CLINICAL STUDY

Phenotype and *HFE* genotype in a population with abnormal iron markers recruited from an Endocrinology Department

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Abstract

Objective: The aim of this study was to describe *HFE* genotype in a population of patients with altered iron markers recruited in an Endocrinology Department and to define the possible phenotype–genotype relationships.

Methods: A total of 156 patients with high serum ferritin concentrations (> 300 ng/ml) or transferrin saturation (> 45%) (I group), and a control group of 106 healthy subjects (C group) underwent *HFE* genotyping (classical C282Y and H63D mutations). We also examined the main genetic features of subgroups in I according to the presence (D) or the absence (ND) of diabetes.

Results: (1) The genotypes were significantly different in the I and C groups ($P < 0.001$), with an increased frequency of major 282Y allele in the I group (35% vs 7.5%), but not of minor 63D allele (17 vs 18.5%). (2) The genotype of D and ND groups also differed ($P < 0.0001$), with a lower frequency of C282 heterozygosity ($P < 0.0001$) in the D group, and a higher prevalence of H63D heterozygosity in the D vs ND groups ($P < 0.01$). (3) The phenotypic comparison of D and ND groups also showed a higher mean body mass index, age, and serum ferritin concentration, as well as an increased proportion of males with increased liver enzymes in the D group.

Conclusion: This population harboring abnormal iron markers had a different *HFE* genotype and a higher 282Y allele frequency than the control population. This suggests that blood iron markers could be checked in etiological investigations of metabolic disturbances to identify patients who should undergo genotyping, since approximately 20% were diagnosed with C282Y homozygosity.

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Introduction

Hereditary hemochromatosis (HH) is an iron-storage disease with a phenotype characterized by biochemical changes reflecting abnormal iron homeostasis (e.g. changes in transferrin saturation (TS) and serum ferritin concentrations) and altered total-body iron burden. The symptoms of HH include cirrhosis of the liver, diabetes, hypogonadism, hypermelanotic pigmentation of the skin, and heart failure. Primary hepatocellular carcinoma, complicating cirrhosis, is responsible for about one-third of deaths in affected homozygotes. Iron overload increases insulin resistance, accentuating the risks for cardiovascular complications (1). The *HFE* gene was cloned in 1996, and homozygosity for the 845G-A (C282Y) mutation has been found in over 80% of patients diagnosed with iron overload due to HH. Compound heterozygotes for the

C282Y and 187C-G (H63D) mutations are also over-represented in the HH population. This disease is one of the most common genetic disorders in northern Europe: about 5 in every 1000 northern Europeans are homozygous for the C282Y mutation. Because the homozygous genotype for hemochromatosis is common, and because iron overload is readily treated by phlebotomy, the mutation has been regarded as ideally suited for population screening. However, although most patients of European origin who have been clinically diagnosed with HH have either the C282Y/C282Y or the C282Y/H63D genotype, there is scant and conflicting information about type and number of people in the general population with this genotype and its clinical manifestations. Therefore, the relevance of systematically studying iron metabolism in the general population is still debated, since the penetrance of *HFE* mutations is low (2–4). Moreover, recent studies have

emphasized the role of ferritin as a marker of insulin resistance (5). In western countries, most health authorities recommend an early identification of mutated patients based on clinical symptoms and iron biological parameters, coupled with a family inquiry. The issue is that the clinical phenotype of HH is very heterogeneous and phenotype–genotype correlation is not well known because of the recent availability of genetic tools. Therefore, the aim of this study was to describe the *HFE* genotype in a population of patients recruited in an Endocrinology Department with abnormal iron markers and to define the possible phenotype–genotype relationships.

Research design and methods

Patients

Patients with abnormal iron markers (I group).

One hundred and fifty six subjects, recruited in the Endocrinology and Metabolism Department of Lille University Hospital, corresponding to about 5% of our total recruitment, were included for abnormal iron levels (serum ferritin > 300 ng/ml or TS > 45%). Iron metabolism investigations were performed either because of general symptoms (fatigue, weight loss, arthralgia), diabetes, hepatomegaly, disturbed liver enzymes, or for hypogonadism. Clinical, biochemical and genotype information was collected before treatment. Informed consent was obtained from all the patients before genotype sampling. The population was divided into two groups according to their diabetic status as defined by the WHO criteria: Group D included 81 diabetic patients and group ND 75 non-diabetic subjects. We also compared 3 subgroups of patients divided according to their level of serum ferritin level ($n=148$): < 500 ng/ml ($n=73$), > 500 but < 1000 ($n=34$), > 1000 ($n=41$). Finally, we compared 4 subgroups of patients divided according to their *HFE* genotype: *C282Y* homozygote ($n=33$), *C282Y* heterozygote ($n=34$), *H63D* homo- and heterozygote, double heterozygote ($n=50$), no mutation ($n=39$).

Control group (C group). The C group included 106 healthy Caucasian subjects, without family history of diabetes or iron overload.

Methods

Clinical data. Clinical data were registered as follows: gender, age, ethnic origin, Body Mass Index (BMI), alcohol intake, arthralgia, melanodermy, hepatomegaly, cardiopathy, and diabetes defined as at least two fasting blood glucose levels above 1.25 g/l (7 mmol/l), or one random blood glucose level above 2 g/l (11 mmol/l) or

current treatment for diabetes (insulin or oral anti-diabetic drugs). Most of these patients had type 2 diabetes with positive C peptide.

Biological data. *Sampling times.* Blood samples for iron parameters were collected from fasting patients.

Routine measurements. Glycemia (Glc; mmol/l), plasma alkaline phosphatases (UI/l), gamma-glutamyl and amino-transferases (UI/l) were measured with routine laboratory methods. HbA1c was assessed by means of high-performance liquid chromatography.

Measurement of serum iron, transferrin and ferritin. Serum iron levels were measured using a standard colorimetric method, and serum transferrin levels were determined by rate immunoturbidimetry on an automated analyzer. Serum TS values were calculated as follows: ((serum iron/2) $\times$ serum transferrin) $\times$ 100. Serum ferritin levels were measured by chemiluminescence immunoassay (Immulite 2000, DPC, France).

Identification of the C282Y and H63D mutations in the HFE Gene. Genotyping consisted of analyzing *HFE* genotypes. DNA was extracted from whole blood samples. The two *HFE* mutations were detected with PCR assays followed by restriction-enzyme digestion with *RsaI* for the *C282Y* mutation and *BclI* for the *H63D* mutation.

Statistical analysis. Results were expressed as means $\pm$ s.d. A Student *t* test was used to compare means between two groups with $n > 30$. Group-wise comparisons were made using analysis of variance (ANOVA) followed by the Wilcoxon test or Kruskal–Wallis test. Least squares analysis (Pearson *r* value) was used to identify correlation in the overall population ($n=156$). Comparison of percentages was performed by the chi-square test with Yate's correction. The level of significance was $P < 0.05$.

For allelic comparisons, *P* values were corrected (*P_c*) by the number of tests. Odd ratios were calculated by the method of Woolf (6) and Haldane (7). The power of the study was 82% for a theoretical sample of 148 persons with an α risk of 0.01.

Results

Characteristics of the I Group

Clinical characteristics, serum liver enzymes, iron markers levels of the entire population are shown in Table 1, and genetic results in Table 2.

Table 1 Main clinical characteristics of the whole group of patients with abnormal iron markers and comparison of diabetic and non-diabetic subgroups.

	Whole group	Comparison of diabetic and non-diabetic groups	
		Diabetic	Non-diabetic
Number of subjects	156	81	75
Males (%)	68%	81%***	53%***
Age (years; mean $\pm$ s.d.)	52 $\pm$ 17	58 $\pm$ 13***	46 $\pm$ 18***
BMI (kg/m ² ; mean $\pm$ s.d.)	27 $\pm$ 6	28 $\pm$ 6*	25 $\pm$ 5*
Gamma-glutamyl/amino-transferases > 40 U/l (% of patients)		80%***	30%***
Blood iron level (μ g/dl; mean $\pm$ s.d.)	145 $\pm$ 56	139 $\pm$ 57	151 $\pm$ 56
Blood ferritin level (ng/ml; mean $\pm$ s.d.)	910 $\pm$ 1304	1345 $\pm$ 1521***	463 $\pm$ 831***
Transferrin saturation (%; mean $\pm$ s.d.)	58 $\pm$ 28	60 $\pm$ 30	56 $\pm$ 25

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Ethnic origin. 96.2% of patients were Caucasian. The non-Caucasian patients were native of North Africa. Among them was one homozygote for *H63D* mutation.

Clinical symptoms. 11% of patients were melanodermic. 51% were diabetic with mean glycated hemoglobin of $8.7 \pm 2.8\%$ ($N < 6.5\%$); 24% admitted to excessive alcohol intake (> 30 g/day); 26% had hepatomegaly; 10% spontaneously complained of arthralgia; and 10% suffered from heart disease.

I Group correlation

Iron markers did not correlate with any of the clinical or biological parameters (age, BMI, aminotransferase, glycemia) in the overall population.

Group comparison (Tables 1 and 2)

Comparison of the genotype and allelic frequency (Table 2) in the I and C groups. Genotypes differed significantly in the I and C groups (χ^2 ; $P < 0.001$). The frequency of major allele *C282Y* (35% vs 7.5%) was

significantly higher in the I group than in the C group. Minor allele *H63D* (17 vs 18.5%) did not differ between the two groups.

Diabetic and non-diabetic groups. The genotype of D and ND groups also differed significantly from the C group with an increased frequency of mutated major allele *282Y*, but not of minor allele *63D* (17% vs 18.3%). The genotype of the D group also differed from the ND group ($P < 0.01$) for both mutated alleles, with a lower frequency of the mutated major allele ($P < 0.0001$) but a higher frequency of the minor allele ($P < 0.01$) in the D group. The D and ND groups also differed significantly for age ($P < 0.001$), BMI ($P < 0.05$), ferritin ($P < 0.001$), gender ($P < 0.001$), increased levels of amino-transferases or gamma-glutamyl transferase ($P < 0.001$) (Table 1), *C282Y* heterozygosity ($P < 0.001$), and *H63D* heterozygosity ($P < 0.05$) (χ^2). We did not, however, find any statistical difference between groups for mean TS, frequency of *C282Y*, *H63D* homozygosity and composite heterozygosity or lack of *HFE* mutation.

Table 2 Genetic profile of the I (abnormal iron parameters), diabetic (D), non-diabetic (ND), and control (C) groups.

Genotypes	C	I	D	ND
Number of subjects	106	156	81	75
Homozygosity <i>282Y</i>	0%	33 (21%)	13 (16%)	20 (26.6%)
Composite heterozygosity	2 (1.8%)	10 (6.4%)	5 (6.2%)	5 (6.7%)
Heterozygosity <i>282Y</i>	14 (13.2%)	34 (21%)	11 (13.6%)	23 (30.6%)
Homozygosity <i>63D</i>	7 (6.6%)	5 (3.2%)	4 (5%)	1 (1.3%)
Heterozygosity <i>63D</i>	23 (21.6%)	33 (21%)	23 (28.4%)	10 (13%)
No mutation	60 (57%)	41 (26%)	25 (31%)	16 (21%)
Alleles				
2n number of subjects	212	312	162	150
<i>282Y</i>	16 (7.5%)	110 (35%)	42 (26%)	68 (45%)
<i>282C</i>	196 (92.5%)	202 (65%)	120 (74%)	82 (55%)
<i>63D</i>	39 (18.3%)	53 (17%)	36 (22%)	17 (11%)
<i>63H</i>	173 (81.7%)	259 (83%)	126 (78%)	133 (89%)

Genotype: Whole I vs C: ($P < 0.0001$); D vs ND ($P < 0.01$); D vs C ($P < 0.0001$) ND vs C ($P < 0.0001$) Alleles: Whole I vs C: *282Y* ($P < 0.0001$, OR = 13); *63D* (NS); D vs C: *282Y* ($P < 0.0001$, OR = 6); *63D* (NS); ND vs C: *282Y* ($P < 0.0001$, OR = 54); *63D* (NS); D vs ND: *282Y* ($P < 0.0001$, OR = 0.11); *63D* ($P < 0.01$, OR = 2.73). OR, odds ratio; NS, not significant.

Comparison of subgroups according to ferritin level (<500, 500–1000, and >1000 µg/dl) (Table 3). The 3 subgroups differed significantly for age (ANOVA; $P<0.001$), gender, diabetes, increased liver enzyme (χ^2 ; $P<0.001$), and C282Y heterozygosity frequency (χ^2 ; $P<0.01$). There was no difference, however, for BMI, arthralgia cardiopathy, or frequency of other genotypes. Mean age was also significantly lower (Student test) in subgroup <500 compared to the other 2 subgroups ($P<0.001$): younger patients had lower ferritin levels. The frequency of male gender, diabetes ($P<0.01$), and liver enzymes levels >40 UI/l ($P<0.01$) was higher in subgroup >1000 than in the other 2 subgroups. C282Y heterozygosity was more frequent in group <500 than in the 2 other subgroups.

Comparison of groups according to HFE genotype (Table 4). Statistical differences were found among the four groups as regards means of serum iron level, TS ($P<0.001$ for both), and blood ferritin level ($P<0.01$) (ANOVA), as well as diabetes, and hepatomegaly frequency ($P<0.01$), melanodermy ($P<0.05$), and arthralgia frequency ($P<0.001$) (χ^2). There was no significant difference between the four groups as regards age, mean BMI, gender, alcohol intake, cardiopathy, or increased liver enzyme frequency. Details of the comparison between subgroups two by two are given in Table 3. The C282 homozygote genotype was mainly characterized by hepatomegaly, arthralgia and melanodermy. Diabetes was found in about 40 cases, with a frequency nearly similar to the group C282Y

heterozygosity. The highest frequency of diabetes was found in groups “H63D” and “no mutation”.

Discussion

The profile of patients with abnormal iron markers recruited in an endocrinological environment is not well known because of the clinical heterogeneity of HH and the recent availability of genetic tools. Our aim was definitely not to determine the frequency of diabetes in HH, but rather to describe the HFE genotype in a population of patients with abnormal iron markers recruited in an Endocrinology Department to determine the yield of HFE genotyping, especially according to diabetic phenotype.

First, we compared a control group and the group with abnormal iron parameters. This last group was recruited mainly on metabolic or endocrinological disturbances and included liver burden only in a quarter of cases, usually associated with another non-hepatological symptom. Despite peculiar recruitment, the genotype and allelic frequency of C and I groups were different, with an increased frequency of major C282Y in the I group, in accordance with the already known role of C282Y HFE mutation in iron metabolism (8). This finding was also demonstrated in I subgroups according to the presence (D) or the absence (ND) of diabetes. These results emphasize the fact that iron marker assessment is a useful tool in an endocrinological recruitment.

The second point was to determine the genotype in D and ND groups. Interestingly, the D group showed a lower frequency of C282Y homozygosity and

Table 3 Comparison of subgroups according to blood ferritin level.

Ferritin (µg per 100 ml) Number of subjects	Low <500 73	Mild 500 to 1000 34	High >1000 41	** $P<0.01$ *** $P<0.001$
Age (year)	46 ± 19	59 ± 14	59 ± 12	ANOVA*** Low/Mild*** Low/High***
Gender (% male)	54.8	76.5	90.2	χ^2 ***
BMI (kg/m ²)	26.1 ± 6.7	28.6 ± 5.4	26.8 ± 5.3	NS
Diabetes (%)	28.8	61.8	80.5	χ^2 *** Low/Mild** Mild/High***
Arthralgia (%)	11.4	8.8	12.2	NS
Cardiopathy (%)	8.6	5.9	17.1	NS
Liver enzymes >40 UI/l (%)	35.6	67.6	82.8	χ^2 *** Low/Mild** Mild/High***
C282 homozygosity	17.8	20.6	31.7	NS
C282Y heterozygosity	35.6	8.8	12.2	χ^2 ** Low/Mild** Mild/High***
Composite heterozygosity	4.1	14.7	4.9	NS
H63D homozygosity	2.7	5.9	2.4	NS
H63D heterozygosity	15.1	26.5	29.3	NS
No mutation	26	23.5	19.5	NS

Table 4 Comparison of subgroups according to genotype.

Genotype	C282Y homozygosity	C282Y heterozygosity	Composite, H63D homo-/heterozygosity	No mutation	
Gender (% male)	69.7	55.8	72	71.8	NS
Age (years)	54 ± 18	50 ± 17	55 ± 17	50 ± 15	NS
BMI (kg/m ²)	25.0 ± 5.1	28.4 ± 6.2	27.6 ± 5.3	26.9 ± 7.1	NS
Alcohol intake (%)	25.0	12.5	24.0	33.3	NS
Diabetes	39.4	32.3	66.0	61.5	<i>P</i> < 0.01 b* d** e*
Hepatomegaly (%)	46.9	15.6	14.3	35.9	<i>P</i> < 0.01 a** b** f*
Cardiopathy (%)	9.4	12.5	12	5.1	NS
Arthralgia (%)	34.4	0	4	7.7	<i>P</i> < 0.001 a*** b*** c**
Melanodermy (%)	28.1	3.1	8	7.1	<i>P</i> < 0.01 a** b* c*
Increased liver enzymes (%)	48.5	44.1	64	64.1	NS
Serum iron levels (µg/dl)	186.8 ± 54.2	128.2 ± 51.5	135.7 ± 48.5	137 ± 459.9	<i>P</i> < 0.001 a*** b*** c**
Transferrin Saturation (%)	84.9 ± 30.7	44.5 ± 18.4	53.2 ± 21.1	52.7 ± 25.5	<i>P</i> < 0.001 a*** b*** c***
Ferritin (µg/dl)	1577 ± 2115	557 ± 947	779 ± 580	799 ± 1145	<i>P</i> < 0.001 a* b*

ANOVA test was used to compare means between the four groups, and Wilcoxon test, to compare subgroups two by two. χ^2 was used to compare frequencies between the groups. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Comparison of a: (C282Y homozygosity) and (C282Y heterozygosity), b: (C282Y homozygosity) and (composite, H63D homo- or heterozygosity), c: (C282Y homozygosity) and (absence of mutation), d: (C282Y heterozygosity) and (composite H63D homo- or heterozygosity), e: (C282Y heterozygosity) and (no mutation), f: (composite, H63D homo- or heterozygosity) and (no mutation) subgroups.

heterozygosity than the ND group. Our D group (mainly type 2 diabetes) may have exhibited abnormal iron parameters due to insulin resistance, and not to iron overload mediated by a higher frequency of *HFE* C282Y mutation (5). The fact that the prevalence of the *H63D* allele was higher in the D group than in the ND group (22% vs 11%), but not in the C group (22% vs 18.3%) remains unexplained. The frequent *63D* allele was mainly heterozygous in the D group. Such a finding has already been reported with a mild OR of 1.2 for homo- (9) but also heterozygous *63D* in a series of C282Y non-homozygous patients with physiopathologically proven hepatic iron overload associated with an insulin resistance syndrome (30% vs 17% of *H63D* genotype disturbances in the general population) (10). However, the prevalence of *H63D* genotype being high in the general population, these results have to be taken with caution even if, for unclear reasons, the frequency of the *63D* allele seems higher in patients with diabetes and/or insulin resistance, according to different studies.

The third aim of this study was to determine the phenotype-genotype correlation of our population of patients with abnormal iron markers. The comparison of phenotypes in D and ND groups showed a higher mean BMI, age, and serum ferritin concentration, as well as a higher ratio of males with increased liver enzymes in the D group. Note that the proportion of excessive drinkers did not differ between the two groups. Table 3 shows that the higher the ferritin level, the higher the frequency of diabetes (despite similar BMI), the proportion of males, and the prevalence of C282Y homozygosity and curiously *H63D* heterozygosity. These results are concordant with those of Mendler *et al.* (10), as stated above. Interestingly, despite the fact that we did not check the presence of liver iron overload

in our I patients, we found similar results in terms of phenotype-genotype correlation. This means that blood iron markers, much less invasive to obtain than a liver biopsy, should be checked in etiological investigations of diabetes to identify patients who should undergo genotyping.

The interest of studying iron metabolism in the etiological diagnosis of diabetes is still debated, since results of systematic *HFE* genotyping in diabetes are controversial. However, most studies on *HFE* genotyping in diabetes do not take iron status into account. In this study, in terms of yield, once diabetes with abnormal iron markers was identified, 69% of the patients had an abnormal *HFE* genotype (mainly *H63D* heterozygosity corresponding to 40% of the D group). The ratio was not different from the 79% found in the ND group, in which nearly 60% of the patients were at least C282Y heterozygous. Approximately 20% of patients for whom iron parameters had been measured on account of clinical symptoms – excluding isolated liver burden – were diagnosed with HH. Nevertheless the yield of this screening is a bit less profitable in D than in ND patients since their iron parameters are probably in part altered by insulin resistance itself. By contrast, only 43% of the control group had an abnormal *HFE* genotype, with no case of HH and the same prevalence of *H63D* heterozygosity than in the D group. When we compared our data to other genotype analyses according to diabetes status (11–19), we found a much higher frequency of *HFE* gene mutations (21% C282Y homozygote) in the Lille series. This can be explained by the fact that we did not perform genotyping systematically, but only when iron parameters were disturbed. This finding emphasizes the relevance of investigating iron metabolism in patients identified in

an endocrinological recruitment to improve the effectiveness of genotyping, and perhaps prevent morbidity-mortality (20–25).

Last, patients in the I genotype subgroups differed significantly. The C282 homozygous genotype was mainly characterized by hepatomegaly, arthralgia and melanodermy. The prevalence of diabetes was similar (about 40%) in C282Y homo- and heterozygous groups, whereas its highest prevalence (60%) was found in the 'H63D' and 'no mutation' groups. However, ferritin levels were different in these 4 groups, arguing for other factors involved in insulin resistance expression (iron overload, increased liver enzymes, alcohol, BMI, steatosis, inflammatory syndrome, alcohol intake, etc.).

These results raise the question of the role of the C282Y mutation in diabetes. Some studies suggest that the frequency of *HFE* mutations is increased in type 2 diabetes, probably in relationship with liver iron overload (11–16). Nevertheless, the Rotterdam study has not found an increase of the frequency of *HFE* mutation in patients with glucose intolerance or type 2 diabetes by comparison with general population or in case control studies (9, 17, 18). By contrast, the frequency of iron overload in type 2 diabetes is about 40%. The presence of hyperferritinemia, which is considered to be a marker of iron overload, is linked to the incidence of type 2 diabetes in prospective studies (16) and associated with the presence of metabolic syndrome and insulin resistance (5, 6, 11, 16, 19). Therefore, the 282Y mutation does not seem to play a direct role in diabetes genesis, but perhaps an indirect one by modulating the iron supply of the organism. The level of ferritin would be controlled by at least two factors: the *HFE* genotype and the degree of insulin resistance. It is probably the reason why, despite a higher level of blood ferritin in D group (linked to both insulin resistance and *HFE* genotype), the frequency of 282Y mutation was higher in ND than D group, allowing an increased level of ferritin despite the lack of insulin resistance.

In conclusion, knowledge and strategies in iron overload are a work in progress. Thanks to genotyping, hemochromatosis can now be diagnosed earlier, decreasing the occurrence of complications. The recent national French recommendations for iron overload (ANAES 2004) help achieve early identification of a propositus based on clinical symptoms, followed by iron markers assessment, then genotyping and family inquiry. This study emphasizes the fact that assessment of iron markers is of interest in patients with metabolic disturbances, their *HFE* genotype being significantly different from a control group with an increased frequency of mutated major allele 282Y. Moreover, the genotype differed according to the phenotype with a lower frequency of C282Y and a higher frequency of H63D heterozygosity in the D group. The phenotypic comparison of D and ND groups also showed a higher mean BMI, age, and serum ferritin concentration, as well as an increased proportion of males with increased liver enzymes in the D group. This means

that blood iron markers, much less invasive to obtain than liver biopsy, could be checked in etiological investigations of diabetes to identify patients who should undergo genotyping.

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