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Prevalence of the C282Y, H63D, and S65C Mutations of the *HFE* Gene in 1,146 Newborns from a Region of Northern Spain

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ABSTRACT

In Spain, 85% of patients with genetic hemochromatosis (GH) are homozygous for the C282Y mutation of the *HFE* gene. H63D and S65C mutations of *HFE* may also play some role in the disease. The aim of this study was to establish the prevalence of C282Y, H63D, and S65C mutations of the *HFE* gene in newborns in Catalonia, Spain. One thousand one hundred forty-six newborn screening cards were selected randomly. DNA from these cards was extracted and *HFE* mutations were analyzed with the LightCycler equipment (Roche Diagnostics GmbH, Mannheim, Germany). Sufficient DNA sample was obtained to screen for the three mutations in 1,043 cases (91%). The allelic frequencies of C282Y, H63D, and S65C mutations were 0.03 (IC 95% 0.022–0.037), 0.2 (IC 95% 0.19–0.22), and 0.01 (95% confidence interval [CI] 0.006–0.015), respectively. The frequency of C282Y homozygous newborns was 0.001 (95% CI 0.0005–0.0014). The frequencies of newborns doubly heterozygous for C282Y/H63D and C282Y/S65C were 0.01 (95% CI 0.005–0.02) and 0.002 (95% CI 0.0002–0.01), respectively. The allelic frequency of C282Y mutation is similar to that observed in Southern France, in the Czech Republic and in some areas of Italy. The allelic frequency of H63D mutation in Catalonia is the highest reported to date. Nevertheless, S65C is infrequent. These data should be kept in mind when designing hemochromatosis genotypic screening programs in Catalonia.

INTRODUCTION

GENETIC HEMOCHROMATOSIS (GH) is a disease characterized by increased absorption of intestinal iron leading to its accumulation in some organs over the years. It is a frequent disorder in Caucasians, with an estimated prevalence of 3–5 cases per 1000 individuals. In 1996, Feder *et al.* (1996) described a single mutation, C282Y in the *HFE* gene, which accounts for 80–90% of all diagnosed cases in populations of north-western European ancestry. This figure is similar in Spain, and 85% of patients (Sanchez *et al.*, 2000) who fulfilled classic criteria of GH are homozygous for this mutation. The role of another frequent mutation in this gene, H63D, is under debate. A third mutation of the *HFE* gene, S65C, has been reported as a cause of

GH in combination with C282Y in French patients (Mura *et al.*, 1999). The aim of this study was to establish the prevalence of these three *HFE* gene mutations and their clinically relevant combinations in a representative random sample of newborns in Catalonia, Spain. Such data are essential to calculate cost-effectiveness of genotypic GH screening in our country.

MATERIALS AND METHODS

During 2003, 1146 newborn screening cards (Guthrie cards) from the Catalan Newborn Screening Program were anonymously and randomly selected for the study. We previously calculated that 1000 was the number of newborns needed to cal-

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culate the allelic frequency of C282Y mutation with a precision of 0.01, and processed some additional samples to prevent the inclusion of noninformative cases. A 5-mm circle was punched out from the dried blood spots on filter paper and placed in individual tubes. DNA extraction was performed following the protocol described by Jinks *et al.* (1989). The presence of three mutations of the *HFE* gene, C282Y, H63D and S65C, was tested in the LightCycler™ (Roche Diagnostics GmbH, Mannheim, Germany). Amplification of the H63D and S65C variant fragment was performed with primers 63F (5'-GCT CTG TCT CCA GGT TCA CAC TC-3') and 63R (5'-CCC TCT CCA CAT ACC CTT GC-3'). The site of the C282Y substitution was amplified with primers 282F (5'-TGG CAA GGG TAA ACA GAT CC-3') and 282R (5'-CTC AGG CAC TCC TCT CAA CC-3'). The cycle conditions were as follows: denaturation of the template DNA for 1 cycle of 95°C for 10 min, programmed temperature transition rate of 20°C per second, amplification of the target DNA for 45 cycles of 95°C for 10 sec, 55°C for 10 sec (each with a temperature transition rate of 20°C per second) and 72°C for 18 sec (20°C per second). The melting curve analysis was performed in 1 cycle of 95°C for 5 sec and 45°C for 15 per second, each with a temperature transition rate of 20°C per second, and then ramping to 85°C for 0 sec at 0.1°C per second. The C187G transversion at codon 63 was then monitored with the 5'-LightCycler-Red 705-labeled anchor (5'-CTG GTA TCC ACG TAG CCC AAA GCT TCA A-3') and the 3'-fluorescein-labeled sensor (5'-CGG CGA CTC TCA TGA TCA TAG AAC ACG AAC A-3') probe. Because the mutation causing the S65C variant is located only six nucleotides away from the H63D change, it is detected in the same experiment. The G845A transition at codon 282 is simultaneously analyzed with the 3'-fluorescein-labeled sensor (5'-GAG ATA TAC GTG CCA GGT GGA GCA C-3') and the 5'-LC-Red640-labeled anchor probe (5'-AGG CCT GGA TCA GCC CCT CAT TGT-3'). The study was approved by the Institutional Ethics Committees.

Statistical methods

Allelic frequencies for each mutation and CI 95% of these frequencies were calculated using SPSS® statistical software (SPSS Inc., Chicago, IL), implementing the formula described by Miettinen (1970) as a "macro." Proportions were compared using Fisher's exact test. The frequency of C282Y and S65C homozygous individuals was estimated using the Hardy-Weinberg equilibrium formula. The results were considered statistically significant when *p* values were less than 0.05.

RESULTS

A total of 1146 newborns were screened. We had sufficient DNA to perform the three screened mutations in 1043 newborns (91%). Overall results are summarized in Table 1. The allelic frequencies of C282Y, H63D and S65C mutations were estimated to be 0.03 (95% CI, 0.022–0.037), 0.2 (95% CI, 0.19–0.22) and 0.01 (95% CI, 0.006–0.015), respectively. One newborn was C282Y homozygous, and the proportion of this genotype was estimated at 0.001 (95% CI, 0.0005–0.0014). Forty newborns, 0.04 (95% CI, 0.03–0.05), were H63D ho-

TABLE 1. ALLELIC FREQUENCIES AND GENOTYPES OF 1043 SCREENED CATALAN NEWBORNS

	Cases	Frequency	95% CI
Allelic frequencies			
C282Y	61	0.03	0.022–0.037
H63D	424	0.2	0.19–0.22
S65C	21	0.01	0.006–0.015
<i>HFE</i> Genotypes			
C282Y homozygous	1	0.001 ^a	0.0005–0.0014 ^a
C282Y heterozygous	47	0.045	0.033–0.06
H63D homozygous	40	0.04	0.03–0.05
H63D heterozygous	325	0.31	0.28–0.34
S65C homozygous	0	0.0001 ^a	0–0.0002 ^a
S65C heterozygous	10	0.01	0.005–0.02
C282Y/H63D	10	0.01	0.005–0.02
C282Y/S65C	2	0.002	0.0002–0.01
H63D/S65C	9	0.009	0.004–0.016

^aThe frequency of C282Y and S65C homozygous individuals was estimated using the Hardy-Weinberg equilibrium formula.

mozygous. No newborns were S65C homozygous. A proportion of 0.01 (95% CI, 0.005–0.02) and 0.002 (95% CI, 0.0002–0.01) newborns were double-heterozygous for C282Y/H63D and C282Y/S65C mutations respectively. Nine newborns, 0.009 (95% CI, 0.004–0.016) were H63D/S65C.

Ninety-six newborns had non-Caucasian parents (with a low incidence of C282Y mutations). The allelic frequency of this mutation (0.016) was lower in this selected population (95% CI, 0.003–0.04) without achieving statistical significance (*p* = 0.27). The allelic frequency of H63D (0.13) was significantly inferior in this group (95% CI, 0.09–0.2 *p* = 0.02), and the S65C mutation was absent. Allelic frequencies of newborns with Caucasian parents did not differ significantly from those found in the group as a whole.

DISCUSSION

Two screening methods for the preclinical detection of early stage GH are available: serum iron measures and molecular testing for mutations in the *HFE* gene. These phenotypic and genotypic screening tests are particularly interesting taking into account that simple periodic phlebotomies can be used to prevent iron accumulation and clinical complications. GH might represent the first adult-onset genetic disorder for which universal population-based screening would be appropriate (Adams *et al.*, 2000). However, universal screening for GH has not been recommended because of the uncertainty regarding the real frequency of the disease in each country and discrepancies observed among population studies about the clinical penetrance of the disease (Ajioka *et al.*, 2003; Beutler, 2003).

The main objective of this work was to obtain accurate data, from a specific region of Spain, to answer the first question (prevalence of causal mutations). There are some important concerns about the study population chosen for this screening. Some ethics experts advise against genetic screening for adult-onset diseases in the newborn population, specially when the penetrance

of disease is incomplete as in GH. Their argument is based on two main points: firstly, that newborns can not give informed consent, and secondly, that the individual may be labeled with a preexisting condition that could be prejudicial yet never come to pass. Nevertheless, this is the only screening system that guarantees diagnosis for the whole population. Moreover, in our opinion, a genotype compatible with GH represents a risk factor to develop iron overload in the future rather than a true disorder in itself. A C282Y homozygote does not require treatment at diagnosis and is unlikely to need it in the future. Probably all that is needed is the recommendation to become a blood donor in adult life, to control body iron stores. Because treatment is always successful and free of important side effects, there are no grounds for such a diagnosis to affect insurance policies, at least in countries with a sound national health system as in the European Union or Canada. The present paper shows such screening is feasible, using the same logistic approach as that used to diagnose other disorders in the newborn population in our country.

The prevalence of C282Y mutation is similar to that first reported (Baiget *et al.*, 1998) and subsequently confirmed in Catalan blood donors (Sanchez *et al.*, 2003) and in newborns from southern France (Aguilar-Martinez *et al.*, 2001), the Czech Republic (Cimburova *et al.*, 2002), and some areas of Italy with inhabitants of northern European ancestry (Salvioni *et al.*, 2003). Nevertheless, the allelic frequency of this mutation in Catalan newborns seems to be lower than that recorded for Caucasian newborns in the United States (Steinberg *et al.*, 2001), Ireland (Byrnes *et al.*, 2001), Canada (Girouard *et al.*, 2002), Denmark (Merryweather-Clarke *et al.*, 1999), northern Portugal and northern France (Jouanolle *et al.*, 1998; Cardoso *et al.*, 2001), but higher than that observed in Italy in general (Restagno *et al.*, 2000) and southern Portugal (Cardoso *et al.*, 2001). Frequencies may vary in other regions of Spain with a different genetic background, as has been observed in Portugal, Italy, and France.

An interesting result of this work concerns the allelic frequency of H63D mutation, because, to the best of our knowledge, it is the highest reported to date. The relationship between this mutation and hemochromatosis has been unclear ever since this mutation was discovered. Some authors consider it to be only a polymorphism, but all cohorts of hemochromatosis patients are enriched with the double heterozygous C282Y/H63D mutation, and its relationship with iron overload has been shown in animal models (Tomatsu *et al.*, 2003). If eventually, this mutation is found to play a role in clinically relevant iron overload, even though its influence on iron metabolism would be less important than that caused by C282Y, the epidemiologic impact on the Catalan population would be significant.

Mutation S65C has been described as a cause of hemochromatosis in combination with C282Y (Mura *et al.*, 1999). Nevertheless, the low frequency of this mutation observed in the present study supports our hypothesis that this mutation is not relevant in diagnosing hemochromatosis in Catalonia (Remacha *et al.*, 2000).

Finally, as was to be expected, the frequencies of HFE mutations are lower in newborns with non-Caucasian parents with a low prevalence of these mutations. Although these findings do not significantly alter the frequencies observed in the total population, they should be taken into account, specially if immigration continues to increase at the current rate.

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