissue damage and causes cirrhosis of the liver, diabetes mellitus, arthropathy, cardiomyopathy, endocrine abnormalities and an increased risk of hepatocellular carcinoma (Powell et al. 1994; Bothwell et al. 1995).

Homozygosity for the C282Y mutation in *HFE* is the most common disease genotype and has been identified as the cause of HH in over 80% of individuals of European descent with HH (Feder et al. 1996; Worwood et al. 1997). Other mutations in the *HFE* gene account for the disease phenotype in approximately 2%–10% of cases (reviewed by Pointon et al. 2000). Non-*HFE* related forms of haemochromatosis are less common but have also been reported and include mutations in the transferrin receptor 2 gene (*TFR2*) (Camaschella et al. 2000; Roetto et al. 2001), the solute carrier family 40 (proton-coupled divalent metal ion transporter) member 1 gene (*SLC40A1*), which is also known as the solute carrier family 11 (proton-coupled divalent metal ion transporter) member 3 gene (*SLC11A3*), ferroportin 1 gene (*FPN1*), iron-regulated transporter 1 gene (*IREG1*) or metal transporter 1 gene (*MTP1*; Njajou et al. 2001; Montosi et al. 2001; Devalia et al. 2002; Roetto et al. 2002; Wallace et al. 2002; Cazzola et al. 2002; Hetet et al. 2003; Jouanolle et al. 2003; Gordeuk et al. 2003; Rivard et al. 2003) and the hepcidin antimicrobial peptide gene (*HAMP*), which is also known as the

Table 1 Oligonucleotide primers used for amplifying PCR products subjected to dHPLC analysis (T_m melting temperature, A_{mm} annealing temperature)

| Gene | Exon | Primers | T_m (°C) | | PCR fragment size (bp) | Ann (°C) |
|-------------------------|------|------------------------------|-----------------|--------------------------------|------------------------|----------|
| | | | Forward (5'-3') | Reverse (5'-3') | | |
| ^a <i>HFE</i> | 1 | GGACACTGGATCACCTAGTGTT | 66 | CTAGTTTCGATTTTCCAGCCC | 355 | 58 |
| | 2 | ACATGGTTAAGGCCTGTTGC | 60 | CAGCTGTTTCCTTCAAGATGCA | 373 | 54 |
| | 3 | AAATAGGGACCTATTCCTTTGGT | 64 | GTGCCCTGCAACCTCCTCCA | 393 | 58 |
| | 4 | TGGCAAGGGTAAACAGATCC | 60 | CTCAGGCACCTCCTCAACC | 390 | 58 |
| | 5 | GTATGTGACTGATGAGAGCCA | 62 | CAGAGGTACTAAGAGACTTC | 310 | 54 |
| | 6 | TAGTGCCCAAGTCTAAATTG | 58 | TGAGTCTTAGTTTTGTCTCC | 201 | 58 |
| <i>HAMP</i> | 1 | AGCAAAGGGAGGGGGCTCAGACCAC | 80 | TCCCATCCCTGCTGCCCTGCTAAGGAC | 292 | 60 |
| | 2+3 | TTGCCGGGAGCCAGTCTCAGAGGTCCAC | 60 | TGCAAGGCAGGGTCAGGACAAGCTCTTAGC | 497 | 63.1 |
| <i>CYBRDI</i> | 1 | AGACAGCCCCAAGAAGTCC | 56 | TCCTCCAGCACTTCCTCAG | 407 | 62.3 |
| | 2 | GAGGGGAGAAAGCAAAAGCCAAAG | 56 | AAGATCCAGCACTGCACCTC | 476 | 63.1 |
| | 3 | GTAGTGGAACTGGAGCGAG | 56 | TGTGAGACTTTTTGGTCTTC | 496 | 62.1 |
| | 4i | CTGTTTTGCATGTGCTGTATC | 62 | ATCTGTGCCAGCCTCATC | 352 | 58 |
| | 4ii | AGGTTCCATGCCAGCCTACTC | 66 | ATCTATTCTGCACAAGGCACCG | 406 | 58 |

^aPrimer sequences supplied by Prof. C Camaschella^b $T_m = 2(nA+nT) + 4(nG+nC)$

liver-expressed antimicrobial peptide gene (*LEAP1*) or hepcidin gene (*HEPC*; Krause et al. 2000; Nicolas et al. 2001; Park et al. 2001; Pigeon et al. 2001; Roetto et al. 2003).

The products of these genes have various roles in iron metabolism. HFE binds to the transferrin receptor (TFR) and reduces its affinity for iron-loaded transferrin. TFR2 mediates the cellular uptake of transferrin-bound iron. SLC40A1 encodes a transmembrane protein involved in the release of iron from duodenal epithelial cells and mediates iron efflux in the presence of a ferroxidase. HAMP acts as a signalling molecule involved in the maintenance of iron.

A complex spectrum of phenotypic expression has been observed for individuals with HH (Adams et al. 1997; Rhodes et al. 1997; De Villiers et al. 1999; Milani and Kotze 1999; Sachot et al. 2001), which has raised the

possibility that other modifier genes contribute to the clinical variability observed in HH. Digenic inheritance of haemochromatosis involving mutations in both the *HFE* and *HAMP* genes has also been described, with the severity of the *HAMP* mutation determining the severity of disease (Merryweather-Clarke et al. 2003).

In this study, the genes involved in iron storage, transport and regulation including *HFE*, *SLC40A1*, *HAMP* and *CYBRD1*, were analysed in patients referred for a molecular diagnosis of HH in the South African population. These individuals comprised a subpopulation of individuals who were included in a previous study (Kotze et al. 2004) and who either tested negative for the *HFE* mutation C282Y or were heterozygous for this common mutation underlying HH.

Table 2 Allele frequencies of the variants identified both in the patient and in population-matched control groups in the diverse South African population (*WHH* Caucasian haemochromatosis patient group, *WC* Caucasian control group, *BHH* Black haemochromatosis patient group, *BC* Black control group, *P* probability value, *NS* not significant)

| Gene | Exon/ intron | Variant | Allele frequencies ^a | | <i>P</i> | Allele frequencies ^a | | <i>P</i> |
|----------------|-----------------|---|---------------------------------|--------------------------------|-------------------|---------------------------------|-------------------------------|----------------------|
| | | | <i>WHH</i> (2 <i>n</i> =112) | <i>WC</i> (2 <i>n</i> =100) | | <i>BHH</i> (2 <i>n</i> =22) | <i>BC</i> (2 <i>n</i> =40) | |
| <i>HFE</i> | 2 | IVS2+4T→C ^b (Rochette et al. 1999) | 0.39 | 0.31 | NS | 0.18 | 0.43 | 0.05 ^f |
| | 2 | H63D ^c (Feder et al.1996) | 0.17 | 0.15 | NS | – | – | NS |
| | 2 | S65C ^{c, d} (Henz et al.1997) | 0.03 | – | – | – | – | – |
| | 4 | C282Y ^c (Feder et al.1996) | 0.08 | 0.10 | NS | – | – | – |
| | 4 | IVS4-44T→C ^b (Beutler and West 1997) | 0.10 | 0.06 | NS | 0.18 | 0.03 | 0.04 ^e |
| | 4 | IVS4-50A→G ^b (De Villiers et al. 1999) | 0.06 | 0.15 | NS | 0.14 | 1.00 | <0.0001 ^f |
| <i>SLC40A1</i> | 5 | IVS5-47G→A ^b (Beutler and West 1997) | 0.50 | 0.46 | NS | 0.15 | 0.65 | 0.0001 ^f |
| | 1 | (CGG) ₇ ^b (Lee et al. 2001) | 0.35 | 0.22 | NS | 0.50 | 0.78 | – |
| | 1 | (CGG) ₈ ^b (Lee et al. 2001) | 0.65 | 0.78 | NS | 0.23 | 0.10 | 0.08 |
| | 1 | (CGG) ₉ ^b (Lee et al. 2001) | – | – | – | 0.27 | 0.12 | 0.07 |
| | 1 | g.-23A→G ^b (This study) | – | – | – | 0.36 | 0.03 | 0.0007 ^e |
| | 1 | g.-8A→G ^c (Douabin-Gicquel et al. 2001) | 0.15 | 0.15 | NS | – | 0.08 | NS |
| | 1 | g.-98G→C ^c (Douabin-Gicquel et al. 2001) | 0.20 | 0.15 | NS | – | 0.08 | NS |
| | 1 | IVS1-24G→C ^b (Devalia et al. 2002) | 0.84 | 0.88 | NS | 0.82 | 0.28 | <0.0001 ^e |
| | 2 | IVS2+21T→C ^{c, d} (This study) | – | – | – | 0.04 | – | NS |
| | 3 | IVS3+111A→G ^{c, d} (This study) | – | – | – | 0.04 | – | NS |
| | 4 | I109 ^c (This study) | 0.008 | – | NS | – | 0.03 | NS |
| | 4 | L129 ^c (This study) | – | – | – | 0.23 | 0.05 | 0.04 ^e |
| | 6 | V221 ^c (Devalia et al. 2002) | 0.21 | 0.08 | 0.03 ^e | 0.14 | – | 0.04 |
| | 7 | D270V ^{c, d} (This study) | – | – | – | 0.04 | – | – |
| <i>HAMP</i> | 3 | Exon3+33C→T ^{c, d} (This study) | 0.008 | – | – | – | – | – |
| <i>CYBRD1</i> | 1 | IVS1-4C→G ^{c, d} (This study) | 0.03 | – | – | – | – | – |
| | 2 | IVS2+8T→C ^b (This study) | 0.67 | 0.64 | NS | 0.77 | 0.88 | NS |
| | 3 | IVS3-32G→C ^b (This study) | – | – | – | 0.18 | – | – |
| | 4 | R226H ^{c, d} (This study) | 0.008 | – | – | – | – | – |
| | 4 | S266N ^b (McKie et al. 2001) | 0.76 | 0.86 | NS | 0.77 | 0.88 | NS |

^aAllele frequency of polymorphic allele denoted

^bIdentified in both a heterozygous and homozygous state

^cIdentified only in a heterozygous state

^dVariants identified only in the patient group

^eStatistically significant over-representation of the polymorphic allele in the patient compared with the control groups

^fStatistically significant under-representation of the polymorphic allele in the patient compared with the control groups

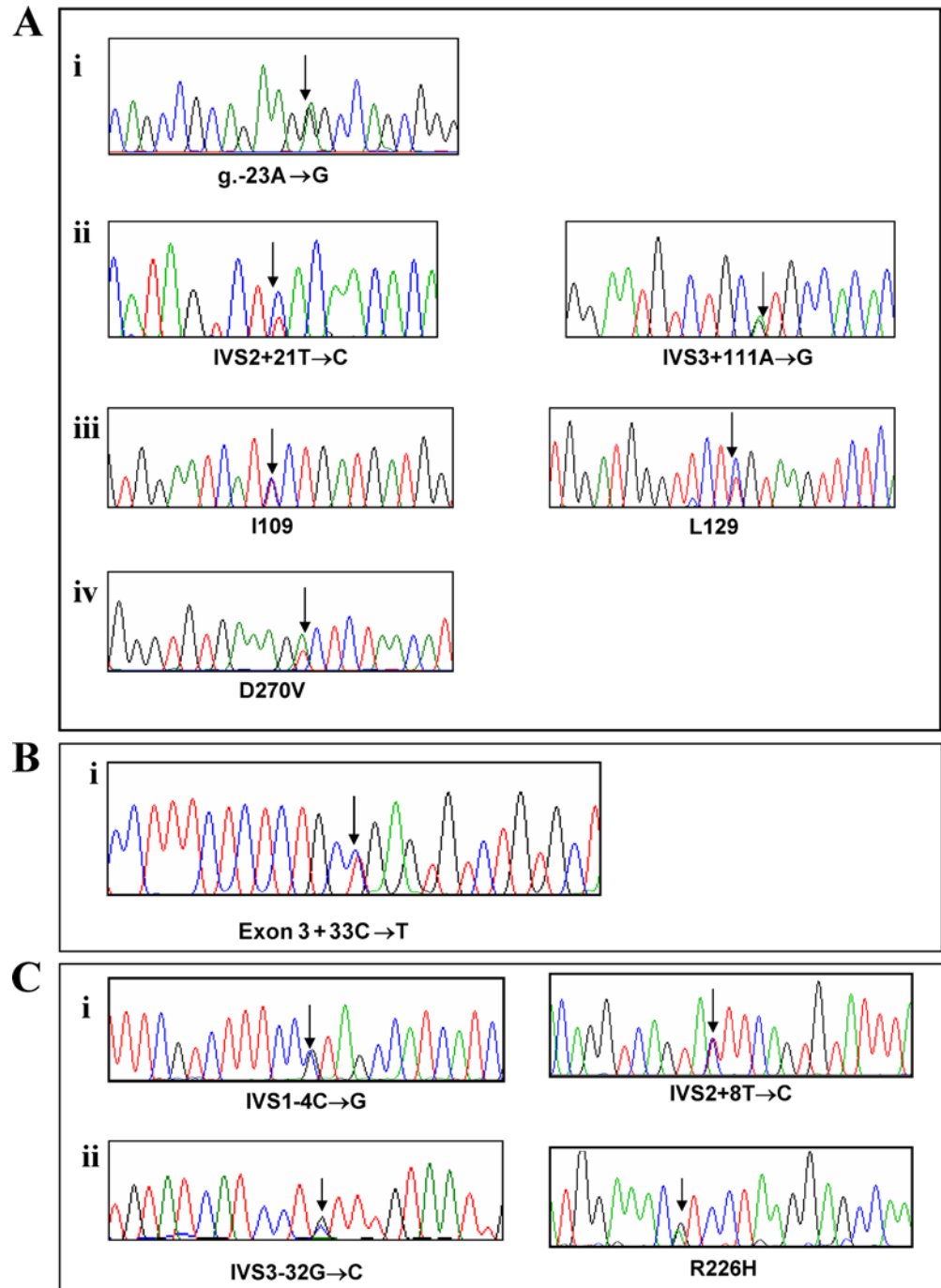
Materials and methods

Subjects

The study population initially included 161 apparently unrelated patients who were referred for HH mutation screening based on abnormal iron parameters (Bacon and Sadiq 1997) in the absence of secondary causes for elevated ferritin and transferrin saturation levels or a family history of HH (Kotze et al. 2004). Serum iron parameters were not available for the majority of cases. After exclusion of all C282Y homozygous patients, a

subpopulation including 58 individuals without the C282Y mutation and nine C282Y heterozygotes were selected for further analysis. The patients and controls were from the South African Black (11 patients and 20 control individuals) and Caucasian (56 patients and 50 control individuals) populations, with "Caucasian" referring to an individual of European descent, mainly of Dutch, French, German or British origin and "Black" referring to South Africans of central African descent. Iron status was unknown in these healthy control individuals.

Fig. 1A–C Direct sequencing analysis of the novel variants identified (arrows points of variation, red thymidine or T, blue cytidine or C, green adenosine or A, black guanosine or G). **A** In the *SLC40A1* gene in exon 1 (i), introns 2 and 3 (ii), exon 4 (iii) and exon 7 (iv). **B** In the *HAMP* gene in the 3' untranslated region. **C** The *CYBRD1* gene in introns 1 and 2 (i) and intron 3 and exon 4 (ii).



Denaturing high-performance liquid chromatography analysis of the *HFE*, *SLC40A1*, *HAMP* and *CYBRD1* genes

Deoxyribonucleic acid was extracted from whole blood by using a standard method (Miller et al. 1988). The various exons of the *HFE*, *SLC40A1* (Njajou et al. 2001), *HAMP* and *CYBRD1* genes were amplified by polymerase chain reaction (PCR) amplification with the intronic oligonucleotide primers listed in Table 1. The samples were heteroduplexed by heating at 95°C for 10 min followed by cooling to 20°C at 2°C/min steps and analysed by denaturing high-performance liquid chromatography (dHPLC) on a WAVE DNA Fragment Analysis System (Transgenomic). The temperatures of experimental runs used for dHPLC analysis are available upon request. Semi-automated DNA sequencing was performed on PCR products in an ABI 3100 PRISM automated sequencer.

Statistical analysis

Allele and genotype frequencies were estimated and statistical differences between patient and control groups were tested for significance by the Fisher exact test and/or chi-squared (χ^2) analysis with Yates' correction. A probability value smaller than 0.05 was regarded as statistically significant.

Results

The variants identified in both the patient and control groups, their allele frequencies and statistically significant differences detected between the groups are presented in Table 2. The sequencing traces of novel variants are shown in Fig. 1.

HFE gene

All variants observed in the *HFE* gene had been previously described (Rochette et al. 1999; Höhler et al. 1999; De Villiers et al. 1999; Beutler and West 1997) and no additional mutations were identified by dHPLC technology (Table 2). Nine of the 56 (16.1%) Caucasian patients included in the study were heterozygous for C282Y and six (10.7%) of these patients also had the H63D mutation. The S65C mutation was present in three of 56 (5.4%) Caucasian patients and was absent in the respective control group. One individual with the S65C mutation also had the H63D mutation.

The C282Y, H63D and S65C mutations were absent in the Black population, only previously described polymorphisms being identified in this population group (Table 2). Statistically significant associations were, however, observed for various polymorphisms in the Black patient group compared with the population-matched control group. These associations were observed

when comparing allele frequencies for IVS2+4T→C ($P<0.05$, χ^2 with Yates' correction = 2.59), IVS4-44T→C ($P<0.04$, χ^2 with Yates' correction = 2.83) and IVS5-47G→A ($P<0.0002$, χ^2 with Yates' correction = 11.42). A statistically significant association was observed with IVS4-50A→G for both allele ($P<0.0001$, χ^2 with Yates' correction = 43.35) and genotype ($P<0.0001$, χ^2 with Yates' correction = 30.00) frequencies. The variants IVS2+4T→C, IVS4-50A→G and IVS5-47G→A were under-represented and variant IVS4-44T→C was over-represented in the Black patient group compared with the population-matched control group. This finding is indicative of linkage disequilibrium with functional variants upstream of exon 1 affecting the expression of *HFE*.

SLC40A1 gene

Several previously described polymorphisms were identified (Table 2). Six novel variants were also identified: an A to G substitution at nucleotide position 23 upstream of the initiating ATG in exon 1 (g.-23A→G); a T to C transversion within the second intron (IVS2+21); an A to G transversion within the third intron (IVS3+111); synonymous substitutions in the codons for I109 (g.327C→T) and L129 (g.387C→T). We found a novel mutation (g.808A→T) in exon 7 resulting in the substitution of aspartic acid 270 with valine (D270V). This mutation is in the large cytoplasmic region between transmembrane domains 4 and 5 in the model of Devalia et al. (2002).

No clear correlation could be made between the number of CGG repeats in the promoter region and dHPLC chromatograms and thus it was decided to perform direct sequencing analysis for the patient and control groups on this region. The majority of the variants were identified only in the Black South African population, including (CGG)₉, g.-23A→G, IVS2+21T→C, IVS3+111A→G and L129. The intronic variants IVS2+21T→C and IVS3+111A→G occurred together in a Black patient and were absent in the control population. The nucleotide changes were not expected to alter gene splicing and were not investigated further. The missense mutation D270V was identified in a single Black patient and was absent from the control population.

Statistical analysis was performed for the various polymorphisms identified and the only significant association achieved in the Caucasian population was for the polymorphism within exon 6 (V221) (allele frequency: $P<0.03$, χ^2 with Yates' correction = 3.14; genotype frequency: $P<0.02$, χ^2 with Yates' correction = 3.98) when comparing the patient group with the population-matched control group.

Several statistically significant associations were observed for the polymorphisms identified in the Black population when comparing the patient group with the population-matched control group. These associations included the following variants: g.-23A→G (allele fre-

quency: $P < 0.0007$, χ^2 with Yates' correction = 10.53; genotype frequency: $P < 0.001$, $\chi^2 = 12.82$), IVS1-24G→C (allele frequency: $P < 0.0001$, χ^2 with Yates' correction = 14.71; genotype frequency: $P < 0.001$, $\chi^2 = 13.09$), L129 (allele frequency: $P < 0.04$, χ^2 with Yates' correction = 2.86; genotype frequency: $P < 0.02$, $\chi^2 = 5.10$) and V221 (allele frequency: $P < 0.04$, χ^2 with Yates' correction = 3.15; genotype frequency: $P < 0.03$, χ^2 with Yates' correction = 3.32).

HAMP gene

Mutation analysis of the *HAMP* gene revealed a novel variant identified in the region 3' of the poly-A addition site, causing a C to T substitution 33 nucleotides after the end of exon 3 (exon 3+33). This variant was present in an individual who was also heterozygous for both the C282Y and H63D mutations of the *HFE* gene.

CYBRD1 gene

The previously described polymorphism S266N (McKie et al. 2001) within exon 4 of *CYBRD1* was identified at a high frequency in both study populations, with similar frequencies in the respective control populations (Table 2). Additionally, we identified a new polymorphism within intron 2, causing a T to C transversion (IVS2+8), which was also common in our study population. Three novel sequence changes were restricted to the patient group: a C to G transversion within intron 1 (IVS1-4C→G); a G to A substitution at codon 226 (g.776G→A) resulting in an arginine to histidine substitution (R226H); a G to C transversion within intron 3 (IVS3-32). The IVS1-4C→G variant was identified in four of 56 (7.1%) and mutation R226H was identified in one of 56 (1.8%) Caucasian patient referrals. Variant IVS3-32G→C was identified in four of 11 (36.4%) Black HH patients and was absent in the population-matched control group. A poly-T region within intron 2 showed high variability in our study population and ranged from 12 to 21 Ts in this region. No statistically significant associations were observed for the polymorphisms identified in the *CYBRD1* gene, although the mutations only identified in patients and not in controls might contribute to the disease phenotype.

Discussion

Polymorphisms are sequence variations present in the general population without an obvious effect on protein function. However, several studies have recently demonstrated the involvement of low-penetrance polymorphisms in the clinical expression of complex diseases (Borrego et al. 1999; Cambien et al. 1999; Cargill et al. 1999; Fitze et al. 1999; Yang et al. 2001; Rosenberg et al. 2002). In this study, statistically significant associations have been observed for many of the polymorphisms identified,

especially in the Black South African population. Because of small sample size, these observations could be attributable to chance, although the possibility of mutations with low penetrance contributing to the disease phenotype cannot be ruled out. The likelihood that the polymorphisms identified may be in linkage disequilibrium with other disease-causing loci should also be considered. Functional studies need to be performed to elucidate the role of these polymorphisms, which potentially contribute to the haemochromatosis phenotype in the Black South African population.

Mutations identified in genes other than *HFE* that were absent from the control population were identified in 11 of 67 (16%) patients investigated by dHPLC analysis. These included six of 56 (11%) in the Caucasian population, viz. *HAMP*: exon 3+33C→T (one of 56, 2%), *CYBRD1*: IVS1-4C→G (four of 56, 7%) and R226H (one of 56, 2%), and five of 11 (45%) in the Black population, viz. *SLC40A1*: D270V (one of 11, 9%) and *CYBRD1*: IVS3-32G→C (four of 11, 36%). These mutations were identified in different individuals and none had a combination of the variants identified.

The patient with the D270V mutation, a 27-year-old Black female presenting with fatigue, had high transferrin saturation (78%, reference range 15–50%) and serum-iron (48.3 $\mu\text{mol/l}$, reference range 6.6–26 $\mu\text{mol/l}$) levels upon biochemical analysis. As her serum ferritin level was below 1,000 $\mu\text{g/l}$, she has not had a liver biopsy. Since she is a young female of childbearing age, her serum ferritin and transferrin saturation levels are being closely monitored. She has not been venesected. Her menstrual cycle has become irregular and, as a consequence, she has now become anaemic.

The D270V mutation occurs between the fourth and fifth transmembrane domains in the model of Devalia et al. (2002). Many of the *SLC40A1* mutations identified to date map to potential external loops in this model. One exception is the mutation described by Jouanolle et al. (2003), G490D, found in a 54-year-old Asian female who presented with asthenia and a very high serum ferritin concentration (8,943 $\mu\text{g/l}$) with a transferrin saturation of 53%. The Q248H polymorphism found in African Americans recently described by Gordeuk et al. (2003) maps to the same cytoplasmic loop as does the D270V mutation described here. The phenotype associated with these two sequence changes is different. Our patient, a young adult female, has raised transferrin saturation (78%), whereas the patients described by Gordeuk et al. (2003) has a mean transferrin saturation of 24%. The Q248H polymorphism is not associated with raised transferrin saturation and may be associated with mild anaemia and a tendency to iron loading (Gordeuk et al. 2003), whereas our patient has raised serum iron indices and no anaemia. Our patient also presented with high serum γ -glutamyl transferase (37 U/l, reference range 0–32 U/l) and serum-bilirubin (unconjugated; 12 $\mu\text{mol/l}$, reference range 0–10 $\mu\text{mol/l}$).

A likely candidate for contributing to iron overload in the Black South African population is the (CGG)_n

microsatellite identified in the 5' untranslated region (5'UTR) of the *SLC40A1* gene. Here, we have identified seven [(CGG)₇], eight [(CGG)₈] and nine [(CGG)₉] repeats in the Black South African population and a marginally significant association has been observed for this polymorphism. Of particular interest is the g.-23A→G polymorphism, identified in eight of 11 (72.7%) Black HH patients and one of 20 (5%) controls in the same population; this polymorphism appears to be in *cis* with the (CGG)₇ allele. It would be interesting to determine whether an allelic effect similar to that recently observed for the -237C→T polymorphism in the *SLC11A1* gene applies, where expression of allele 3 of the promoter (GT)_n repeat is reverted in the presence of this variant, resulting in expression profiles similar to that of allele 2 (Zaahl et al. 2004). The possible effect of silent mutations identified in the *SLC40A1* gene, viz. I109 and L129, should also be considered. Although these mutations are normally classified as neutral polymorphisms, some of these silent mutations have been shown to have an effect on the mRNA level and therefore affect the translated product (Cartegni et al. 2002).

The patient with the *HAMP* exon3+33C→T mutation, a 23-year-old Caucasian male, presented with high-normal serum ferritin levels of 226.8 µg/l and was found to be heterozygous for both the C282Y and H63D mutations, which are rarely (<1% of cases) associated with iron overload. Subsequent analysis showed that his father and younger brother were H63D heterozygotes and his mother and other brother were heterozygous for both the C282Y and H63D mutations. The *HAMP* exon3+33C→T mutation was also present in the proband's father (H63D heterozygote) who presented with normal iron status. This sequence variant is therefore unlikely to influence iron status.

Intronic mutations identified in the *CYBRDI* (IVS1-4C→G, IVS3-32G→C) gene may cause splicing defects resulting in the abolishment or at least a reduction in the amount of mature mRNA generated. It has been estimated that 15% of all point mutations in an intron result in a mRNA splicing defect, causing human disease (Krawczak et al. 1992). The *CYBRDI* IVS3-32G→C variant could affect splicing since it is included within a branch-site area (Breathnach and Chambon 1981; Mount 1982; Padgett et al. 1986). The IVS1-4C→G variant identified in *CYBRDI* precedes an exon encoding for a region of b561 that is thought to be related to substrate binding or recognition and variant IVS3-32G→C precedes the exon encoding the sixth transmembrane domain.

Alternative splicing of many pre-mRNAs is also affected by the intracellular concentrations of antagonistic splicing factors of the SR family and hnRNAPA1 (Zahler et al. 1993; Caceres et al. 1994; Muro et al. 1999). Predictive results for the effect of the R226H variant, located at the C-terminal of the *CYBRDI* gene, on these factors were generated by using the ESEfinder (ESE—exonic splice element) program (<http://exon.cshl.edu/ESE/>). A SF2/ASF and SC35 binding site was abolished in the presence of the mutated allele (A), which might

result in generating aberrant mRNAs that are either unstable or code for defective or deleterious protein isoforms. *CYBRDI* is responsible for duodenal ferric reductase activity, with protein and mRNA levels increasing under iron deficiency conditions and decreasing upon iron loading, thereby regulating iron status (McKie et al. 2001, 2002). The mutations identified in *CYBRDI* could possibly disrupt the gene product, resulting in an inability of the gene to respond to intracellular iron levels. Functional studies need to be performed to elucidate the role of all the mutations identified and to confirm the results obtained with the ESEfinder program.

Autosomal recessive inheritance is well known as being associated with *HFE* mutations (Feder et al. 1996), whereas mutations in the *SLC40A1* gene are associated with an autosomal dominant form of inheritance (Njajou et al. 2001; Montosi et al. 2001; Wallace et al. 2002; Devalia et al. 2002; Roetto et al. 2002). Iron overload in African patients could not be ascribed to the common *HFE* mutations but possible mutations in the *HFE* promoter region, affecting regulation of the gene, and the possible involvement of *SLC40A1* and *CYBRDI* warrant further investigation in this context. The data presented are in accordance with various other studies, demonstrating the significance of multiple genes in iron regulation.

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References

- Adams PC, Campion ML, Gandon G, Le Gall J-Y, David V, Jouanolle A-M (1997) Clinical and family studies in genetic hemochromatosis: microsatellite and *HFE* studies in five atypical families. *Hepatology* 26:986–990
- Bacon BR, Sadiq SA (1997) Hereditary hemochromatosis: presentation and diagnosis in the 1990s. *Am J Gastroenterol* 92:784–789
- Beutler E, West C (1997) New diallelic markers in the HLA region of chromosome 6. *Blood Cells Mol Dis* 23:219–229
- Borrego S, Saez ME, Ruiz A, Gimm O, Lopez-Alonso M, Antinolo G, Eng C (1999) Specific polymorphisms in the *RET* proto-oncogene are over-represented in patients with Hirschsprung disease and may represent loci modifying phenotypic expression. *J Med Genet* 36:771–774
- Bothwell TH, Charlton RW, Motulsky AG (1995) Hemochromatosis. In: Scriver CR, Scriver AL, Sly WS, Valle D (eds) *The metabolic and molecular basis of inherited disease*, 7th edn. McGraw-Hill, New York, pp 2237–2269
- Breathnach R, Chambon P (1981) Organisation and expression of eukaryotic split genes coding for proteins. *Annu Rev Biochem* 50:349–383
- Caceres JF, Stamm S, Helfman DM, Krainer AR (1994) Regulation of alternative splicing *in vivo* by overexpression of antagonistic splicing factors. *Science* 265:1706–1709
- Camaschella C, Roetto A, Cali A, De Gobbi M, Garozzo G, Carella M, Majorano N, Totaro A, Gasparini P (2000) The gene *TFR2* is mutated in a new type of haemochromatosis mapping to 7q22. *Nat Genet* 25:14–15

- Cambien F, Poirier O, Nicaud V, Herrmann SM, Mallet C, Ricard S, Behague I, Hallet V, Blanc H, Loukaci V, Thillet J, Evans A, Ruidavets JB, Arveiler D, Luc G, Tired L (1999) Sequence diversity in 36 candidate genes for cardiovascular disorders. *Am J Hum Genet* 65:183–191
- Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N, Shaw N, Lane CR, Lim EP, Kalyanaraman N, Nemesh J, Ziaugra L, Friedland L, Rolfe A, Warrington J, Lipshutz R, Daley GQ, Lander ES (1999) Characterisation of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet* 22:231–238
- Cartegni L, Chew SL, Krainer AR (2002) Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev* 3:285–298
- Cazzola M, Cremonesi L, Papaioannou M, Soriani N, Kioumi A, Charalambidou A, Paroni R, Romtsou K, Levi S, Ferrari M, Arosio P, Christakis J (2002) Genetic hyperferritinaemia and reticuloendothelial iron overload associated with a three base pair deletion in the coding region of the ferroportin gene (*SLC11A3*). *Br J Haematol* 119:539–546
- De Villiers JN, Hillermann R, Loubser L, Kotze MJ (1999) Spectrum of mutations in the *HFE* gene implicated in haemochromatosis and porphyria. *Hum Mol Genet* 8:1517–1522
- Devalia V, Carter K, Walker AP, Perkins SJ, Worwood M, May A, Dooley JS (2002) Autosomal dominant reticuloendothelial iron overload associated with a 3-base pair deletion in the ferroportin 1 gene (*SLC11A3*). *Blood* 100:695–697
- Douabin-Gicquel V, Soriano N, Ferran H, Wojcik F, Paliere E, Tamim S, Jovelin T, McKie AT, Le Gall JY, David V, Mosser J (2001) Identification of 96 single nucleotide polymorphisms in eight genes involved in iron metabolism: efficiency of bioinformatic extraction compared with a systematic sequencing approach. *Hum Genet* 109:393–401
- Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R Jr, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Wolff RK (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* 13:399–408
- Fitze G, Schreiber M, Kuhlisch E, Schackert HK, Roesner D (1999) Association of *RET* protooncogene codon 45 polymorphism with Hirschsprung disease. *Am J Hum Genet* 65:1469–1473
- Gordeuk VR, Caleffi A, Corradini E, Ferrara F, Jones RA, Castro O, Onyekwere O, Kittles R, Pignatti E, Montosi G, Garuti C, Gangaidzo IT, Gomo ZAR, Moyo VM, Rouault TA, Macphail P, Pietrangelo A (2003) Iron overload in Africans and African-Americans and a common mutation in the *SLC40A1* (ferroportin 1) gene. *Blood Cell Mol Dis* 31:299–304
- Henz S, Reichen J, Liechti-Gallati S (1997) HLA-H gene mutations and haemochromatosis: the likely association of H63D with mild phenotype and the detection of S65C, a novel variant in exon 2. *J Hepatol* 26:57
- Hetet G, Devaux I, Soufir N, Grandchamp B, Beaumont C (2003) Molecular analyses of patients with hyperferritinemia and normal serum iron values reveal both L ferritin IRE and 3 new ferroportin (*slc11a3*) mutations. *Blood* 102:1904–1910
- Höhler T, Leininger S, Schneider PM (1999) A new polymorphism in the human *HFE* gene. *Immunogenetics* 49:823–824
- Jouanolle AM, Douabin-Gicquel V, Halimi C, Loreal O, Fergelot P, Delacour T, Lajarte-Thirouard AS de, Turlin B, Le Gall JY, Cadet E, Rochette J, David V, Brissot P (2003) Novel mutation in ferroportin 1 gene is associated with autosomal dominant iron overload. *J Hepatol* 39:286–289
- Kotze MJ, Villiers JNP de, Bouwens CSH, Warnich L, Zaahl MG, Merwe S van der, Oberkanins C (2004) Molecular diagnosis of hereditary hemochromatosis: application of a newly-developed reverse-hybridization assay in the South African population. *Clin Genet* 65:317–321
- Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, Adermann K (2000) LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. *FEBS Lett* 480:147–150
- Krawczak M, Reiss J, Cooper DN (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum Genet* 90:41–54
- Lee PL, Gelbart T, West C, Halloran C, Felitti V, Beutler E (2001) A study of genes that may modulate the expression of hereditary hemochromatosis: transferrin receptor-1, ferroportin, ceruloplasmin, ferritin light and heavy chains, iron regulatory proteins (IRP)-1 and -2, and hepcidin. *Blood Cells Mol Dis* 27:783–802
- McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A, Peters TJ, Raja KB, Shirali S, Hediger MA, Farzaneh F, Simpson RJ (2001) An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* 291:1755–1759
- McKie AT, Latunde-Dada GO, Miret S, McGregor JA, Anderson GJ, Vulpe CD, Wrigglesworth JM, Simpson RJ (2002) Molecular evidence for the role of a ferric reductase in iron transport. *Biochem Soc Trans* 30:722–724
- Merryweather-Clarke AT, Cadet E, Bomford A, Capron D, Viprakasit V, Miller A, McHugh PJ, Chapman RW, Pointon JJ, Wilmhurst VLC, Livesey KJ, Tanphaichitr V, Rochette J, Robson KJH (2003) Digenic inheritance of mutations in *HAMP* and *HFE* results in different types of haemochromatosis. *Hum Mol Genet* 12:2241–2247
- Milani MY, Kotze MJ (1999) Molecular diagnosis of hereditary haemochromatosis—identify an affected person and save a family. *S Afr Med J* 89:263–264
- Miller S, Dykes D, Polesky H (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215
- Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Cassanelli S, Trenor CC, Gasparini P, Andrews NC, Pietrangelo A (2001) Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (*SLC11A3*) gene. *J Clin Invest* 108:619–623
- Mount S (1982) A catalogue of splice junction sequences. *Nucleic Acids Res* 10:459–472
- Muro AF, Caputi M, Pariyarath R, Pagani F, Buratti E, Baralle FE (1999) Regulation of fibronectin EDA exon alternative splicing: possible role of RNA secondary structure for enhancer display. *Mol Cell Biol* 19:2657–2671
- Nicolas G, Bennoun M, Devaux I, Beaumont C, Grandchamp B, Kahn A, Vaulont S (2001) Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proc Natl Acad Sci USA* 98:8780–8785
- Njajou OT, Vaessen N, Joosse M, Berghuis B, Dongen JW van, Breuning MH, Snijders PJ, Rutten WP, Sandkuijl LA, Oostra BA, Duijn CM van, Heutink P (2001) A mutation in *SLC11A3* is associated with autosomal dominant hemochromatosis. *Nat Genet* 28:213–214
- Padgett R, Grabowski P, Konarska M, Seiler S, Sharp P (1986) Splicing of messenger RNA precursors. *Annu Rev Biochem* 55:1119–1150
- Park CH, Valore EV, Waring AJ, Ganz T (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* 276:7806–7810
- Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O (2001) A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem* 276:7811–7819
- Pointon JJ, Wallace D, Merryweather-Clarke AT, Robson KJH (2000) Uncommon mutations in the hemochromatosis gene. *Genet Test* 4:151–161

- Powell LW, Jazwinska EC, Halliday JW (1994) Primary iron overload. In: Brock JH, Halliday JW, Pippard MJ, Powell LW (eds) Iron metabolism in health and disease. Saunders, London, pp 228–270
- Rhodes D, Raha-Chowdhury R, Cox TM, Trowsdale J (1997) Homozygosity for the predominant Cys282Tyr mutation and absence of disease expression in hereditary haemochromatosis. *J Med Genet* 34:761–764
- Rivard SR, Lanzara C, Grimard D, Carella M, Simard H, Ficarella R, Simard R, D'Adamo AP, De Braekeleer M, Gasparini P (2003) Autosomal dominant reticuloendothelial iron overload (HFE type 4) due to a new missense mutation in the *FERROPORTIN 1* gene (*SLC11A3*) in a large French-Canadian family. *Haematologica* 88:824–826
- Rochette J, Pointon JJ, Fisher CA, Perera G, Arambepola M, Arichchi DS, De Silva S, Vandwalle JL, Monti JP, Old JM, Merryweather-Clarke AT, Weatherall DJ, Robson KJ (1999) Multicentric origin of hemochromatosis gene (*HFE*) mutations. *Am J Hum Genet* 64:1056–1062
- Roetto A, Totaro A, Piperno A, Piga A, Longo F, Garozzo G, Cali A, De Gobbi M, Gasparini P, Camaschella C (2001) New mutations inactivating transferrin receptor 2 in hemochromatosis type 3. *Blood* 97:2555–2560
- Roetto A, Merryweather-Clarke AT, Daraio F, Livesey K, Pointon JJ, Barbabietola G, Piga A, Mackie PH, Robson KJ, Camaschella C (2002) A valine deletion of ferroportin 1: a common mutation in hemochromatosis type 4. *Blood* 100:733–734
- Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, Loukopoulos D, Camaschella C (2003) Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. *Nat Genet* 33:21–22
- Rosenberg N, Murata M, Ikeda Y, Opare-Sem O, Zivelin A, Geffen E, Seligssohn U (2002) The frequent 5,10-methylenetetrahydrofolate reductase C677T polymorphism is associated with a common haplotype in whites, Japanese, and Africans. *Am J Hum Genet* 70:758–762
- Sachot S, Moirand R, Jouanolle AM, Mosser J, Fergelot P, Deugnier Y, Brissot P, Gall JY le, David V (2001) Low penetrant hemochromatosis phenotype in eight families: no evidence of modifiers in the MHC region. *Blood Cells Mol Dis* 27:518–529
- Wallace DF, Pedersen P, Dixon JL, Stephenson P, Searle JW, Powell LW, Subramaniam VN (2002) Novel mutation in ferroportin 1 is associated with autosomal dominant hemochromatosis. *Blood* 100:692–694
- Worwood M, Shearman JD, Wallace DF, Dooley JS, Merryweather-Clarke AT, Pointon JJ, Rosenberg WMC, Bowen DJ, Burnett AK, Jackson HA, Lawless S, Raha-Chowdhury R, Partridge J, Williams R, Bomford A, Walker AP, Robson KJH (1997) A simple genetic test identifies 90% of UK patients with hemochromatosis. *Gut* 41:841–844
- Yang Z, Zhu T, Ma G, Yin H, Qian W, Zhang F, Cao K, Ma W (2001) Apolipoprotein E polymorphism in the early onset of coronary heart disease. *Chin Med J* 114:983–985
- Zaahl MG, Robson KJH, Warnich L, Kotze MJ (2004) Differential expression of the 5'-(GT)_n repeat in the *SLC11A1* gene: opposite allelic effect in the presence of the -237C→T polymorphism. *Blood Cells Mol Dis* 33:45–50
- Zahler AM, Neugebauer KM, Lane WS, Roth MB (1993) Distinct functions of SR proteins in alternative pre-mRNA splicing. *Science* 260:219–222