ORIGINAL ARTICLE

The 16189 variant of mitochondrial DNA occurs more frequently in C282Y homozygotes with haemochromatosis than those without iron loading

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Background: Patients with hereditary haemochromatosis (HH) are usually homozygous for the C282Y mutation in the *HFE* gene. They have variable expression of iron overload and present with a variety of complications, including liver disease, diabetes, arthropathy, fatigue, and cardiomyopathy. The mitochondrial 16189 variant is associated with diabetes, dilated cardiomyopathy, and low body fat at birth, and might contribute to genetic predisposition in further multifactorial disorders. The objective of this study was to determine the frequency of the 16189 variant in a range of patients with haemochromatosis, who had mutations in the *HFE* gene.
Methods: Blood DNA was analysed for the presence of the 16189 variant in British, French, and Australian C282Y homozygotes and controls, with known iron status, and in birth cohorts.
Results: The frequency of the mitochondrial 16189 variant was found to be elevated in individuals with haemochromatosis who were homozygous for the C282Y allele, compared with population controls and with C282Y homozygotes who were asymptomatic (42/292 (14.4%); 102/1186 (8.6%) ($p = 0.003$); and 2/64 (3.1%) ($p = 0.023$), respectively).
Conclusions: Iron loading in C282Y homozygotes with HH was exacerbated by the presence of the mitochondrial 16189 variant.

INTRODUCTION

Hereditary haemochromatosis (HH) (OMIM 235200) is an autosomal recessive disorder of iron metabolism. The HH associated mutation, C282Y, in the *HFE* gene, is common in Northern Europeans, among whom approximately 1/300 are C282Y homozygotes (Genbank accession U60319)[1]). The C282Y mutation disrupts formation of the disulphide bond in the α3 domain of the HFE protein, thus preventing its normal association with β_2-microglobulin (β_2M).[2] This dramatically reduces cell surface expression of the complex.[3 4] The HFE/β_2M complex normally interacts with the transferrin receptor, which enables the cell to regulate iron uptake.[5–9] In persons with haemochromatosis, iron eventually accumulates in parenchymal cells, causing a number of complications such as chronic fatigue, arthritis, diabetes, heart disease, skin pigmentation, and liver fibrosis and cirrhosis.[10]

Over 90% of patients with HH in the UK are homozygous for the C282Y mutation.[11] In France and Australia, between 72% and 100% of people with HH are C282Y homozygotes.[12–14] A small proportion of iron loaded patients are compound heterozygotes for C282Y and a much milder mutation, H63D.[2] Not all C282Y homozygotes become iron loaded.[15] Many studies demonstrate incomplete penetrance in HH,[16–19] and these suggest that most people who are C282Y homozygotes will have increased transferrin saturation, but only some will develop an increased ferritin concentration. A smaller proportion will have clinical signs of the disease.

Modifier genes have been shown to influence penetrance in a number of conditions.[20] Such genes can enhance or reduce the phenotypic expression in many diseases. Dietary and environmental factors have been shown to influence haemochromatosis, and studies investigating penetrance of haemochromatosis in mouse models have demonstrated that heritable factors influence iron homeostasis.[21–24] We have recently described digenic inheritance of juvenile haemochromatosis in an individual with the C282Y mutation in *HFE* and the Met50del IVS2+1(-G) mutation in the hepcidin gene.[25]

Iron deposition and/or loading is associated with impaired mitochondrial function in subjects with a variety of diseases.[26–29] Chronic fatigue is a typical symptom of both HH[30] and mitochondrial disease.[31 32] Diabetes is associated with HH; the presence of a mitochondrial DNA (mtDNA) variant at position 16189 appears to be a risk factor for developing type 2 diabetes.[33–35] The 16189 variant is situated in the hypervariable, non-coding region of the mitochondrial genome (mtDNA), HV-1, within the D-loop. The variant is characterised by a T→C substitution at position 16189, which produces a highly variable poly C tract.[34] It is therefore frequently accompanied by heteroplasmic length variation. The 16189 variant is common in some Pacific populations, with a frequency of 96%,[36] and diabetes has become almost epidemic in these populations as they are increasingly exposed to Western lifestyles.

Some aspects of mitochondrial function may be important for oxygen sensing,[37–39] which in turn influences iron loading. It therefore seemed appropriate to investigate the role of mitochondria in relation to HH and iron homeostasis. We have begun by examining the frequency of the mtDNA 16189 variant in several groups of people with conditions involving mutations in the *HFE* gene.

Abbreviation: HH, hereditary haemochromatosis

METHODS

Study subjects

A total of 1585 subjects of UK, French, and Australian origin were tested for the presence of the 16189 variant in the D-loop of mtDNA. These subjects fell into five groups, two of which had iron overload (groups 1 and 2, a total of 335 patients) and three of which did not (groups 3–5).

Group 1 comprised C282Y homozygotes with haemochromatosis, who had a transferrin saturation of more than 40% and a serum ferritin concentration of more than 300 μg/l, in the absence of any other cause of iron loading. The age range was from 29 to 89 years, (mean 49.5 years, standard deviation (SD) 13.9). Two subjects had type 1 diabetes (β cell destruction), and 10 had type 2 diabetes. The majority of these participants had completed iron depletion treatment and so had had more than 5 g of iron removed by quantitative phlebotomy.

Group 2 comprised subjects with intermediate iron loading. These were French patients with haemochromatosis, who were compound heterozygotes for the H63D and C282Y mutations in *HFE*. The age range was from 27 to 64 years (mean 39 years, SD 7.5). Five participants had type 2 diabetes and two had diabetes that was neither type 1 nor type 2. The iron overload in this group was less severe than in group 1, and less than 4 g of iron had been removed by quantitative phlebotomy.

Group 3 included C282Y homozygotes without haemochromatosis; their serum ferritin concentrations fell within the normal range. These samples were either identified as part of an anonymous blood donor study in South Wales[17] or as part of a free national health check in France.[19] The age range of the samples was from 22 to70 years (mean 40 years, SD 1.5). No one in this group was known to have diabetes.

Group 4 comprised two subgroups with normal serum ferritin—that is, random blood donors and H63D homozygotes. The 89 random blood donors were from South Wales and had normal iron indices.[17] The genotypes in this subgroup included HH/CC (57), HD/CC (18), HH/CY (11), HD/CY (2), and DD/CC (1), demonstrating allele frequencies of 6.2% and 11.2% for C282Y and H63D, respectively. The second subgroup comprised H63D homozygotes, 26 of whom were picked up as part of a free national health check in France and 165 from the Welsh blood donor survey .[17 40] Their ages ranged from 27 to 91 years (mean 40.3 years, SD 15.7).

The birth cohorts (group 5) were all of North West European ancestry, and were chosen with no recruitment bias with respect to iron stores. We involved, as UK Caucasian controls, a birth cohort from the Avon area that had previously been typed for the 16189 variant. The French birth cohort from Amiens was typed for *HFE* mutations, but the British and Australian birth cohorts were not. The *HFE* allele frequencies (5.0% for C282Y and 18.0% for H63D) in the French birth cohort did not differ from those in a healthy adult control population from the same area.

All patient samples were maternally unrelated and were tested for the C282Y and H63D mutations.[41–43] Our study forms part of a larger study investigating phenotype/genotype relationships in haemochromatosis. Samples were collected locally under procedures approved by the appropriate ethical committee.

Detection of the 16189 polymorphism

DNA was extracted using standard methods. Polymerase chain reaction (PCR) amplification of the D-loop of mtDNA was carried out as previously described.[44] Digestion of PCR products with *Mnl*I took place at 37℃ for more than four hours, according to the manufacturer's specifications (New England Biolabs, Beverly, MA). Digested products were resolved by electrophoresis in 2% agarose gels. In the wild type sequence, there was a restriction site at position 16189, giving a major fragment of 323bp. In the mutant allele, the *Mnl* I site at 16189 was absent, giving a major fragment of 358bp.

Sequence analysis

Sequence analysis, using an ABI 377 automated sequencer (Applied Biosystems, Warrington, UK), was carried out on samples that were positive for the 16189 variant. A standard protocol was followed using ABI Big Dye terminators. A Strata Prep® purification kit was employed to purify the first round PCR amplification products used as template. Because of possible heteroplasmic length variation, all samples lacking the *Mnl*I site were sequenced in both directions. The sequences were compared with a reference sequence, and assigned to particular mtDNA haplogroups on the basis of diagnostic motifs.[45 46]

Statistical analysis

Statistical analysis was performed using the SPSS package. Logistic regression was necessary to fit a proportion parameter for each country and each group at the same time. One tailed significance was used because the null hypothesis was that the 16189 variant would be more frequent in affected than unaffected individuals, consistent with a role in exacerbating the phenotype.

RESULTS

The results are shown in tables 1 and 2. The frequency of the 16189 variant was highest, at 14.4% (42/292), in those C282Y homozygotes with haemochromatosis (group 1). This was significantly higher than for the birth cohort controls, where the frequency was 8.6% (78/906, p = 0.029) (group 5). The lowest frequency of 3.1% (2/64) was observed for group 3, C282Y homozygotes who did not have haemochromatosis. This was significantly lower than the frequency in individuals with haemochromatosis who were C282Y homozygotes (p = 0.023) (group 1 *v* group 3).

The other group expressing symptoms of iron overload, group 2 (compound heterozygotes with HH), also had higher 16189 frequencies (11.6%) than the birth cohort control group, but this difference was not significant. There was no significant difference between groups 1 and 2.

Table 1 Comparison of *HFE* genotype with the frequency of the 16189 variant in subjects with haemochromatosis and healthy controls

Subjects	British (%)	French (%)	Australian (%)	Total (%)	Group
C282Y homozygotes with increased iron	22/157 (14.0)	15/112 (13.4)	5/23 (21.7)	42/292 (14.4)	1
Patients with intermediate iron		5/43 (11.6)		5/43 (11.6)	2
C282Y homozygotes with normal iron	1/43 (2.3)	1/21 (4.8)		2/64 (3.1)	3
Controls with normal iron	23/254 (9.1)	1/26 (3.7)		24/280 (8.6)	4
Birth cohort	48/545 (8.8)	19/204 (9.3)	*11/157 (7.0)	78/906 (8.6)	5
Total	94/999 (9.4)	41/406 (10.1)	16/180 (8.8)	151/1585 (9.52)	

*There were, in addition, three participants with both the 16189 variant and heteroplasmy for a C to T transition at bp 16193. The p values are barely altered when these are included.

Table 2 Odds ratios, 95% (one sided) confidence intervals, and one sided p values for the presence of the 16189 variant in patients homozygous for the C282Y mutation and iron loading (group 1), compared with those with normal serum ferritin concentrations (groups 3 and 4) and the birth cohort controls (group 5)

Group	British	Total
Odds loaded (group 1) *v* unloaded (groups 3 and 4)	1.85 (1.11–∞) p=0.025	2.05 (1.29–∞) p=0.006
Odds loaded (group 1) *v* birth cohort (group 5)	1.69 (1.07–∞] p=0.029	1.77 (1.26–∞) p=0.003

The H63D allele is much more common than the C282Y allele in European populations, having allele frequencies between 10 and 20%.[41] Up to 5% of persons with haemochromatosis are compound heterozygotes, and less than 1% of persons with iron overload in the UK are H63D homozygotes.[11] We compared the 191 H63D homozygotes in group 4 (age range from 27 to 91 years) with the birth cohort (group 5). The overall frequency of the 16189 variant in the H63D homozygotes (group 4) was 9.4% (18/191). This was not significantly different from the birth cohort (group 5) (8.6%).

The British component of group 5 was a birth cohort from the Avon area in which the 16189 variant had previously been found to be present in 8.8% (48/545) of samples,[47] consistent with other European Caucasian studies.[4 49] The frequency of the 16189 variant was slightly lower in the 89 blood donors from South Wales (6.7%) than in the birth cohorts (8.6%).

In table 2, the odds ratios for the presence of the 16189 variant were significantly greater for the British participants who were C282Y homozygotes with iron loading than for those with normal ferritin concentrations ($p = 0.025$). Logistic regression was used to calculate the odds ratios for comparisons between the totals adjusting for the countries, and gave odds ratios of 2.05 and 1.77 for the presence of the variant in subjects who were C282Y homozygotes with iron loading, compared with unloaded individuals and birth cohorts, respectively (see tables 1 and 2). Logistic regression of the proportion of participants who were positive for the 16189 variant on iron loading (group 1 *v* group 2 *v* group 3) for the UK and France was carried out. Adjusting for country, this showed that there was a significant ($p = 0.03$) downward linear trend in the log of the odds ratios from the French and British patients with increased to intermediate to normal iron loading (with multiplicative steps in odds ratios of 0.58 downwards, or 1.735 upwards from 3 to 2 to 1).

DISCUSSION

In this study we demonstrated an increased frequency of the 16189 variant in C282Y homozygotes with haemochromatosis compared with individuals with the same *HFE* genotype who did not show signs of iron overload (14.4% and 3.1%, respectively, compared with 8.4% of controls). Although we did not obtain our baseline frequency for the 16189 variant using matched controls, mtDNA haplotyping was able to exclude the possibility of a spurious association due to the known C282Y founder effect. MtDNA analysis demonstrated that the 16189 variant had arisen on several different mitochondrial haplogroups (table 3). These data were consistent with other studies in which H and U are the commonest European haplotypes.[45 46] Moreover, the excess of individuals with the 16189 variant among those with haemochromatosis was not due to a single mitochondrial haplotype. Together, these results suggest that the association we demonstrated was due to an effect of the 16189 variant itself, rather than of another pathogenic mtDNA mutation when cosegregating with it.

Table 3 shows a comparison of the mitochondrial DNA haplogroup associated with the 16189 variant in all the different groups. Haplogroup B is derived from Asia, Oceania, and native American populations, whereas the remaining haplogroups originate mainly from Europe, North Africa, and Western Asian Caucasians. Those in groups 1 and 2 have haemochromatosis and intermediate iron overload, respectively. Group 3 includes asymptomatic C282Y homozygotes, and group 4 consists of asymptomatic H63D homozygotes and healthy blood donors. Group 5 comprises the birth cohort controls.

We postulate that, in haemochromatosis, mitochondrial function might influence iron loading. There was a clear association of the 16189 variant with iron loading in the presence of the C282Y mutation. Diabetes and chronic fatigue are features of both HH and mitochondrial disease. Patients with both C282Y and the 16189 variant may be subject to these complications. Further studies are needed to confirm our hypothesis that the 16189 variant influences iron loading in C282Y homozygotes, and to a lesser extent in C282Y heterozygotes with disease.

Some aspects of mitochondrial function may be important in oxygen sensing,[37–39] which is central to iron homeostasis. Oxygen levels contribute to transcriptional and translational control of several genes involved in iron homeostasis such as transferrin, transferrin receptor, ferritin, ceruloplasmin, and heme-oxygenase 1. Hypoxia inducible factor (HIF-1) is a key regulator in this process.[50–54] Hepcidin levels are reduced by hypoxia[55] and may be inappropriately low in untreated patients with haemochromatosis.[56] Mutations in hepcidin can result in juvenile haemochromatosis,[57] and may play a key role in modifying the severity of haemochromatosis in C282Y heterozygotes and homozygotes.[25] Hepcidin is a type II acute phase protein.[58] The effects of impaired mitochondrial function and of the presence of the 16189 variant on hepcidin levels and thence on iron loading are currently unknown. Involvement of mitochondria in iron balance, perhaps because of a role in iron sensing, would be biologically plausible: iron is required by mitochondria for the synthesis of cytosolic proteins required for the normal assembly of the electron transport complexes.

We suggest that the 16189 variant produces a subtle defect in mitochondrial function. It lies close to conserved sequences that contain the origin of mtDNA replication of the heavy strand (O_H), as well as both promoters. Hence it is

Table 3 Comparison* of haplogroups† within each subject group

Group	B	H	I	J	K	T	U	V	W	X	Unknown
1	5.3 (2)	42.1 (16)	0	2.6 (1)	0	5.3 (2)	34.2 (13)	0	0	7.9 (3)	2.6 (1)
2	0	0	0	0	0	0	60 (3)	0	20 (1)	20 (1)	0
3, 4, 5	0	36.7 (18)	10.2 (5)	2.0 (1)	2.0 (1)	8.2 (4)	22.5 (11)	2.0 (1)	0	12.2 (6)	4.1 (2)

*Results are expressed as %, with the number of samples sequenced in brackets.
†B to X, the haplogroups associated with the 16189 variant.

possible that this region of mtDNA is involved in regulating mtDNA copy number.[59] Previous investigators have used organello dimethyl sulphate footprinting assays to demonstrate that proteins bind to sites in this region of mtDNA.[60] Preliminary data suggest that the 16189 variant affects cell function,[61] and that in some tissues the presence of the 16189 variant may result in reduced mitochondrial copy number (J Poulton, unpublished data). This might impair a cell's maximum rate of oxidative phosphorylation.

The combination of the 16189 variant and excess iron due to the presence of C282Y *HFE* alleles may be cumulative in causing disease in some haemochromatosis patients. Too much or too little iron can induce mitochondrial DNA damage, oxidative stress, and mitochondrial dysfunction.[62] Our results suggest that iron loading in these patients is exacerbated by the presence of the 16189 variant, which may confer a subtle reduction in mitochondrial function.

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ELECTRONIC-DATABASE INFORMATION

Accession numbers and URLs for data in this article are as follows: Genbank, http://www.ncbi.nlm.nih.gov/Genbank/ (for haemochromatosis gene HFE); Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim/(for HH, MIM 235200).

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ECHO

Heart disease common in Leber's optic neuropathy

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All patients with Leber's hereditary optic neuropathy should be screened for cardiac abnormalities, according to a team of investigators from London who screened 24 consecutive patients and found that eight had cardiac symptoms, 14 had ECG abnormalities and five had myocardial hypertrophy. The five patients with myocardial hypertrophy, defined as a left ventricular wall thickness of 15mm or more on two dimensional echocardiography, were all from the same family, and carried the same mitochondrial DNA point mutation, known as 3460. Patients with the other two mitochondrial DNA mutations associated with Leber's hereditary optic neuropathy (11778 and 14484) had no myocardial hypertrophy. Nor did six other members of the family carrying the 3460 mutation. Experts have long suspected that Leber's hereditary optic neuropathy could affect the heart, and at least one previous study suggests an association with left ventricular pre excitation. This latest study is probably the first describing hypertrophic cardiomyopathy in patients with the disease.

The three mitochondrial DNA mutations 3460, 11778, and 14484 account for 90% of Leber's hereditary optic neuropathy. All alter the encoding of complex 1, a major protein complex in the mitochondrial oxidative phosphorylation system. It's unclear exactly why patients with the 3460 mutation seem more prone to hypertrophic cardiomyopathy than patients with the other mutations, although the three mutations affect different protein subunits in complex 1. Mutation 3460, for example, alters the ND1 subunit which causes a 30–35% decrease in ATP synthesis. Mutation 11778 alters the ND4 subunit, and mutation 14484 alters the ND6 subunit. More research is needed to investigate the impact of these different alterations on the myocardium.

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