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α-Thalassemia 2, 3.7 kb deletion and hemoglobin AC heterozygosity in pregnancy: a molecular and hematological analysis

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Summary α-Thalassemia is a synthesis hemoglobinopathy with a worldwide distribution. α-thalassemia-$2^{3.7kb}$ (α-Thal$2^{3.7kb}$) was investigated by PCR and standard hematologic analysis techniques in 106 pregnant women – 53 heterozygous for hemoglobin (Hb) A and C (AC) and 53 homozygous for the normal Hb A (AA) with similar ages and race ancestry. Eleven (21%) of AC women were α-Thal$2^{3.7kb}$ heterozygous and 1 (2%) was homozygous, while 12 AA women (23%) were heterozygous. In the AA group, the MCV differed among those with normal α genes and those with α-Thal$2^{3.7kb}$ ($P = 0.031$). Statistical analysis of AC group patients with normal α genes and α-Thal$2^{3.7kb}$ carriers showed differences in MCV ($P = 0.001$); MCH ($P = 0.003$) and Hb C concentrations ($P = 0.011$). Analysis of AA and AC group patients with normal α genes showed differences in RBC ($P = 0.033$), Hb concentration ($P = 0.003$) and MCHC ($P < 0.0001$). There were no statistically significant differences for any hematologic parameters between AC and AA group patients with the α-Thal$2^{3.7kb}$ genotype. The AC α-Thal$2^{3.7kb}$ homozygous women had low hematologic parameters. Serum ferritin levels were normal among the groups studied. These results emphasize the importance of diagnosis and follow-up of patients with hemoglobinopathy carriers during pregnancy in order to administer adequate therapy and avoid further complications for mothers and newborns.

Keywords Hemoglobinopathies, hemoglobin C, pregnancy, thalassemia, hematologic parameters

Introduction

The S and C Hb variants have a worldwide distribution (Weatherall & Provan, 2000). The S variant occurs in frequencies of 40–50% in African populations (Livingstone, 1967), and has also been described in high frequency among non-African populations in southern Turkey, Saudi Arabia, the northern Mediterranean, Sicily, Cyprus and Greece (Campbell & Oski, 1977). In the US, the S trait (AS) has a frequency of 6% among African-Americans, and a similar gene distribution exists in Central and South America and the West Indies (Bunn & Forget, 1986). In contrast, The β^C allele is found almost exclusively among black Americans and West Africans from Northern Ghana and Volta territory and to a lesser degree in Western Nigeria. Although it has been found

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rarely in individuals from Italy, particularly from Sicily, its geographic distribution when compared with other β variant chains such as β^S,β^D and β^E is quite localized (Boehm *et al.*, 1985). In USA, the Hb C frequency reaches 3% among African-Americans (Morrison, 1979). In Brazil, it is the second most frequent variant Hb and Hb A and C heterozygosity (AC) was described in up to 3.5% (Zago *et al.*, 1983; Zago & Costa, 1985; Nascimento *et al.*, 1986).

Inherited Hb synthesis disorders can affect any globin gene, with a high prevalence of α- and β-thalassemias (Lee *et al.*, 1992). α-thalassemia (α-Thal) is one of the most important Hb disorders and is characterized by absent or reduced synthesis of α-globin chains (Baysal & Huisman, 1994). The α-globin gene cluster has been mapped to the terminal portion of the short arm of chromosome 16 (16p13.3) and is arranged in the order 5′-ζ-ψζ-ψα1-α2-α1-θ1−3′ (Dodé *et al.*, 1992; Ko *et al.*, 1998). Normal individuals have four functional α-globin genes, two per haploid genome (αα/αα) (Williams *et al.*, 1996). α-thalassemias are frequently caused by deletions involving one or both α genes. Deletions of 3.7 and 4.2 kb of DNA in only one α gene in each homologous chromosome (-α/αα or -α/-α) is the most common form of α-thalassemia 2 or α^+, and deletion in both α genes (–/α α or –/–) is designed α-thalassemia 1 or α^0 (Fichera *et al.*, 1997). The heterozygous state of α^0 and the homozygous state of α^+ are both characterized by mild hypochromic and microcytic anemia (Williams *et al.*, 1996), with heterogeneous phenotypic expression (Fichera *et al.*, 1997).

Pregnancy is a critical time, as the vascular bed and blood volume expands, and fetal growth accelerates metabolic drain. There are several reports emphasizing the clinical effects of inherited disorders of Hb structure such as Hb S and C on the course of pregnancy, labor, delivery and the puerperium (Fiakpui & Moran, 1973; Milner, Jones & Döbler, 1980). In women with sickle cell disease, it is frequently associated with clinical complications (Perkins, 1971; Hendrickse *et al.*, 1972; Horger 1974; Milner, Jones & Döbler, 1980; Larrabe & Monga, 1997). Few reports have addressed the association between α-Thal, Hb C, and pregnancy (Morrison, 1979). In the present report, we studied α-thalassemia $2^{3.7kb}$ (α-Thal$2^{3.7kb}$) in groups of pregnant women with AC and normal Hb A (AA) patterns, analyzing haematologic, hemoglobin and serum ferritin characteristics. This study was conducted in a Brazilian population from Salvador (Bahia state, north-east of Brazil), a city with a high prevalence of Hb abnormalities and a high rate of race admixture, represented by a strong component of African genes (Krieger *et al.*, 1965; Azevedo *et al.*, 1980).

Materials and methods

We conducted a case-controlled study with 106 pregnant women from Salvador who attended the Tsylla Balbino public maternity ward. These women were previously selected from a cross-sectional study of 1953 pregnant women at the same institution. The 53 eligible AC women, aged 13–39, were selected from the 64 AC women identified in the previous study; AA women of similar age (±4 years of the respective case) visiting the facility during the study period were selected as controls. These studies were previously approved by the Gonçalo Moniz Research Institute's (CPQGM–FIOCRUZ) Institutional Ethical Committee.

Ten milliliters of peripheral blood were obtained from each woman; 5 ml were ethylenediaminetetraaceticacid (EDTA) anticoagulated and used for hematologic analysis, hemoglobin genotyping, and DNA extraction. The other 5 ml were collected without additive and used for serum ferritin analysis (180 Ferritin, Chiron Diagnostics ACS Massachusetts, U.S.A.) reference values 10–120 ng/ml. Hematological parameters were obtained using an automated cell counter (Coulter Count T 890 Florida, U.S.A.). Hemoglobin C was quantified by Hb elution from cellulose acetate strips, and fetal Hb (Hb F) levels were determined by alkali denaturation (Dacie & Lewis, 1984). DNA was isolated from leukocytes using a GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech New Jersey, U.S.A.) and the molecular characterization of α-Thal$2^{3.7}$ was determined by polymerase chain reaction (PCR) using primer combinations for mutant (A + B) and normal α-genes (A + C) as previously described (Baysal & Huisman, 1994; Smetanina & Huisman, 1996). Each set of reactions included positive and negative controls. To determine the α-gene genotype, two sets of reactions were conducted in a PCR buffer containing 1.7 mM $MgCl_2$, 50 mM Tris–HCl pH 8.9, 200 μM dNTPs (G + C), 100 μM dNTPs (A + T), 13% glycerol, 2.5 U Taq DNA Polymerase (Gibco-BRL, Life Technologies Sao Paulo, Brazil), 25 pmoles of each specific primer and 500 ng of genomic DNA (Foglietta *et al.*, 1996). The PCR reaction mix was heated at 98 °C for 3 min and at 85 °C for 3 min, followed by 35 cycles: five of 98 °C for 30 s, 66 °C for 1 min 30 s and 72 °C for 30 s, and 30 of 96 °C for 30 s, 66 °C for 30 s and 72 °C for 2 min. After cycling, the tubes were kept at 72 °C for 10 min, and subsequently analyzed by gel electrophoresis (1% agarose gel, stained with ethidium bromide) in TAE 1X and UV visualized (Figure 1). The reactions were performed in a Perkin–Elmer Thermal Cycler model 2400 (Perkin Elmer Cetus Corporation, Norwalk, CT, USA).

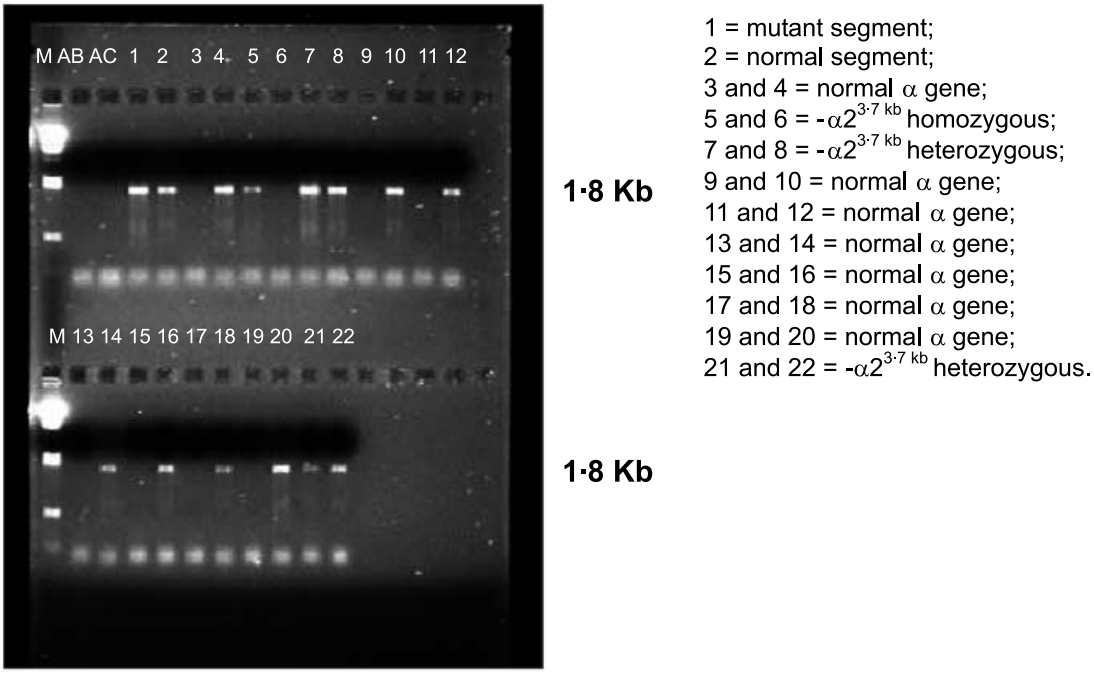

Figure 1. Identification of the amplification products by gel electrophoresis. One percent agarose gel in 1X TAE buffer pH 8.3, showing the PCR reaction for the α-Thal-$2^{3.7kb}$ investigation with normal and deleted fragments. For the 3.7-kb deletion, primers A + B amplified the abnormal fragment (1.8 kb) and primers A + C amplified the normal fragment (1.8 kb). M, molecular weight marker (λ *Hind*III); AB and AC lines are negative controls for PCR reactions; lines 1 show a PCR amplification of mutant segment of α gene with A + B primers, and line 2 shows a PCR amplification of normal segment of α gene with A + C primers. Lines 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21 show PCR reaction by A + B primers, and lines 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 show PCR amplification by A + C primers.

EPI Info Software version 6.04 (Dean, Dean & Coulumbier, 1994) was used for statistical analysis. Means were compared using analysis of variance (ANOVA) for normally distributed data only. A *p*-value of less than 0.05 was considered statistically significant.

Results

Among the 53 pregnant women with AC Hb pattern, 41 (77%) had normal α genes and 12 (23%) were α-Thal$2^{3.7kb}$ carriers, 11 (21%) heterozygous (-α/αα) and 1 (2%) homozygous (-α/-α). In the AA group, 12 (23%) were heterozygous for α-Thal$2^{3.7kb}$. Analysis of hematologic data from the AA group indicated that α-Thal$2^{3.7kb}$ carriers showed significantly lower MCVs than those with normal α genes ($P = 0.031$; Table 1). Within the AC group, α-Thal$2^{3.7kb}$ carriers demonstrated lower MCVs ($P = 0.001$), lower Hb C levels ($P = 0.011$), and lower MCHs ($P = 0.003$; Table 2). Statistical comparisons between AA and AC genotypes in patients with normal α genes indicated that AC patients had higher mean RBC counts ($P = 0.033$), Hb levels ($P = 0.003$), PCVs

Table 1. Hematologic and Hb results of AA women with α-Thal-$2^{3.7kb}$ and α normal genes

Hematologic parameters and Hb types	α genes (N) genotype		*P*-value
	αα/αα (41)	$-\alpha^{3.7}$/αα (12)	
RBC ($\times 10^{12}$)/l	3.80 ± 0.50	4.01 ± 0.50	0.164
Hb (g/dl)	10.80 ± 1.62	10.90 ± 1.40	0.802
PCV	0.33 ± 0.04	0.33 ± 0.04	0.861
MCV (fl)	88.32 ± 7.27	84.01 ± 4.03	0.031
MCH (pg)	28.50 ± 3.17	28.05 ± 2.30	0.660
MCHC (g/dl)	32.27 ± 1.57	32.40 ± 1.19	0.797
Hb A_2 (%)	2.90 ± 1.02	2.81 ± 1.17	0.795
Hb F (% in adults)	1.39 ± 1.16	1.39 ± 0.89	0.985
Ferritin levels (ng/ml)	22.31 ± 27.57	32.69 ± 39.67	0.429

Table 2. Hematologic and Hb results of AC women with α-Thal-$2^{3.7kb}$ and α normal genes

Hematologic parameters and Hbs types	α-gene (N) genotype		
	αα/αα (41)	−α/αα (11)	*P*-value
RBC (× 10^{12}/l)	4.02 ± 0.49	4.12 ± 0.42	0.432
Hb (g/dl)	11.94 ± 1.80	11.16 ± 1.53	0.850
PCV	0.35 ± 0.04	0.34 ± 0.04	0.230
MCV (fl)	88.52 ± 7.03	82.83 ± 5.53	0.001
MCH (pg)	29.67 ± 2.87	27.46 ± 2.60	0.003
MCHC (g/dl)	33.47 ± 1.33	32.61 ± 1.21	0.012
Hb C (%)	37.12 ± 5.77	32.09 ± 4.97	0.011
Hb F (% in adults)	1.42 ± 1.52	1.76 ± 1.89	0.546
Ferritin levels (ng/ml)	22.17 ± 24.04	25.59 ± 31.05	0.661

Table 3. Hematologic and Hb results for AA and AC women with normal α genes

Hematologic parameters and Hb types	Hemoglobin patterns (N)		
	AA (41)	AC (41)	*P*-value
RBC (× 10^{12}/l)	3.80 ± 0.50	4.02 ± 0.49	0.033
Hb (g/dl)	10.80 ± 1.62	11.94 ± 1.80	0.003
PCV	0.33 ± 0.04	0.35 ± 0.04	0.039
MCV(fl)	88.32 ± 7.27	88.52 ± 7.03	0.896
MCH (pg)	28.50 ± 3.17	29.67 ± 2.87	0.083
MCHC (g/dl)	32.27 ± 1.57	33.47 ± 1.33	<0.01
Hb F (% in adults)	1.39 ± 1.16	1.42 ± 1.52	0.906
Ferritin levels (ng/ml)	22.31 ± 27.57	22.17 ± 24.04	0.733

Table 4. Hematologic and Hb results for AA and AC women with α-Thal-$2^{3.7kb}$

Hematologic parameters and Hb types	Hb patterns (N)		
	AA (12)	AC (11)	*P*-value
RBC (× 10^{12})	4.01 ± 0.50	4.12 ± 0.42	0.194
Hb (g/dl)	10.90 ± 1.40	11.16 ± 1.53	0.461
PCV	0.33 ± 0.04	0.34 ± 0.04	0.583
MCV (fl)	84.01 ± 4.03	82.83 ± 5.53	0.296
MCH (pg)	28.05 ± 2.30	27.46 ± 2.60	0.263
MCHC (g/dl	32.40 ± 1.19	32.61 ± 1.21	0.392
Hb F (% in adults)	1.39 ± 0.89	1.76 ± 1.89	0.560
Ferritin levels (ng/ml)	32.69 ± 39.67	25.59 ± 31.05	0.715

($P = 0.039$) and MCHCs ($P < 0.0001$; Table 3). Comparison between the AA and AC genotypes in patients with α-Thal$2^{3.7kb}$ did not show statistically significant differences for any of the hematologic parameters (Table 4). Hematologic data from the AC α Thal$2^{3.7kb}$ homozygous women were not included in the statistical analyses; however, these women were found to have RBC counts of 2.89×10^{12} cells/l; Hb of 4.9 g/dl; PCV of 16.6%; MCV of 57.4 fl; MCH of 16.8 pg; MCHC of 29.3 g/dl; Hb C of 27.84%; and Hb F of 1.61%. There were no statistically significant differences between any of the groups with respect to serum ferritin concentrations (Tables 1–4).

Discussion

We studied the presence of α-Thal$2^{3.7kb}$ among groups of pregnant Brazilian women (from Salvador) with AA and AC Hb patterns. Comparison between AA women with normal α genes and those who were α-Thal$2^{3.7kb}$ carriers showed statistical differences in MCV. In the study sample, this finding could be attributed to presence of α-Thal$2^{3.7kb}$, as serum ferritin levels were normal in carriers (Lee *et al.,* 1992). However, AC women with normal α genes and AC α-Thal$2^{3.7kb}$ carriers were found to have statistically significant differences with respect to MCV,

MCH and Hb C concentrations. The presence of α-Thal2$^{3.7kb}$ appeared to result in lower Hb C concentrations. In those who were heterozygous for positively charged Hb variants (e.g. Hb S, C, D and E), the amount of variant hemoglobin has previously been found to decrease when the quantity of α-chain becomes limiting. This observation has been attributed to a preferential affinity of the α-globin chain for the negatively-charged β^A chain during Hb assembly (Steinberg, 1991; Lee *et al.*, 1992; Giordano *et al.*, 1997). The fact that lower concentrations of Hb C were observed in the α-Thal2$^{3.7kb}$ carriers supports this hypothesis. The high Hb C concentration observed in the normal α-gene carriers might be attributed to the low solubility of Hb C in water when compared with Hb A, binding preferentially to the band 3 protein on the inner surface of the erythrocyte membrane (Liu *et al.*, 1996). The life-span of erythrocytes with Hb C is shorter, and the rate of total hemoglobin production is 2.5–3 times higher (Beutler *et al.*, 1990). The higher values for RBC, Hb, PCV and MCHC in the AC group could be also attributed to the high viscosity of the Hb C erythrocyte (Bunn & Forget, 1986). However, these differences were not observed when comparing AA and AC α-Thal2$^{3.7kb}$ carriers, possibly because the presence of α-Thal2$^{3.7kb}$ modifies hematologic parameter values in both groups. On the other hand, AC α-Thal2$^{3.7kb}$ homozygous women showed very low hematologic parameter values, indicating that α-Thal2$^{3.7kb}$ can interfere with the course of pregnancy and Hb concentration is an important factor for the maintenance of placental weight and placental ratio (Las & Wong, 1997). In addition, erythrocytes containing Hb C have a low oxygen affinity, which decreases the intracellular pH (for reasons as yet unknown), and probably shifts the oxygen dissociation curve of whole blood to the left – the combination of these effects results in normal tissue oxygenation (Murphy, 1976). The intracellular Hb C aggregates limit cell deformability by increasing internal viscosity, thereby predisposing to fragmentation, spherocyte formation, and splenic sequestration. The lower concentrations of Hb C found in AC α-Thal2$^{3.7kb}$ carriers compared with AC women with normal α genes, which result from α-chain imbalances, may have a beneficial effect on the course of pregnancy. This hypothesis is supported by the hematologic and Hb characteristics of the population studied (Table 2). Few papers have described the association of Hb C heterozygosity and α-Thal (Giordano *et al.*, 1997). α-Thalassemia is associated with microcytosis and a hypochromic blood picture, similar to iron deficiency anemia. Anemia in pregnancy is most often caused or aggravated by a concomitant iron deficiency (Benjamin, Bassen & Meyer, 1961). In same cases, folic acid deficiency may also play a pathogenetic role (Low, Johnston & Mc Bnde, 1965), and it is therefore appropriate to give preventative iron and folic acid supplements to pregnant women. However, even in well-treated pregnant women, anemia becomes moderately severe in the third trimester as a result of dilutional anemia, with Hb concentrations occasionally below 10 g/dl. Concomitant α-Thal. may improve the anemia of pregnancy (Beutler *et al.*, 1990).

The results of the present report emphasize the importance of hemoglobinopathy screening in women of reproductive age. Although an association between the presence of variant Hb (e.g. Hb S and/or C), the occurrence of α-Thal, and the administration of iron therapy during pregnancy remains controversial, a well-established hemoglobinopathy screen will help prevent clinical complications for mothers and newborn babies.

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