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Frequencies of CD39, IVS1-1, IVS1-6 and IVS1-110 mutations in beta-thalassemia carriers and their influence on hematimetric indices

Frequências de mutações CD39, IVS1-1, IVS1-6 e IVS1-110 em portadores de betatalassemia e suas influências nos valores hematimétricos

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ABSTRACT

Introduction: Beta-thalassemia is caused by a deficient synthesis of the ß-chain of hemoglobin, which leads to a chronic, microcytic and hypochromic anemia. More than 200 mutations have already been associated with this type of thalassemia, and their frequencies may vary according to the population. **Objectives:** The objectives of this study were to determine the frequencies of CD39, IVS1-1, IVS1-6 and IVS1-110 mutations in people with beta-thalassemia from the city of Franca, São Paulo, and to evaluate the influence of the genotypes on hematological alterations. **Methods:** Venous blood samples were collected from 25 volunteers previously diagnosed with beta-thalassemia. Complete blood counts (CBC) were performed, and the identification of the mutations was carried out using the polymerase chain reaction (PCR). **Results:** The CD39 mutation was found in 11 (44%) individuals, followed by IVS1-6 (9; 36%) and IVS1-110 (4; 16%). One patient (4%) did not present any of these mutations. IVS1-6 mutation was inversely correlated to red cell distribution width (RDW) ($r_s = -0.44; p = 0.034$), and CD39 was correlated to lower mean corpuscular volume (MCV) ($r_s = -0.44; p = 0.034$). Multivariable linear regression models showed that the CD39 mutation carriers have lower levels for hemoglobin (ß = -0.61; $p = 0.044$) and hematocrit (ß = -2.1; $p = 0.018$). **Conclusion:** The results showed a high frequency of the CD39 mutation in the city of Franca, and the correlations observed between the presence of CD39 mutation and the hematological alterations suggest a genotype influence on the phenotype of beta-thalassemia, which would contribute to the clinical variations of this hemoglobinopathy.

Key words: beta-thalassemia; hemoglobinopathies; genotype.

INTRODUCTION

Hemoglobin (Hb) disorders are the most common inherited and monogenic diseases as 7% of the world population is believed to be carrier of a mutant gene for thalassemias or other hemoglobinopathies[1, 2]. These mutations may cause qualitative or quantitative damages to Hb, depending on the affected site[3-5].

The group of thalassemias comprehends the genetic disorders in which the Hb synthesis is quantitatively impaired[6-8]. In beta-thalassemia, one of the most relevant types of the disease, the β-chain of Hb is absent or reduced due to mutations in the *HBB* gene, located in chromosome 11[9]. Based on clinical features and on the genotype of the individuals, this anemia is divided into three forms, from the mild to the severe type: beta-thalassemia minor, intermedia and major[9, 10].

Thalassemias, in general, are more frequent in the Mediterranean region and their occurrence in Brazil is mainly due to the miscegenation in the country[11]. In each population group there are specific mutations for beta-thalassemia that are regularly found in the patients. In Brazil, four mutations are frequently reported in the genetic studies: CD39 C>T (HBB: c.118C>T), IVS1 nt1 G>A (HBB: c.92+1G>A), IVS1 nt6 T>C (HBB:c.92+6T>C) and IVS1 nt110 G>A (HBB: c.93-21G>A)[9, 12].

The mutations of intron 1 in the *HBB* gene are non-deletional alterations that affect the messenger RNA (mRNA) processing[13]. The IVS1-1 mutation stops mRNA splicing because the replacement of

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guanine by adenine (G>A) alters the splice junction and generates a non-functional mRNA which is degraded inside the nucleus, resulting in a β^0 phenotype, i.e., the affected gene does not synthesize the β-chain[13, 14]. IVS1-6 mutation affects the splice site consensus sequence, causing a decrease in the normal splicing process, but not the complete blockade, leading to the phenotype β^{++}, a situation where the β-chain synthesis is reduced but not absent[15]. IVS1-110 mutation, on the other hand, occurs in a region not normally used in the mRNA (cryptic sites), but the G>A change creates an alternative splice site, which is preferably used during the mRNA processing, generating aberrant transcripts; however, the correct splicing can still occur, so this mutation is classified as β^{+}[13].

The CD39 mutation affects the codon 39 and is classified as a nonsense mutation. The replacement of cytosine by thymine (CAG>TAG) results in a premature stop codon, interrupting the translation of the mRNA. In this case, there is no synthesis of β-chain by the affected RNA, leading to β^0 phenotype[13, 14].

Different frequencies of these mutations are reported in studies from Brazil[12, 16-20], which reinforces the importance of investigating the molecular bases of beta-thalassemia in several regions of the country. Thus, this study was conducted in the city of Franca, located at the northeastern region of São Paulo state, and the objectives were to identify the frequency of the CD39, IVS1-1, IVS1-6 and IVS1-110 mutations among beta-thalassemia carriers, and to evaluate their influence on hematological parameters to a better understanding of the clinical variations of this hemoglobinopathy.

METHODS

Sample collection

All participants were referred by a hematologist as beta-thalassemia carriers, since they were previously diagnosed by the physician through clinical examinations allied to blood tests, i.e., complete blood counts (CBCs) and Hb electrophoresis. Twenty-five voluntary unrelated individuals, residents (but not necessarily born) in the city of Franca, São Paulo, were included in the study after signing a free and informed-consent form. Participants were at least 18 years old by the time of their recruitment. Samples of venous blood were collected in two evacuated tubes containing K_2EDTA (Vacutainer®; BD, Franklin Lakes, NJ, USA). One tube was used for hematological evaluation while the other was used for deoxiribonucleic acid (DNA) isolation.

The study was approved by the Ethics Committee of the Universidade de Franca and conducted in accordance with the Declaration of Helsinki[21].

Hematological evaluation

CBCs were performed in an automated counter (BC-3200; Mindray®, Shenzhen, China). Erythrocytes morphology was evaluated in blood smears with Leishman stain under an optical microscope.

DNA isolation and genotyping

Genomic DNA was isolated from peripheral blood using the GenElute™ BloodGenomic DNA kit (Sigma-Aldrich, Saint Luis, MO, USA), according to the manufacturer's instructions. DNA concentrations were measured in spectrophotometer (NanoVue™; GE Healthcare, Little Chalfont, UK), then the samples were diluted to 20 ng/µl and stored at -20ºC until analyses.

The CD39, IVS1-1, IVS1-6 and IVS1-110 mutations were genotyped using the polymerase chain reaction (PCR) in a T100™ thermocycler (BioRad, Hercules, CA, USA)[22-24]. In order to avoid false-positives or false-negatives, blank solutions containing all reagents except DNA were analyzed concurrently with samples; all reactions were performed in triplicates. Primers sequences and the length of the PCR products fragments were: 5'-GACTCAAAGAACCTCTA-3' (CD39; 439 bp); 5'-GTAACCTTGATACCAAA-3' (IVS1-1; 268 bp); 5'GTCTTGTAACCTTGATG3' (IVS1-6; 273 bp); 5'-GGGTGGGAAAATAGACT-3' (IVS1-110; 337 bp); 5'-GGCTGTCATCACTTAGACCTCA-3' (Internal control 1; 659 bp); 5'-AGAAGGGGAAAGAAAACATCAA-3' (internal control 2; 659 bp). The School of Pharmaceutical Sciences of Ribeirão Preto-Universidade de São Paulo (USP) gently provided all primers.

PCR reactions were performed in 200 µl microtubes in which were added 2.5 µl of 10× PCR buffer (Life Technologies, Carlsbad, CA, USA), 1.0 µl of 50 mM $MgCl_2$ (Life Technologies, Carlsbad, CA, USA), 2.0 µl of 50 mM deoxynucleotide solution mix (dNTPs) (Life Technologies, Carlsbad, CA, USA) 2.5 µL of primer C1, 2.5 µl of primer C2, 2.5 µl of each mutation primer (in separate reactions), 0.25 µl of Taq DNA polymerase (500 U/l, Life Technologies, Carlsbad, CA, USA), 5 µl of DNA samples at 20 ng/µl and 6.75 µl of ultrapure water. The tubes were placed in the thermal cycler under the following conditions: 3 minutes at 94ºC for pre denaturation; 32 cycles of 50 seconds at 94ºC for denaturation, 50 seconds at 54ºC for the annealing of CD39, IVS1-1 and IVS1-6 primers and at 58ºC for IVS1-110, 50 seconds at 72ºC for extension; 7 minutes at 72ºC for one last extension cycle.

The amplification products were analyzed in a 1.5% agarose gel stained with GelRed™ (Biotium, Inc; Fremont, CA, USA) after a one hour run at 80 volts.

Statistical analyses

First, descriptive analyses were performed in order to assess the general characteristics of the participants, and the variations of the hematological parameters according to gender were evaluated by Student's *t*-test. Spearman correlations were performed between the CD39, IVS1-6 and IVS1-110 mutations and Hb levels, erythrocytes, Ht, red cell distribution width (RDW), hematimetric indices and gender.

Finally, multivariate general linear models were employed to evaluate the impact of the genotypes on the hematological parameters. Univariate models were employed and the variables were included in the multivariate model if they showed a p value < 0.20. The values for mean corpuscular Hb concentration (MCHC) and RDW were transformed through the analyses. Normality of data was assessed through Shapiro-Wilk test, which is indicated to small sample sizes. Data were considered as normally distributed if the significance value of Shapiro-Wilk test was greater than or equal to 0.050.

Results were defined as statistically significant when $p \leq 0.050$. All the analyses were performed using SPSS®20 Statistics software (IBM; Armonk, NY, USA).

RESULTS

General characteristics

From the 25 patients included in the study, 24 were previously diagnosed with beta-thalassemia minor and one with beta-thalassemia major, according to their medical records. **Table 1** presents the general characteristics for the participants, including the results of the CBCs. Eighteen individuals (72%) were female and seven (28%) were male. Mean ± standard deviation (SD) for Hb, grouped by gender, were 11.1 ± 0.875 g/dl for women and 12.4 ± 1.43 g/dl for men ($p = 0.016$). Erythrocytes levels and Ht were also statistically different according to gender ($p = 0.010$ and $p = 0.0060$, respectively).

It is important to highlight the presence of microcitosis and hypochromia, as demonstrated by the low values for mean corpuscular volume (MCV) (67.7 ± 7.0 fl) and mean corpuscular Hb (MCH) (21.2 ± 2.83 pg), respectively. A significant anisocytosis was observed on blood smears, besides being evidenced by the high RDW index (15.0 ± 1.58%).

TABLE 1 – General characteristics of study participants

X ± SD		Women	Men	p^a
		X ± SD	X ± SD	
Hb (g/dl)	11.5 ± 1.17	11.1 ± 0.875	12.4 ± 1.43	0.016*
Erythrocytes (mi/mm³)	5.43 ± 0.774	5.19 ± 0.759	6.04 ± 0.389	0.01**
Ht (%)	36.0 ± 3.57	34.9 ± 2.69	39.0± 4.02	0.006**
MCV (fl)	67.7 ± 7.7	68.4 ± 8.87	65.8 ± 2.91	0.454
MCH (pg)	21.2 ± 2.83	21.8 ± 3.02	19.9 ± 1.78	0.14
MCHC (%)	31.8 ± 0.923	31.9 ± 0.803	31.5 ± 1.22	0.415
RDW (%)	15.0 ± 1.58	14.6 ± 1.33	15.8 ± 1.93	0.17

Hb: hemoglobin; Ht: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; X: arithmetic mean; SD: standard deviation; [a]: Student's t*-test to compare the differences between genders.*

**p ≤ 0.050; **p ≤ 0.010*

Genotyping results and correlation with phenotype

The results for genotyping are described in **Table 2**. The CD39 mutation was the most frequent among the participants, found in 11 individuals (44%). Following, IVS1-6 mutation was found in nine samples (36%) and IVS1-110 mutation was positive for four samples (16%). The IVS1-1 mutation was not found and one sample (4.0%) was not positive for any of these four mutations.

The analyses of blood smears revealed many poikilocytosis in all the samples and most of them were dacryocytes (in 68% of samples) and target cells (in 72% of samples). Moreover, it was possible to observe an occurrence of elliptocytes (28%), spherocytes (16%) and stomatocytes (4%).

Regarding statistical analyses, two individuals were not included in the tests, that is, the one diagnosed with beta-thalassemia major and the one who was not positive for the mutations studied, in order to minimize statistical bias. Spearman correlations were performed for the CD39, IVS1-6 and IVS1-110 mutations and Hb levels, erythrocytes, Ht, RDW, hematimetric indices and gender. Gender was correlated to Hb ($r_s = 0.53$; $p = 0.010$), erythrocytes ($r_s = 0.54$; $p = 0.0080$) and Ht ($r_s = 0.56$; $p = 0.0050$). The presence of IVS1-6 mutation was inversely correlated to RDW ($r_s = -0.44$; $p = 0.034$), which shows a less intense anisocytosis in the carriers of this mutation. On the other hand, the CD39 mutation was correlated to a lower MCV ($r_s = -0.44$; $p = 0.034$). However, since genotypes are included as nominal variables, these data may not represent a consistent result, which reinforces the importance of the multivariable linear regressions, and results are described in **Table 3**. The CD39 mutation was associated to lower Hb ($\beta = -0.61$; $p = 0.044$) and lower Ht ($\beta = -2.1$; $p = 0.018$) levels. These data indicate that the presence of this

particular mutation may contribute to a more intense anemia, even in individuals with beta-thalassemia minor. Since this is a nonsense mutation, one allele is completely silenced and only 50% of the β-chain is produced.

TABLE 2 – Frequencies of the mutations found in the study

Mutation	Frequency
CD39 (C>T)	11 (44%)
IVS1-6 (T>C)	9 (36%)
IVS1-110 (G>A)	4 (16%)
IVS1-1 (G>A)	0 (0%)
Other	1 (4%)
Total	25 (100%)

TABLE 3 – Multivariate linear regressions between genotypes and hematological parameters

Genotype	Hb[a]	Erythrocytes[b]	Ht[c]	RDW[d]	MCV[e]	MCH[f]	MCHC[g]
CD39	-0.61*	0.034	-2.1*	0.065	-2.8	-0.99	0.002
IVS1-6	0.42	-0.094	1.4	-0.071	2.3	0.61	-0.002
IVS1-110	0.35	0.097	1.2	0.005	1.1	0.67	0.001

[a]: Hb = α + β1 × gender + β2 × erythrocytes + β3 × genotype; [b]: erythrocytes = α + β1 × gender + β2 × genotype; [c]: Ht = α + β1 × gender + β2 × erythrocytes + β3 × genotype; [d]: RDW = α + β1 × genotype; [e]: MCV = α + β1 × genotype; [f]: MCH = α + β1 × genotype; [g]: MCHC = α + β1 × gender + β2 × genotype.

Hb: hemoglobin; Ht: hematocrit; RDW: red cell distribution width; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

*$p \leq 0.050$.

DISCUSSION

In Brazil, beta-thalassemia is more prevalent in the Southeast region, with 66% of all the cases concentrated in this area[25]. This fact may be due to the intense immigration of peoples from the Mediterranean to the Southeastern states of Brazil, which occurred between the 19th and 20th centuries[26] and contributed to the insertion of thalassemic genes in this population. Some studies show that CD39, IVS1-110 and IVS1-6 mutations are the most frequent in different regions of Italy[27, 28], as well as in certain population groups in Portugal[29, 30].

In the present study, the mutations found in the city of Franca follow the pattern generally seen among the population from the Southern and Southeastern Brazil[16-18, 22]. Although scarce, the studies performed in some cities from the state of São Paulo show that the CD39 mutation accounts for about 50%-60% of beta-thalassemia cases[17, 18, 22]. We also demonstrated a significant influence of the genotype on the anemia clinical condition, since the CD39 mutation carriers had lower Hb concentrations and lower Ht and MCV values. In addition, the IVS1-6 mutation was correlated with lower RDW values, indicating a less intense anisocytosis when compared to other genotypes. Some studies have found a relationship between hematological parameters and the different mutations, supporting the results evidenced here. Rund *et al*. (1992)[31] investigated 18 mutations in heterozygotes and found a decreased MCV and MCH values in individuals with β^0 mutations. Similar results were described by Stefanis *et al*. (1994)[32], who reported higher values for MCV in the presence of the IVS1-6 mutation. However, none of these studies found or investigated the correlation between the genotypes, Hb and Ht, which reinforces the need to carry out further studies in order to accurately determine the existing genotype-phenotype relationship in beta-thalassemia.

In general, the heterogeneity of the clinical forms of beta-thalassemia can be explained by the molecular bases involved in the origin of the disease. The β^0 mutations, such as CD39 and IVS1-1, are associated with the complete blockade of the β-chain synthesis and also lead to a greater formation of free α-chains precipitates, which causes an early destruction of erythrocytes[8]. However, studies related to genotype-phenotype interaction are still scarce and the different distribution of mutations in each region of the country makes it difficult to compare unrelated populations. Furthermore, beta-thalassemia should be studied more intensively, in order to achieve a more realistic picture of this hemoglobinopathy in the Brazilian population.

CONCLUSION

This was the first study conducted in Franca, São Paulo, aiming to determine the main mutations related to beta-thalassemia in the city. It was possible to identify three mutations generally associated with populations from the Mediterranean region and we also found correlations between the genotypes and phenotype, suggesting an influence of the molecular basis on the hematological alterations found in this anemia.

The study had limitations, particularly the small sample size and the absence of complementary tests, such as Hb electrophoresis and screening for alfa-thalassemia. Further studies with a larger sample size and more detailed data would be helpful to the assessment of the genotype-phenotype interactions that were discussed in our work.

RESUMO

Introdução: *A betatalassemia é causada pela síntese deficiente da cadeia ß da hemoglobina, o que leva à ocorrência das anemias crônica, microcítica e hipocrômica. Mais de 200 mutações já foram associadas a esse tipo de talassemia, mesmo que diferentes populações apresentem frequências variadas para cada uma delas.* ***Objetivos:*** *Os objetivos deste estudo foram determinar as frequências das mutações CD39, IVS1-1, IVS1-6 e IVS1-110 em indivíduos betatalassêmicos da cidade de Franca, São Paulo, e avaliar a influência dos genótipos sobre alterações hematológicas.* ***Métodos:*** *Amostras de sangue venoso foram coletadas de 25 voluntários previamente diagnosticados com betatalassemia. Foram realizados hemogramas completos, e a identificação das mutações foi feita utilizando a técnica de reação em cadeia da polimerase (PCR).* ***Resultados:*** *A mutação CD39 foi encontrada em 44% dos indivíduos, seguida por IVS1-6 (36%) e IVS1-110 (16%). Um paciente (4%) não apresentou nenhuma das mutações. A mutação IVS1-6 correlacionou-se inversamente ao* red cell distribution width *(RDW) (r_s = -0,44;* p = *0,034), enquanto a presença da mutação CD39 mostrou-se correlacionada a menores valores de volume corpuscular médio (VCM) (r_s = -0,44;* p = *0,034). Modelos de regressão linear multivariados mostraram que os portadores da mutação CD39 possuem menores valores de hemoglobina (ß = -0,61;* p = *0,044) e hematócrito (ß = -2,1;* p = *0,018).* ***Conclusão:*** *Os resultados mostraram alta frequência da mutação CD39 na cidade de Franca, e as correlações observadas entre a presença da mutação CD39 e as alterações hematológicas sugerem influência do genótipo sobre o fenótipo da betatalassemia, o que poderia contribuir para as variações clínicas dessa hemoglobinopatia.*

Unitermos: *betatalassemia; hemoglobinopatias; genótipo.*

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