THE C282Y AND H63D MUTATIONS IN THE HAEMOCHROMATOSIS GENE AMONG SICKLE CELL ANEMIA PATIENTS FROM THE NORTHEAST OF BRAZIL

ESTUDO DAS MUTAÇÕES C282Y E H63D DO GENE DA HEMOCROMATOSE HEREDITÁRIA EM PACIENTES COM ANEMIA FALCIFORME DO NORDESTE DO BRASIL

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Hereditary haemochromatosis (HH) is an autosomal recessive disorder. C282Y and the H63D mutations in the HFE gene have been associated to HH. AIM: To evaluate the frequencies of the C282Y and H63D mutations in sickle cell anemia patients (SS) and a reference group of newborns from Salvador, Bahia, Brazil. RESULTS: Of the 130 sickle cell anemia patients analyzed, one (1%) was heterozygous for the C282Y mutation and 22 (16.9%) were heterozygous for the H63D mutation. The reference group showed a similar allelic frequency of mutations. Among the patient group, ferritin serum levels were high in 23 (38.3%) samples. The most frequent b^s- globin gene genotypes were CAR/BEN, BEN/BEN and CAR/CAR. The mutations investigated seem to play an important role in vaso-occlusive crises (p=0.037) among sickle cell patients.

Keywords: Hereditary haemochromatosis, C282Y mutation, H63D mutation, sickle cell anemia patients.

A Hemocromatose Hereditária (HH) é uma doença autossômica recessiva. As mutações C282Y e H63D no gene HFE têm sido associadas à HH. Objetivo: Avaliar as frequências das mutações C282Y e H63D em pacientes com anemia falciforme (SS) e em recém-nascidos de Salvador, Bahiaa, Brasil. Resultados: dos 130 pacientes SS analisados, hum (1%) foi heterozigoto para a mutação C282Y e 22 (16,9%) foi heterozigoto para H63D. O grupo controle apresentou frequência alélica similar para as mutações estudadas. Entre os pacientes 23 (38.3%) apresentaram níveis séricos de ferritina elevados. Os haplótipos mais frequentes para o gene da globina b^s foram CAR/BEN, BEN/BEN e CAR/CAR. As mutações investigadas parecem ter um papel importante nas crises de vaso-oclusão (p=0.037) entre os pacientes SS.

Palavras-chave: hemocrromatose hereditária, mutação C282Y, mutação H63D, anemia falciforme.

Hereditary haemochromatosis (HH) is an autosomal recessive disorder characterized by abnormal iron absorption, resulting in an iron overload followed by an accumulation of iron in organs such as the liver, heart, pancreas and endocrine system[8 15 19 47 41].

The haemochromatosis human gene is located very close to the HLA-A gene complex on chromosome number 6[25]. The HFE gene codifies a MHC class I protein homologous, it is not an iron carrier, however, interacts with the transferrin receptor located on the cellular surface and is responsible for the regulation of transferrin iron uptake. This regulation mechanism is not completely understood however, recent research into the metabolism of iron has described the involvement of the HFE protein in the control of iron absorption[36].

Feder et al.[43] described two mutations in the HFE gene which may be responsible for haemocromatosis, the mutation G>A at nucleotide 845 (C282Y) replacing the amino acid cysteine for tyrosine at position 282 in the protein's chain and the C>G (H63D) at nucleotide 187 that replaces the amino acid histidine with aspartic acid at position 63 in the protein's chain. The C282Y mutation is very frequent among Northern European descendants (10%) but is rare or absent in African, Asian south pacific and aboriginal Australian population[43].

The homozygous state for C282Y mutation has been associated with the most severe haemocromatosis picture and it has been found in 83% of North American patients with haemocromatosis[20] and in more than 90% patients from the UK and France[34]. The H63D mutation has been associated with haemochromatosis when found in heterozygousity with the C282Y mutation (C282Y/H63D)[6].

African-descendant HH carriers present an increase in iron in macrophages and hepatic parenchymal cells[24]. The most frequent mutations found among North European descendants have not been seen in African populations. Recent studies have shown the possibility of different mutations playing an important role in iron overload in these individuals[24]. Additional HFE exon and intron mutations have been reported, such as the E168X, E168Q, V53M, V59M, S65C, G168T, G169T, G93R, I105T, T281K; but only the C282Y, H63D and S65C mutations in the HFE gene have been associated to

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an abnormal iron status[14 32 48]. Several mutations in the HFE gene have been associated with HH in different populations.

Salvador is a city in Bahia, a state located in the Northeast region of Brazil. It has a high rate of racial admixture with a strong African influence and Africans descendants make up the largest racial group in the population[5]. The state of Bahia has the highest prevalence of sickle cell anemia in Brazil[2].

The disease is characterized by severe hemolytic anemia, vaso-occlusive crises and other clinical complications, such as chest acute thoracic syndrome and infections described among patients[45]. The disease displays a phenotypic variability due to several modifying factors such as co-inheritance of α_2-thalassemia, HbF levels and β^S – globin gene haplotypes[18 21 44]. In some cases blood transfusion therapy is administered in cases of a vascular cerebral accident and chest acute syndrome when patients have a hemoglobin (Hb) concentration lower than 5g/dL. In aplastic crises blood transfusion therapy is indicated when there is an alteration in cardiac function, with hemoglobin levels bellow 4 g/dL. It can also be administered in the presence of spleen sequestration crises, priapism and septicemia. However, blood transfusion therapy can contribute to adverse effects such as hyperviscosity, increase in blood volume, hemolytic reactions, hemolytic fever reactions, allergic reactions, infectious illness transmission and iron overload[33 39 40].

The present study investigated the frequencies of the C282Y and H63D HH mutations in sickle cell anemia patients in order to establish the frequency of these mutations and evaluate if these HH mutations could be considered a disease severity marker in a group of sickle cell anemia patients.

Material and Methods

Population

We studied 130 sickle cell anemia patients non consaguineum from the *Fundação de Hematologia e Hemoterapia da Bahia* (HEMOBA) and 100 newborns from the Maternity Hospital Tsylla Balbino in Salvador, Bahia. The mean age of the sickle cell anemia patients was 19.5 (± 13.6), 66 males and 64 females. Of the 100 newborns, 37 were males and 53 females. Consent was obtained to allow the patients' and newborns' participation in the study. The study was approved by the Institutional Ethical Committee at the Centro de Pesquisas Gonçalo Moniz / FIOCRUZ-BA, with protocol number 141.

Methods

The hematological analyses were performed by electronic cell counter (Coulter Count T890 – FL, USA) and the hemoglobin profile was determined by High Performance Liquid Chromatography (HPLC) (Variant™ II BIO-RAD – CA, United State). Five milliliters of peripheral blood and umbilical cord blood anticoagulated with EDTA were obtained from the SS patients and newborns respectively. DNA was isolated from peripheral blood leukocytes using the Kit GFX™ Genomic Blood DNA Purification (Amersham Pharmacia Biotech - NJ, USA).

The C282Y and H63D HH mutations were investigated by PCR-RFLP techniques. The PCR products were digested by Rsa I and MboI restriction enzyme respectively and analyzed in an 8% polyacrilamide gel, stained by ethidium bromide and UV visualized.

The serum ferritin was estimated by electrochemiluminescence immunoassay in the Roche Elecsys 1010/2010 immunoassay analyzer (Roche Diagnostics).

The beta S- globin gene cluster haplotypes in sickle cell anemia patients were investigated by searching for endonucleases restriction sites, using the XmnI, HindIII, Hinc II, HincII, HinfI and HpaI enzymes, as previously described[46].

Clinical histories were collected from patient records. The statistical data analysis was carried out using EPI Info software version 6.04 and a p=0.05 was considered statistically significant.

Results

Hematological and clinical aspects of sickle cell anemia among patients were studied. Among a total of 130 SS patients, the median hemoglobin concentration (Hb) was 7.66 g/dL (± 1.68); the median hematocrit (Hct) 25.6% (± 4.9); the median cell volume (MCV) 93.5 fL (±12.3); the median cell hemoglobin (MCH) 28g/dL (± 4.5) and the median cell hemoglobin concentration (MCHC) 29.9% (+ 2.4).

According to the patients' clinical histories, 83/130 (63.8%) sickle cell anemia patients had been hospitalized; 73/130 (56.2%) had received blood transfusion and 30/73 (41.1%) had received up to five blood transfusions. Forty eight (36.9%) of the 130 patients had had infections, and 20/48 (41.7%) had had an inferior respiratory tract infection, followed by leg ulcers in 17/48 (35.4%) and urinary tract infection in two patients (4.2%). Comorbities were present in 34 (26.8%) of the 130 patients; splenic sequestration was found in nine (26.5%); cerebrovascular accidents (CVA), surgery and acute chest syndrome in four (11.8%) and retinopathy in three (8.8%).

In our study the C282Y mutation had an allelic frequency of 0.025 among the reference group and 0.015 among sickle cell anemia patients; the H63D mutation had an allelic frequency of 0.080 among the reference group and 0.084 among patients. The C282Y and H63D HFE mutations frequences were in Hardy-Weinberg equilibrium. There were no differences among the allelic frequencies of either mutations investigated among sickle cell anemia patients and the reference group (Table 1).

Table 1. C282Y and H63D – HFE mutation distribution among sickle cell anemia patients and the reference group from Bahia, Brazil.

C282Y Mutation	Sickle cell anemia patients - N (%)	Reference group - N (%)
WT/WT	129 (99.2)	99 (99)
C282Y/WT	1 (0.77)	1 (1)
Total	130 (100)	100 (100)
H63D Mutation		
WT/WT	108 (83.1)	84 (84)
H63D/WT	22 (16.9)	16 (16)
Total	130 (100)	100 (100)

Table 2. Distribution of serum ferritin levels and gender among sickle cell anemia patients from Bahia, Brazil.

Serum Ferritin level	Genders of sickle cell anemia patients – n (%)		Total - n (%)
	Female	Male	
Normal	11 (36.6)	26 (86.7)	37 (61.7)
High	19 (63.4)	4 (13.3)	23 (38.3)
Total	30 (100)	30 (100)	60 (100)

Exact test Fisher - p=0.0002. Reference values: Male: 30 – 400 ng/mL; Female: 13 - 150 ng/mL.

Serum ferritin levels were evaluated among 60 sickle cell anemia patients and the female group showed the highest levels (p=0.0002) (Table 2).

As regards age, ten (7.7%) sickle cell anemia patients were over 40 years of age, of thesefive had HH mutations, three were heterozygous for H63D, one was heterozygous for C282Y mutation.

Ferritin levels were estimated in 60 (46.2%) of the 130 sickle cell anemia patients and 23/60 (38.3%) showed high serum levels; 19 (82.6%) were female and four (17.4%) were male. When the high ferritin serum levels and HH mutations in sickle cell anemia patients with vaso-oclusive crisis histories were analyzed, 12 of the 23 had crises and five (41.7%) were heterozygous for the H63D mutation (p=0.037, Exact test Fisher) (Table 3).

Table 3. Distribution of the C282Y and H63D mutations in sickle cell anemia patients with high levels of serum ferritin and a history of vaso-clusive crisis.

Genotype	Crisis – n (%)		Total
	Presence	Absence	
Wild Type	7 (38.9)	11 (61.1)	18
Mutant	5 (100)	0	5
Total	12	11	23

Exact test Fisher: p=0.037.

Analyses of the beta^S-globin gene haplotypes were carried out in 124 sickle cell anemia patients, 64 (51.6%) being Car/Benin; 35 (28.2%) Benin/Benin; 18 (14.5%) were CAR/CAR; two (1.6%) were CAR/Atypical; two (1.6%) Benin/Cameron; one (0.8%) CAR/Cameron; one (0.8%) CAR/Saudi Arabian and one (0.8%) Senegal/Atypical. No association between haplotypes and the presence of HFE gene mutations was found.

Discussion

The genetic bases of HH have been investigated in many populations. Some mutations have been identified, but only the C282Y, H63D and S65C mutations in the HFE gene have been associated with HH[14 32 48]. The frequencies and effects of these mutations, other than C282Y and H63D, have yet to be determined[32]. The influence of iron overload severity and the risk of HH development has been shown among C282Y homozygous and H63D/C282Y and S65C/C282Y double heterozygous individuals[37].

The C282Y allele frequency in the Caucasian population was estimated at 0.063[7]. The association between HH and C282Y homozygous vary according to populations. In studies among European descendants, the C282Y homozygous in haemochromatosis patients ranged from 64% among Italians[12], 100% among Australians[28] and 80-90% among English and French[20].

The C282Y mutation is either absent or has a low allelic frequency in non-Caucasian populations, such as African, Asian, South Pacific and Aboriginal Australians[34 43].

In Brazil the C282Y HFE mutation has been studied in four Brazilian population groups: Caucasians, African descendants, Amerindians and an ethnically mixed group. There was a description of an allelic frequency of 1.4% in the Caucasian group; 1.1% in the African descendant group and 1.1% in the ethnically mixed group and the mutation was not found in the Amerindian group[3]. Pereira et al.[35] found a frequency of 3.7% of C282Y HFE in Euro-Brazilian, 0.7% in a mixed group and 0.5% in Afro-Brazilians, when they studied a highly mixed urban population from São Paulo, a Southeastern Brazilian state.

The allelic frequencies of the HFE mutations between the studied groups here were similar and were in agreement with previous studies[3 35] in the Brazilian population.

The manifestation of HH disease begins in the 4° and 5° decade of life due to progressive iron accumulation[17]. Carru et al.[13] reported that the C282Y mutation is significantly increased in very old (>90 years) Sicilian women, suggesting a role in longevity. To validate and extend these results, they investigated the distribution of the three most common HFE gene mutations (C282Y, H63D and S65C) in Sardinian centenarians and controls; they did not confirm the increase in the C282Y mutation observed before in Sicilian oldest old women. This finding may be due to a feature of HH disease that manifests itself in the 4° or 5° decade of life, a phase when the physician investigate more this mutations.

The biochemistry parameter serum ferritin is very sensitive to iron overload, but it is not specific because it could increase in liver and malignant diseases and inflammation state. Bulaj et al.[10] reported that approximately 25% of heterozygous members of HH patients' family could have high serum ferritin and transferrin iron saturation. Jackson et. al.[27] found a high level of serum ferritin in men who were heterozygous for C282Y mutation.

When we analysed our sickle cell anemia patients group with high levels of the serum ferritin, the presence of HH mutations and histories of vasoocclusion crises, we verified that all five patients heterozygous for the H63D mutation had already had crisis episodes. This goes against previous studies that have shown that heterozygous HH mutations carriers can present high levels of serum ferritin and in this case specifically, it can influence vaso-occlusive crises and can be related to an increase in inflammation and protective antioxidants among these patients, as described by Walter et. al.[49], when they studied the oxidative stress and inflammation in iron-overload patients with hemoglobinopathies.

Many pathologies have been associated with the presence of the HH mutation. The iron overload is highly toxic and plays a decisive role in the generation of reactive oxygen species (ROS)[36]. The ROS are involved in inflammatory, infectious, atherosclerotic and neoplastic conditions. Therefore, the genotype that leads to an increase in iron in the body must be associated with an increased risk of many common diseases[51].

Blood tranfusion therapy has been used in sickle cell anemia patients mainly to prevent strokes. Stroke prevention in sickle cell anemia patients has already reported an increase in serum ferritin in these patients. Disease complications and early death have been associated with an increase in iron overload in patients with hereditary hemochromatosis and β thalassemia[9 16].

On analysis of the HH mutations in sickle cell anemia patients with high levels of serum ferritin with a history of vaso-occlusive crises, we found a statistical difference (p=0.0372). Koduri et al.[30] in a review about iron metabolism and sickle cell anemia described that when iron deficiency lowers, the mean corpuscular deoxyhemoglobin-S concentration (MCHC-S) thereby decreases the sickling tendency and the hemolysis severity. These data explain the presence of a high number of crises in sickle cell anemia with mutations. The crisis can be induced by iron overload and can increase the sickling and the number of crisis events.

The β^S- globin gene haplotypes have been associated as a marker in clinical and anthropological studies of sickle cell anemia patients. The CAR/BEN genotype has the highest frequency due to the fact that Bahia historically received slaves from Western Africa, Central and South Africa. Our results agree with those from previous studies carried out among sickle cell anemia patients from Bahia, in the Northeast of Brazil[1 23 31].

The iron deposition pattern among African and European descendants vary, there is marked iron loading of Kupffer's cell as well as hepatocytes, being frequently associated to other unknown genetic, nutritional and environmental features. The iron overload has been reported in populations of countries of southern, eastern and western Africa. Bahia received a large influx of slaves from Central and Western Africa which is reflected in the predominance of the CAR/BEN genotype in our results. Therefore, we are unable to establish a possible association between the HH mutations in the reference group from a very mixed population and the clinical picture of the sickle cell anemia patients. However, to explain the role of these genetic alterations in hemolytic anemia severity it is necessary to carry out further studies involving other mutations in order to hypothesize about the possible mechanisms involved in iron overload among these patients.

The present study was important to establish a possible association between C282Y and H63D mutations in the HFE gene and the clinical events in a group of sickle cell anemia patients from Bahia. Further studies are needed in order to explain the mecanism of iron overload in Afro-descents from our population and the possible involvement of the other genes in this process.

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