DNA TESTING FOR HAEMOCHROMATOSIS: DIAGNOSTIC, PREDICTIVE AND SCREENING IMPLICATIONS

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Summary
Since 1996, the identification of the HFE gene has enabled DNA testing for hereditary haemochromatosis (HH). The range of DNA testing available includes: (1) diagnostic, (2) predictive (also called presymptomatic testing) and (3) screening. Access to DNA testing has been facilitated by an Australian Medicare rebate, the first available for genetic disorders. Despite the availability of HFE DNA testing in HH, it remains necessary to interpret results in the context of the clinical picture. Traditional markers based on phenotype (transferrin saturation, ferritin and liver biopsy) are still required in some circumstances. We report our experience with HFE DNA testing using a semi-automated approach, which allows multiplexing for the two common mutations (C282Y and H63D). Screening a cohort of β-thalassaemia major and sickle cell anaemia patients of predominantly Mediterranean origin showed that these individuals do not have the common C282Y mutation. This excluded C282Y as a factor in the pathogenesis of iron overload in these haemoglobinopathies. It also showed that the C282Y mutation is of limited value when investigating HH in certain ethnic groups. An Australian family studied illustrated the relative contribution of C282Y and H63D in iron overload. A recently reported third mutation (S65C) in the HFE gene was detected in a low frequency in the populations tested. 

Key words: HFE, haemochromatosis, DNA, C282Y, H63D, S65C, thalassaemia

Abbreviations: HH, hereditary haemochromatosis; C282Y, cysteine changing to tyrosine at amino acid 282; H63D, histidine changing to aspartic acid at amino acid 63; S65C, serine changing to cysteine at amino acid 65; HFE, hereditary haemochromatosis gene.

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INTRODUCTION
Haemochromatosis was first described by Trousseau in 1865.1 It was not until 1935 that the hereditary nature of this disorder was noted by J. Sheldon. In 1996, the gene for haemochromatosis was isolated by workers in a biotechnology company.2 The hereditary haemochromatosis (HH) gene was first called HLA–H because of its position within the HLA complex on the short arm of chromosome 6, and its similarity to HLA class I genes. However, HFE is now the official designation since the gene is not strictly a component of the HLA system, and a pseudogene within the HLA complex had already been named HLA-H. In Australia, the final development in this very brief historical review of HH occurred in 1998, when HFE DNA testing for the C282Y mutation was added to the Medicare schedule. This represented the first acknowledgement by Medicare that DNA testing for genetic disorders existed, although this technology had been in use since the early 1980s. To limit unnecessary DNA testing, pre-requisites were included before C282Y testing would be reimbursed through Medicare. These were: (1) the patient had an elevated transferrin saturation or elevated serum ferritin on testing of repeated specimens, or (2) the patient had a first-degree relative with haemochromatosis or with homozygosity for the C282Y genetic mutation, and (3) only one test was allowed within a three-year period.

HH is inherited as an autosomal recessive trait. However, the mode of inheritance is more complex since the environment and other genetic factors appear to play a role in the natural history of HH. The sex ratio of clinical haemochromatosis is not equal, as would be expected in an autosomal recessive disorder. Female to male ratios varying from 11% (German) to 35% (Canadian) have been reported in those with clinical symptoms.3 The sex difference is thought to reflect the loss of iron through menstruation, although this may not be the total explanation. Another factor that might interfere with the development of HH is regular blood donation. Risk factors for the development of HH (or its complications) include excessive intake of alcohol, iron or vitamin C and racial background.3,4 In respect of the latter, HH occurs worldwide, but is most common in those of northern European background, particularly Nordic or Celtic origin. In Australia, Jazwinska et al. showed a strong founder effect for HH with the majority of patients being of Celtic origin.5

Genetic heterogeneity is also found in HH. A juvenile form of haemochromatosis exists. This differs from the typical HH in its earlier age of onset and subsequent more severe expression. Both sexes are affected equally, and genetic studies have excluded the HLA locus as the site for the disorder indicating that juvenile HH is genetically distinct. The genetic locus for the juvenile form has now been localised to chromosome 1q. The gene at this site remains to be identified.6 There is also some preliminary evidence that the more common adult-onset HH may not always be associated with mutations within the HFE gene. This follows from a study in which the HFE was extensively characterised in a cohort of Italians with HH. Mutation analysis including DNA sequencing of HFE exons

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failed to find any abnormalities. The authors concluded that HH was heterogeneous and perhaps another locus could be involved. An alternative explanation given was that parts of the HFE gene that had not been characterised (intron segments, untranslated regions or regulatory elements) might be the site for mutations.

HH is considered to be one of the most common autosomal recessive disorders in Caucasians. The prevalence of HH varies from about two to five per 1000 in different populations, with a commonly reported carrier frequency of about 10%. Two mutations in the HFE gene (C282Y and H63D) are associated with HH, although studies of clinically affected individuals show that up to a third of these are only heterozygous for one of the two mutations, or had no detected HFE mutation. The geographical distribution of the two mutations is also noteworthy with the highest frequencies for C282Y being reported in northern Europeans consistent with the theory of a north European origin for the mutation. The C282Y mutation is absent in African, Asian, Australian aborigines and Melanesians. On the other hand, the H63D defect is more widely distributed, although its risk for the development of HH is considerably less.

DNA testing for the C282Y mutation, and, in some laboratories the H63D mutation, is now routinely undertaken. Experience gained to date has shown that the genotypic test (i.e. the DNA test) has not totally replaced the more traditional phenotypic tests (transferrin saturation, serum ferritin and liver biopsy), which remain the gold standards for iron overload. A key unresolved issue concerns the clinical significance of the DNA mutations since homozygosity for the C282Y defect does not guarantee that an individual will develop HH. Finally, there is considerable debate within the medical community of the relative merits of widespread population screening using the C282Y mutation to detect preclinically those who are homozygous for C282Y, and so at risk for developing HH.

We report our experience with DNA testing for the two common HFE mutations, as well as a third (S65C), which has recently been described. In addition to patients referred for DNA testing, we have assessed the frequency of HFE mutations in β-thalassaemia major and sickle cell anaemia patients and β-thalassaemia carriers. Some observations on technical aspects of DNA mutation testing have also emerged from this study.

MATERIALS AND METHODS

Patients and specimens

DNA was isolated from anticoagulated blood using the QIAamp 96 Spin Blood Kit (QIAGEN Pty Ltd, Clifton Hill, VIC). With this method approximately 200 μl of blood gives 3–8 μg of DNA, and up to 96 samples can be processed simultaneously. Individuals tested included 146 β-thalassaemia major and eight sickle cell anaemia patients; 50 obligatory β-thalassaemia carriers, an Australian family with HH, and 247 random samples referred to the laboratory for HFE testing.

Mutation detection

The C282Y and H63D mutations were detected simultaneously by a multiplex oligonucleotide ligation assay (OLA). The OLA technique involves a two-step process with an initial DNA amplification followed by hybridisation of probes to the amplicon and ligation of the two adjacent probes. Genomic DNA was amplified with two sets of unlabelled primers to produce templates for the ligation assay. PCR primers for amplification of that segment of exon 4 which contained the C282Y mutation were: 5'-aGAGTTCAACCTCAAAGACGT-3' and 5'-TCTTCATCTCTACCCATAA-3'. For the segment of exon 2 containing the H63D mutation: 5'-TCTCCAGGTTCACTCTCTGC-3' and 5'-CCATAATAGTCCAGAAGTCACAAC-3'. Oligonucleotide probes for the OLA reaction are summarised in Fig. 1. Ligation products could be analysed by capillary electrophoresis in approximately 15 min, and six genotypes produced by the two mutations in the HFE gene were easily distinguished.

Mutation detection for the S65C defect was more conventional and involved amplification of a segment of DNA with the same primers used for the H63D mutation, followed by digestion of the 250-bp product with the restriction enzyme HinfI. The wild-type fragment produced three fragments of 123, 69 and 52 bp while the mutant (S65C) allele, which deletes a HinfI site, produced only two fragments of 192 and 52 bp.

RESULTS

Haemoglobinopathy patients and β-thalassaemia carriers

The results of DNA testing for the C282Y and H63D mutations in 148 patients with β-thalassaemia major and six with homozygous HbS are summarised in Table 1. These confirm that the C282Y mutation is rare (0.32%) in this population group which predominantly involved individuals of Mediterranean origin (80 Greek, 38 Italian, 13 Middle Eastern, 13 Indian/Chinese, 10 unknown). Similar results (1.0%) were obtained in 25 Mediterranean couples unrelated to the haemoglobinopathy patients, but known to be β-thalassaemia carriers. The H63D defect was relatively more common in these two groups, 14.6 and 11.0%, respectively. χ² analysis showed no significant difference in the genotypic profiles between patients with hemoglobinopathy or the β-thalassaemia carrier group when compared to normal Italian or Greek populations reported previously. In the two cohorts tested, there was a statistically significant difference between the distribution of the genotypes in a British population and the local haemoglobinopathy patients (P < 0.001) or the local β-thalassaemia carriers (P < 0.01) (Table 1).

Implications of C282Y and H63D mutations in a family study

An Australian family of Celtic origin was referred for evaluation of HFE testing. The pedigree is shown in Fig. 2. The propositus (II-1) is indicated by →. His ferritin level of 1200 μg/l is consistent with homozygosity for C282Y. He has a brother (II-5) who has a lower ferritin at 300 μg/l and is a double heterozygote for both C282Y and H63D. The father (I–2) is alive and well aged 82, and carries only C282Y mutant allele. Therefore, the deceased mother must have been a double-heterozygote for both mutations (C282Y and H63D). The mother was apparently well until her mid-70s when she developed cardiac failure, which appeared to be refractory to treatment.

S65C mutation in HH

There were 247 samples referred to the laboratory for DNA testing because of clinical/laboratory features that suggested HH or on the basis of a positive family history, and which were not found to be homozygous for the C282Y and H63D defects or double-heterozygotes for C282Y/H63D, were

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tested further for the S65C defect. Seven of 494 chromosomes were shown to be positive for this mutation (frequency 0.01). Some of these were also carriers for the C282Y defect (Table 2). Insufficient clinical information was provided to determine the significance of the S65C defect in relationship to HH. Only three of the haemoglobinopathy and β-thalassaemia carriers tested (total of 408 chromosomes) were heterozygous for the S65C mutation. The three were also heterozygous for the H63D mutation.

**Technical considerations**

There are many different approaches used to identify mutations in DNA. We initially utilised the more conventional enzyme digestion approach to detect HFE defects, and also hybridisation-based methods such as ASO (allele specific oligonucleotides). However, these are difficult to automate, and so the OLA technique is now preferred. This, and the availability of multicolour fluorescence and capillary electrophoresis, has meant that multiplexing is easy to arrange. Should it be necessary to detect the S65C defect on a routine basis, it would not be difficult to develop an oligonucleotide ligation assay using the same amplicon for H63D (Fig. 3). A recent report describing how a polymorphism in intron 4 of the HFE gene can lead to over-estimation of C282Y homozygotes (from an initial 31 homozygotes in 5211 voluntary blood donors to the correct number of 16 homozygotes) is sobering, but contro-

![Fig. 1 Oligonucleotides (P1–6) for the OLA-based PCR test are given. The normal DNA sequences in the vicinity of the C282Y and H63D mutations are boxed. For each mutation, a common probe (P1 and P4) is required, which then allows mutant or wild-type probes to anneal to target DNA. The six various combinations possible with these two mutations are distinguishable either on the basis of colour (TET, HEX and 6FAM dyes attached to the oligonucleotides), or size. For the latter, (– – a) represents a variable number of adenine nucleotides which have been added to oligonucleotides to change the size of different ligation products. Small case letters in the oligonucleotide sequences represent regions in which changes from the wild-type sequence were specifically introduced to enhance the efficiency of ligation by preventing the development of secondary structures. (P or p) 5’ phosphate groups which are required for the ligation reaction.](image-url)

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**Table 1** Genotypic profiles for various population groups tested for the C282Y and H63D mutations in the HFE gene

<table>
<thead>
<tr>
<th>Group/number</th>
<th>HH CC genotype</th>
<th>HD CC genotype</th>
<th>DD CC genotype</th>
<th>HH CY genotype</th>
<th>HD CY Genotype</th>
<th>HH YY genotype</th>
<th>H63D mutant allele frequency</th>
<th>C282Y mutant allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>British population, 413 b</td>
<td>272</td>
<td>90</td>
<td>0</td>
<td>33</td>
<td>16</td>
<td>2</td>
<td>12.8</td>
<td>6.4</td>
</tr>
<tr>
<td>Italian population, 91 b</td>
<td>69</td>
<td>19</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>12.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Greek population, 196 b</td>
<td>144</td>
<td>41</td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>13.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Haemoglobinopathy patients, 154 c</td>
<td>116</td>
<td>29</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>14.6</td>
<td>0.32</td>
</tr>
<tr>
<td>Thalassaemia carriers, 50 c</td>
<td>39</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>11.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

- HH, H63D normal; HD, H63D heterozygote; DD, H63D homozygote; CC, C282Y normal; CY, C282Y heterozygote; YY, C282Y homozygote.
- Data from Merryweather-Clarke et al.
- Results from the present study.
versial.\textsuperscript{12,13} Primers used in our assay did not cover the region in intron 4, which has this polymorphism, and so avoid a potential problem with PCR, which is related to how efficiently primers can bind to target DNA.

**DISCUSSION**

The identification of the *HFE* gene has provided an opportunity for DNA testing in patients considered to have HH. The finding that an individual is homozygous for the C282Y defect places that person at risk for the development of HH. Current estimates are that more than 90% of C282Y homozygotes will develop significant degrees of iron overload compared with fewer than 5% of compound heterozygotes for C282Y and H63D.\textsuperscript{14} Because of environmental and perhaps even genetic factors mentioned previously, the risk for development of *clinical* haemochromatosis based on genotypic (DNA) data cannot be 100%, nor can this type of test predict the severity of iron overload. Follow-up or further phenotypic investigations to determine the iron status (transferrin saturation, serum ferritin) and perhaps liver biopsy to assess for liver damage, are needed before starting treatment. Facts to emerge from DNA testing are summarised in Table 3. In terms of iron overload, the family in Fig. 2 illustrates the relative severity of the homozygous C282Y defect compared to a double-heterozygote for C282Y and H63D. It is interesting to speculate that late-onset cardiac failure in the deceased mother of the propositus also reflected her underlying *HFE* genotype (C282Y/H63D).

The importance of the ethnic background in interpreting the significance of the C282Y mutation is illustrated by the low yield of this defect in our haemoglobinopathy patients as well as the β-thalassaemia carriers. Initially, we studied the haemoglobinopathy group to see if either of the *HFE* mutations might explain why some patients had a more severe or milder clinical picture. However, there is no evidence that *HFE* mutations play a role in pathogenesis in a group of disorders that predispose individuals to iron overload. This is based on the frequencies of the C282Y and the H63D mutations in our patient groups, which were no different to what is found in a control group of normal individuals from the same ethnic backgrounds. A similar finding has been reported in an Italian cohort of thalassaemia major patients.\textsuperscript{15}

We are referred many samples for *HFE* testing where the person has an Asian, Middle Eastern or southern European surname. In these cases, the failure to find the C282Y defect does not exclude the diagnosis of HH. Similarly, the finding of the H63D defect (heterozygous or homozygous) has little clinical significance unless it is associated with the C282Y mutation. It is important that the laboratory provides this information in its reporting format. Since family names within Australia’s multicultural communities are not necessarily helpful in identifying ethnic background, the advice may need to be generic. The very low frequency for the

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**Table 2** The *HFE* S65C mutation in patients referred for DNA testing.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>C282Y</th>
<th>H63D</th>
<th>S65C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC 1117</td>
<td>CY\textsuperscript{a}</td>
<td>HH</td>
<td>SC</td>
</tr>
<tr>
<td>HC 1070</td>
<td>CY</td>
<td>HH</td>
<td>SC</td>
</tr>
<tr>
<td>HC 1042</td>
<td>CC</td>
<td>HH</td>
<td>SC</td>
</tr>
<tr>
<td>HC 1157</td>
<td>CC</td>
<td>HH</td>
<td>SC</td>
</tr>
<tr>
<td>HC 929</td>
<td>CY</td>
<td>HH</td>
<td>SC</td>
</tr>
<tr>
<td>HC 935</td>
<td>CC</td>
<td>HH</td>
<td>SC</td>
</tr>
<tr>
<td>HC 981</td>
<td>CC</td>
<td>HH</td>
<td>SC</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Abbreviations are the same as for Table 1. SC, heterozygote for the S65C defect.
S65C *HFE* defect in the present study in three groups of subjects would not justify its inclusion in the routine protocol. Because S65C has been implicated in mild HH, it might still be worthwhile testing for this mutation in those who are clinically affected, but lack the other two mutations. However, this will need to be determined on an individual basis.

HH is a useful model to discuss the advantages and disadvantages of DNA testing in the detection of genetic disorders. In the advantages category, genotypic (DNA) testing is very versatile because it can be used in a number of situations: (1) *diagnostic* (to assist the clinician in making a diagnosis of haemochromatosis, and perhaps avoid the use of liver biopsy in those who have no evidence of liver damage), (2) *presymptomatic* (to identify at-risk family members who have a relative with HH), (3) *community carrier testing* (to screen communities to detect those who are carriers), and (4) *prenatal diagnosis*. The latter would not be an appropriate use of *HFE* DNA testing. The first two indications are adequately covered by the Medicare pre-requisites for C282Y testing. The place of *HFE* DNA testing in community screening is controversial. The prognosis for haemochromatosis, and most of its complications including hepatocellular carcinoma, depends on the amount and duration of iron overload. Early detection and effective therapy have been shown to be beneficial particularly if the diagnosis is made before there is extensive iron accumulation and organ damage.\(^{14,15}\) This has led to considerations of population screening, including the option to screen neonates as part of the newborn screening programs. However, a consensus remains to be reached with some indicating that population screening should not be implemented until it is clearly known what proportion of individuals with HH (based on DNA testing) will go on to develop serious clinical problems. On the other hand, others point out that HH fulfils the WHO criteria for population screening, and so should be implemented.\(^{18,19}\)

At present, the most persuasive arguments are that genotypic (DNA) screening for HH is not indicated, and should screening be considered appropriate, the relatively cheap and more meaningful phenotypic assays might be preferred.\(^{3,18}\) Should population screening be undertaken, it would be necessary to ask who will be screened and when to screen? Looking for the C282Y defect in southern Europeans and some of the populations identified in Table 3 would not be cost-effective. Testing in the newborn period would make little sense if treatment were not required immediately, or clinical problems would not be expected until mid-adult life. In terms of screening logistics, the most efficient approach adopted in multicultural cities like Sydney and Melbourne might differ from that in regions

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**Table 3** Facts about DNA testing for HH\(^{14,15}\)

- The frequency of the C282Y mutation is highest in individuals of northwestern European origin, e.g., UK, west coast of Brittany, Ireland (10–20% of these populations are carriers for C282Y).
- C282Y is less frequent (2–4%) in southern or eastern European populations, and in some indigenous populations of Africa, Central/South America, Eastern Asia and Pacific islands.
- The H63D mutation is more common than C282Y and has a more global distribution with similar frequencies in most European countries (10–26%), but less in Africa, Indian subcontinent, Asia, the Americas and Pacific islands (0–8%).
- Homozygosity for C282Y is found in 60–100% (average 85%) of white patients with clinically diagnosed HH. The highest rates are in northern Europeans.
- In an Australian study, 17% of subjects homozygous for C282Y did not have other features of HH. There was a predominance of women in this group.
- Current predictions are that >90% of C282Y homozygotes will develop significant degrees of iron overload, compared to <5% of those who are double-heterozygotes for C282Y and H63D. The homozygous H63D defect has rarely been reported in association with HH.
- Unless there are other genetic or environmental factors involved, carriers for the C282Y or H63D defects will not develop iron overload.

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**Fig. 3** Capillary electrophoresis profile of DNA mutation testing for the C282Y and H63D defects. (a) Normal profile, (b) a heterozygote for C282Y, (c) heterozygote for H63D, (d) a double-heterozygote for C282Y and H63D defects, (e) a homozygote for C282Y and (f) a homozygote for H63D. Apart from the different profiles that are produced in this multiplex, the different mutations are also distinguished on the basis of colour, e.g., wild type and mutant alleles for the C282Y mutation appear as green and black peaks respectively (TET and HEX dyes) while the two alleles for H63D are coloured blue (6FAM dye).
with a more homogeneous population of predominantly Anglo-Celtic background.

Another consideration in screening is the knowledge that an asymptomatic individual who is found to be homozygous for C282Y now has a “disease”, although he (and particularly a female in the case of HH) may never develop signs, symptoms or complications of the disorder. This may have relevance to life insurance, job opportunities and perhaps psychological well being. DNA technology such as a combination of OLA and multichannel capillary electrophoresis is available today, and so are not a limitation to screening. However, the results of some well-constructed research studies will be needed before sensible decisions on community screening can be made.

Disadvantages of DNA testing should also be considered. The first is the necessity to interpret the test result, and from this to counsel the patient appropriately. The finding of a raised transferrin saturation and serum ferritin coupled with homozygosity for the C282Y mutation involves a straightforward scenario. On the other hand, the asymptomatic individual who is homozygous for C282Y requires considerably more thought, and long-term follow-up.

Clinicians have a healthy scepticism for pathology results, and will repeat studies that do not fit the clinical picture. However, this is not the case with DNA testing when results are usually accepted without question. This may reflect the fact that DNA testing is frequently undertaken in asymptomatic individuals, thereby taking away clues to the patient’s clinical status. However, false-positive or false-negative DNA test results can have serious consequences for the patient and, just as importantly his/her family since DNA will be shared by relatives. To avoid clerical errors at the collection or processing stages in DNA testing, we recommended that two separate blood samples are referred to the laboratory for DNA testing. Whenever possible, duplicate DNA samples are processed at different times to reduce the potential for technical errors. To avoid specific problems related to mutation detection technology, we utilise two different PCR tests in situations such as prenatal diagnosis when the results of the test can have serious immediate consequences for the parents and the fetus. However, the rapid turnaround time demanded, and high throughput experienced with some tests, such as HFE, means that a single assay is used. Because of the various methods by which DNA tests can be undertaken, and the different approaches that can be adopted, the clinician and pathologist should be aware of the potential drawbacks in DNA testing, and ensure that this information is included in the counselling process.

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References