The C282Y mutation of the *HFE* gene is not found in Chinese haemochromatotic patients: multicentre retrospective study

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Objective. To detect two novel mutations (C282Y and H63D) of the *HFE* gene in Chinese patients with hepatic iron overload.
Design. Multicentre retrospective study.
Setting. Four public hospitals, Hong Kong.
Participants. Fifty Chinese patients who presented from January 1987 through December 1999 with hepatic iron overload from various causes.
Main outcome measures. The DNA from liver biopsy samples was tested for *HFE* mutations by restriction fragment length polymorphism analysis.
Results. The sample DNA quality was unsatisfactory for analysis of the C282Y mutation in one case and the H63D mutation in nine cases. The C282Y mutation was not detected in any of the 49 satisfactory samples. Three of the 41 samples were heterozygous for the H63D mutation and only one was homozygous, giving an allele frequency of 6.1%. Of the three H63D-heterozygotes, one had β-thalassaemia major, one had β-thalassaemia minor, and one had hereditary spherocytosis. None of the 12 patients who were presumed to have primary haemochromatosis were positive for either mutation.
Conclusions. The classical form of human leukocyte antigen–linked hereditary haemochromatosis appears to be absent from this locality. The H63D mutation is found in a minority (9.8%) of the patients, in whom it may act synergistically with an erythropoietic factor.

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Key words: Genes, MHC class I; Hemochromatosis/genetics; Hong Kong; Iron overload/genetics; Mutation, missense

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Introduction

In western countries, iron overload is regarded as a problem that predominantly affects the white population in the form of autosomal recessive human leukocyte antigen (HLA)–linked hereditary haemochromatosis (HHC), which has a prevalence of nearly 0.45%.[1] A major breakthrough in the understanding of HHC was the cloning of the causative gene (originally designated *HLA-H* and now renamed *HFE*) found on chromosome 6p.[2] The gene product is an HLA class I–like protein, which is cell membrane–bound and has been reported to interact with the transferrin receptor to regulate cellular iron uptake.[3,4] A homozygous missense mutation that leads to an amino acid substitution of cysteine by tyrosine at position 282 (C282Y) has been detected in the gene in 83% of 178 American haemochromatosis patients.[2] Although the frequency

of this mutation has been shown to vary in three continents (North America, northern Europe, and Australia), there is now general agreement that 80% to 100% of HHC patients are homozygous for the C282Y mutation.[5-8] A second missense mutation that results in a substitution of histidine by aspartic acid at codon 63 (H63D) has also been identified. This mutation acts synergistically with C282Y in 3% to 5% of the patients, and iron overload can occur in H63D homozygotes.[9-11]

In the Chinese population, severe iron overload is mainly associated with Cooley's anaemia, and primary haemochromatosis appears to be a rare disease. The C282Y mutation has been found to be absent in the general population of China.[12,13] The occurrence of the C282Y mutation among Chinese haemochromatotic patients, however, has not been examined. This study aimed to find the frequency of the C282Y and H63D mutations of *HFE* gene in a group of Chinese patients with iron overload.

Materials and methods

Patients whose liver biopsy results showed increased iron deposition were identified from the computer records from January 1987 through December 1999 of four participating hospitals: the Alice Ho Nethersole Hospital, Caritas Medical Centre, Princess Margaret Hospital, and Queen Elizabeth Hospital. Histological sections of the liver were retrieved and reviewed independently by at least two pathologists. The criterion for inclusion of patients in this study was the presence of a minimum of grade 2 hepatocellular iron, which was assessed in sections that had been stained with Perls' reagent, according to a grading system (on a scale of 0 to 4) modified from Scheuer's method (Table 1).[14,15] Grade 2 iron corresponds to a moderate degree of iron deposition, whereas grades 3 and 4 indicate severe iron overload equivalent to hepatic iron concentrations of 130 to 850 μmol/g dry weight. According to such criteria, patients with the classical form of HHC should have been included. Formalin-fixed, paraffin-embedded liver biopsy samples were analysed for the C282Y and H63D mutations using polymerase chain reaction (PCR) amplification followed by restriction fragment length polymorphism (RFLP) analysis.

DNA extraction and analysis

Tissue sections from each sample were deparaffinised by washing with three changes of xylene, followed by two changes of absolute ethanol, and one change of 95% alcohol. The tissue fragments were incubated with proteinase K at 60°C overnight. An equal volume of a solution of phenol, chloroform, and isoamyl alcohol (in a volume ratio of 25:24:1) was added, and the mixture was vortexed thoroughly and incubated on ice for 15 minutes. The mixture was then centrifuged at 13 000 rpm for 10 minutes, and the upper aqueous layer containing the DNA was transferred to a new tube. An equal volume of a solution of phenol, chloroform, and isoamyl alcohol was added and the DNA purification procedure was repeated. Two microlitres of SeeDNA (Pharmacia Amersham Biotech, Uppsala, Sweden), and 40 μL of 3 M sodium acetate, and 1 mL of absolute alcohol were added to the DNA solution. The mixture was vortexed briefly and centrifuged at 13000 rpm for 5 minutes. The DNA pellet was washed with 1 mL of 70% alcohol and then 1 mL of absolute alcohol. The DNA was vacuum-dried for 15 minutes and resuspended in 50 μL distilled water.

Table 1. Histological grading of hepatocellular iron accumulation[14,15]

Grade	Haemosiderin deposition in hepatocytes
0	Absent
1	<25% of hepatocytes: granules barely discernible
2	25%-75% of hepatocytes: discrete granules in zone 1 and 2
3	75%-100% of hepatocytes: coarse granular deposit with acinar gradient
4	100% of hepatocytes: massive deposit with obliteration of acinar gradient

To detect C282Y and H63D mutations, PCR analysis was performed according to published methods.[16,17] The primers for the detection of the C282Y mutation were: (forward) 5'-GGA AGA GCA GAG ATA TAC GT-3' and (reverse) 5'-CTC AGG CAC TCC TCT CAA CC-3'. The primers for the detection of the H63D mutation were: (forward) 5'-ACA TGG TTA AGG CCT GTT GC-3' and (reverse) 5'-GCC ACA TCT GGC TTG AAA TT-3'. The cycling conditions for both reactions were as follows: heat activation, 10 minutes; 40 cycles of incubation of 94°C for 1 minute (denaturation), 54°C for 1 minute (reannealing), and 72°C for 1 minute (extension); and an extra 7 minutes' extension for the last cycle. The sizes of the PCR products bearing the C282Y and H63D mutations were 392 bp and 208 bp, respectively (Fig). Subsequent digestion of the C282Y-mutated PCR product with the restriction enzyme *Sna*B1 yielded 111-bp and 281-bp fragments of the mutant allele; the wild-type allele was not digested. In contrast, the PCR product containing the H63D mutation yielded 70-bp and 138-bp fragments for the normal allele after digestion with *Nde*II; the mutant allele was not digested.

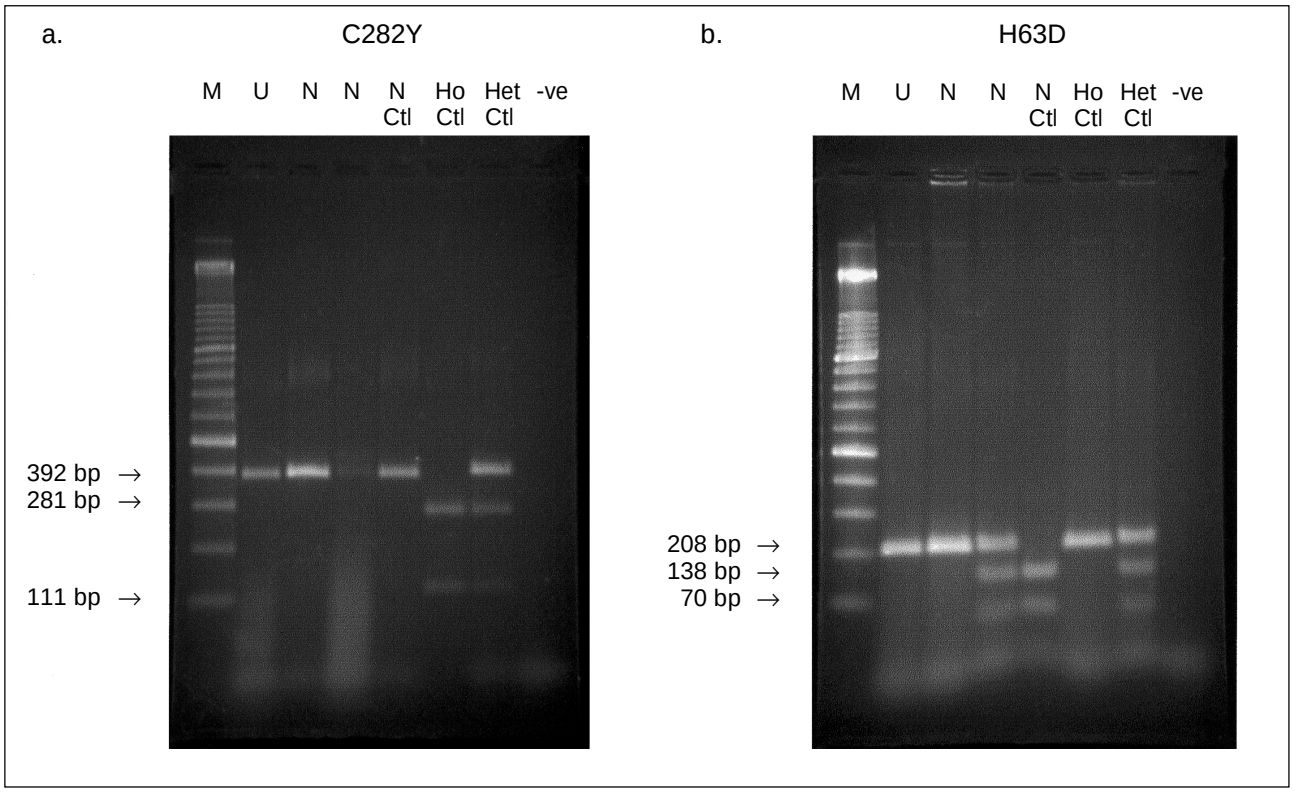

Fig. Example of agarose gel of electrophoresed amplification products to detect C282Y and H63D mutations
Samples were incubated with *Sna*BI or *Nde*II to detect (a) C282Y and (b) H63D mutation, respectively; M = 100-bp molecular weight markers, U = undigested, N = homozygous wild-type, Ho = homozygous mutant, Het = heterozygous, Ctl = control, -ve = negative control

Results

A total of 50 patients (27 males and 23 females) were studied. All patients were Chinese; their mean age was 46.2 years (range, 9-88 years). Their medical records (available in 39 cases) and laboratory data were reviewed, and pertinent clinical data were analysed. According to histological assessment and clinical correlation, the majority of the cases of iron overload were cases of secondary haemochromatosis, which could be divided into the following four disease groups: (1) Cooley's anaemia requiring regular blood transfusion (n=7); (2) haemoglobinopathies or corpuscular abnormalities not requiring transfusion (n=9); (3) alcoholic liver disease and cirrhosis of various causes (n=9); and (4) miscellaneous diseases (eg leukaemia, malignant tumours) requiring occasional transfusion (n=13). A fifth group of patients had idiopathic iron overload and had been regarded from clinical findings as having primary haemochromatosis (n=12). The diagnosis in this group was made because of the combination of an elevated serum ferritin level and iron saturation, compatible histological findings, at least grade 2 siderosis, and an absence of an obvious secondary cause. The family histories of iron overload in this group of patients were not available; their characteristics are summarised in Table 2.

Table 2. Characteristics of patients categorised as having idiopathic haemochromatosis

Characteristic	Value
Sex ratio (M:F)	9:3
Age (years)	
Mean (range)	56.1 (26-71)
Serum ferritin level (ng/mL)*	
Mean (range)	2175 (1098-4471)
Iron saturation (%)†	
Mean (range)	82.5 (60-100)
Liver iron grade	2 (n=5), 3 (n=5), 4 (n=2)

* Normal range: 30-284 ng/mL for males, 6-186 ng/mL for females
† Normal range: 20%-55%; value available for only nine patients

The quality of sample DNA was unsatisfactory for analysis of the C282Y mutation in one case and the H63D mutation in nine cases. No C282Y mutation was detected in the 49 satisfactory samples. Of the 41 samples that were analysed for H63D mutation, three were heterozygous for H63D and only one was homozygous, giving an H63D allele frequency of 6.1%. One heterozygote had β-thalassaemia major, one had β-thalassaemia minor, and the third had hereditary spherocytosis. The characteristics of these three patients are shown in Table 3. Of the four patients with β-thalassaemia minor, the H63D heterozygote had the highest grade of iron deposition. The only H63D-homozygous patient was a 75-year-old woman with carcinoma of cervix, who died from

Table 3. Characteristics of patients who were heterozygous for H63D mutation

Patient	Associated diseases	Sex/age (years)	Haemoglobin level (g/L)	Iron saturation (%)	Serum ferritin level (ng/mL)	Liver iron grade
1	β-Thalassaemia major, hepatitis C virus infection	F/13	99	-	2128	3
2	β-Thalassaemia minor	F/48	95	93	2285	4
3	Hereditary spherocytosis	F/19	125	-	369	2

pneumonia. A para-mortem liver biopsy showed grade 2 siderosis and mild steatosis; iron overload was not suspected while the patient was alive. None of the 12 idiopathic haemochromatotic patients were positive for either mutation.

Discussion

The discovery of the C282Y mutation in the *HFE* gene has had an enormous impact on the diagnosis of haemochromatosis, because a simple PCR-based genotyping assay can be used to identify most patients definitively.[18,19] This situation should be contrasted with other genetic hepatic diseases, such as Wilson's disease,[20] in which many mutations exist throughout the gene of interest and direct genetic testing is limited to specialist research centres. Furthermore, the method of genetic testing for the mutation is relatively straightforward, because the G→A base mutation creates a restriction site for commercially available restriction enzymes. Thus, many hospital laboratories are capable of testing for the mutation.

To the best of our knowledge, this is the first study to analyse *HFE* mutations in Chinese haemochromatotic patients. The main finding is that the C282Y mutation was not detected in our series of patients. The absence of C282Y mutations implies that the classical form of HLA-linked HHC does not exist in the local population. The association of the C282Y mutation with HHC has been established,[2-8] and the mutation is thought to have arisen in a single individual, approximately 60 to 70 generations ago.[21] Thus, the global prevalence of this point mutation varies considerably among the populations studied.[12] It is most prevalent in western European populations of Celtic ancestry,[22] but fewer patients in Italy and southern France are homozygous for the mutation.[23,24] In contrast, the C282Y mutation is absent from Asian, African, and Australian indigenous populations.[12,13,25] The present study confirms the absence of the C282Y mutation among haemochromatotic Chinese patients.

The H63D mutation has been found in diverse ethnic backgrounds and may represent an old mutation.[12,13] Its clinical significance is less clear than that of the C282Y mutation, and there are conflicting data as to whether it contributes to iron overload in C282Y/H63D compound heterozygotes. The single H63D-homozygous patient in our series had iron overload, but to a degree that was not severe enough to cause symptoms or induce fibrosis.

In this series, the majority of cases of iron overload resulted from secondary causes. Haemochromatosis associated with blood transfusion is easy to diagnose, either from the clinical history or histological material. In contrast, haemochromatosis that is acquired through oral intake—for example, haemoglobinopathies not requiring regular blood transfusion—are difficult to diagnose. The histological appearance of the liver in these patients with siderosis is similar, if not identical, to that in patients with HHC. In Hong Kong, iron overload diseases are uncommon and equipment to measure hepatic iron concentration is not generally available, even in research centres. Using the hepatic iron index, which allows physicians in the West to distinguish between HHC and iron overload due to alcoholism,[26] is of little value in this situation. In this study, the cases were associated with haemoglobinopathies, so the degree of iron overload was severe and the indices would have exceeded 1.9. It has been claimed that the minor haemoglobinopathies not requiring regular blood transfusion will not lead to marked iron overload.[27] In fact, this is not the case. Nevertheless, severe siderosis has been recently reported in patients with β-thalassaemia minor or haemoglobin H disease and results in liver dysfunction and systemic symptoms such as diabetes.[28] Given the large number of thalassaemia carriers in Hong Kong, the occurrence of severe iron overload is probably low. One of the patients in this study had been prescribed iron tablets for more than 10 years, and this may be one of the reasons for such an occurrence. Another aggravating factor, as found in this study, is the presence of the H63D mutation. The allele frequency of this mutation in this series was 6.1%, whereas that of the general population ranges from 2.8% to 3.09%.[12,13] The mutation seemed to be associated with an erythropoietic abnormality in three of the 16 patients in disease groups 1 and 2.

The main objective of this study was to examine the genetic alterations in a group of Chinese patients with idiopathic haemochromatosis. However, their disease may represent a phenotypically similar but genotypically different heritable trait. Sequencing the coding region of the entire *HFE* gene in this group of patients may disclose mutations different from the classical ones; such a result has been reported for one non-Caucasian series.[29] On the other hand, there are families from southern Italy who have typical HHC without any detectable mutations in the *HFE* gene.[30] The condition has also been demonstrated to be non–HLA-linked in a Chinese woman.[31] This finding suggests that factors other than mutant *HFE* genes cause HHC. A study of haemochromatosis in an urban African population has suggested the presence of other types of genetic predisposition to iron overload.[32] In addition, Shaheen et al[33] have shown that more than 50% (10/18) of a group of patients with haemochromatosis who lacked the C282Y mutation had other underlying causes of iron overload. In fact, secondary iron overload is the most common cause of haemochromatosis in this locality. The main drawback in this study was the lack of detailed clinical data, the haematological status, or family history of all the patients who were presumed to have primary haemochromatosis. Iron overload as severe as that seen in HHC has been found in patients with alcoholic liver disease, hepatitis C infection, exogenous iron use, or haemoglobinopathies.[34] End-stage cirrhosis alone may also cause substantial iron accumulation, regardless of the aetiology of the liver disease.[35,36] The possibility that the idiopathic haemochromatotic patients in our series had previously unrecognised causes of secondary iron overload cannot be excluded. Prospective studies that carefully exclude secondary causes are thus necessary to identify the cause of disease.

In conclusion, the C282Y mutation is not found in Chinese patients with hepatic iron overload disease. The H63D mutation is found in a minority (9.8%) of patients, in whom it appears to act synergistically with an erythropoietic factor to cause a state of heavy iron overload. For the patients with idiopathic haemochromatosis, the possibility of a secondary cause is high, but a genetic basis cannot be excluded. Further studies, such as the sequencing of the coding region of the entire *HFE* gene, are required to further investigate the genetic basis of hepatic iron overload.

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