UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

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Curso de Pós-Graduação em Patologia

TESE DE DOUTORADO

ANEMIA FALCIFORME EM SALVADOR-BAHIA: CARACTERIZAÇÃO FENOTÍPICA, MOLECULAR E DE SEQÜENCIAS GÊNICAS PONTENCIALMENTE IMPORTANTES NA EXPRESSÃO DOS GENES GAMA DA HEMOGLOBINA FETAL

ELISÂNGELA VITÓRIA ADORNO

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SUMÁRIO

LISTA DE ABREVIATURAS

AC	Heterozigoto para hemoglobina C
AS	Heterozigoto para hemoglobina S
Atp	Haplótipo Atípico
AVC	Acidente vascular cerebral
Ben	Haplótipo Benin
Cam	Haplótipo Camarões
CAR	Haplótipo Bantu
CHCM	Concentração de hemoglobina corpuscular média
Hb	Hemoglobina
HbA1	Hemoglobina 1 do Adulto
HbA2	Hemoglobina 2 do Adulto
HbC	Hemoglobina C
HbD	Hemoglobina D
HbE	Hemoglobina E
HbF	Hemoglobina Fetal
HbS	Hemoglobina S
HCM	Hemoglobina corpuscular média
Hm	Hemácias
HS2	Segundo Sítio hipersensível à DNase I
Ht	Hematócrito
Kb	Kilobases
KD	Kilodáltons
LCR	Região controladora do lócus da globina beta (*LocusRregion Control*)
pb	Pares de base
QTL	Locus quantitativo de características (*Quantitative Trait Loci*)
SC	Heterozigoto duplo para as hemoglobinas S e C
Sen	Haplótipo Senegal
sICAM-1	Molécula de adesão intracelular solúvel
SS	Homozigoto para hemoglobina S
VCAM	Molécula de adesão à célula vascular
VCM	Volume corpuscular médio

LISTA DE SÍMBOLOS

β	Beta
α	Alfa
γ^A	Gama A
γ^G	Gama G
$\gamma_I{}^A$	Cadeia gama A com isoleucina na posição 75
$\gamma_T{}^A$	Cadeia gama A com treonina na posição 75

LISTA DE FIGURAS

10.2 Eletroferograma do sequenciamento automático. A - Substituição T→ C na posição – 157 da região promotora do gene γG, descrita em todos os pacientes analisados, independente do tipo de haplótipo ligado ao grupamento de genes da globina β^S e do nível de HbF; B - Seqüência normal de nucleotídeos da região promotora do gene γG.

10.3 A – Substituição G→ A na posição – 10.677 do HS2-LCR descrita nos pacientes portadores do haplótipo Ben que apresentaram níveis de HbF elevados; B – Seqüência normal de nucleotídeos do HS2-LCR.

RESUMO

ANEMIA FALCIFORME EM SALVADOR-BAHIA: CARACTERIZAÇÃO FENOTÍPICA, MOLECULAR E DE SEQÜÊNCIAS GÊNICAS POTENCIALMENTE IMPORTANTES NA EXPRESSÃO DOS GENES GAMA DA HEMOGLOBINA FETAL. **ELISÂNGELA VITÓRIA ADORNO.** A hemoglobina S (HbS) resulta da troca de nucleotídeo (GAG→GTG) no sexto codon do gene da globina β, levando à substituição do ácido glutâmico por valina na cadeia da globina β. A anemia falciforme ou a homozigose para a HbS, freqüentemente apresenta manifestações clínicas heterogêneas, fortemente relacionadas aos níveis de hemoglobina fetal (HbF). O presente estudo investigou as características fenopíticas e os marcadores moleculares presentes em portadores da anemia falciforme de Salvador-BA, identificando seqüências gênicas potencialmente importantes para a expressão dos genes gama. O perfil de hemoglobinas e o nível de HbF foram determinados por cromatografia líquida de alta performance (HPLC). Informações sobre o perfil clínico dos pacientes foram obtidas através da análise de prontuários. A talassemia $\alpha_2^{-3.7Kb}$ foi investigada pela reação em cadeia da polimerase (PCR) e os haplótipos ligados ao grupo de genes da globina β^S foram investigados por PCR e análise de sítios polimórficos utilizando endonucleases de restrição (RFLP). As regiões promotoras dos genes γG e γA e o HS2-LCR foram amplificados assimetricamente e seqüenciadas no *ABI Prism 3100 prism DNA Sequencer*. As análises estatísticas foram desenvolvidas no *software* EPI-INFO versão 6.04 e a significância foi estabelecida para $p \leq 0.05$. Foram analisados 131 pacientes, dos quais 125 tiveram identificado o genótipo β^S, tendo sido encontrado 64 (51,2%) CAR/Ben; 36 (28,8%) Ben/Ben; 18 (14,4%) CAR/CAR; dois (1,6%) CAR/Aty; dois (1,6%) Ben/Cam; um (0,8%) CAR/Cam; um (0,8%) Car/Arabia-India e um (0,8%) Sen/Aty. A talassemia $\alpha_2^{-3.7Kb}$ foi estudada em 110 pacientes, onde 30 (27,3%) foram heterozigotos e dois (1,8%) homozigotos. O uso de transfusão sangüínea foi maior em pacientes com HbF≤10,0% (p=0,009). Pacientes com genótipos α diferentes apresentaram diferenças para os valores de Hb (p=0,018); Ht (p=0,019); VCM (p=0,0004) e HCM (p=0,039). Os níveis de HbF foram maiores entre os pacientes Ben/Ben que entre os CAR/CAR (p=0,007) e CAR/Ben (p=0,013). A análise das seqüências do HS2-LCR de dez indivíduos demonstrou a substituição G→A na posição −10.677, presente apenas entre os portadores do haplótipo Ben com nível elevado de HbF, sugerindo uma possível associação entre este polimorfismo, a expressão dos genes γ e a síntese da HbF. A análise da região promotora do gene γG demonstrou a substituição T→C na posição -157, que parece ser uma seqüência característica entre os pacientes estudados. Também foi encontrada a deleção de 4 pb na posição −222 a −225 no gene γG e relacionado ao haplótipo Cam. Os dados demonstraram um novo polimorfismo localizado no HS2-LCR e na região promotora do gene γG da globina, justificando a realização de estudos adicionais, associando os níveis de HbF, marcadores biológicos e mecanismos relacionados, visando esclarecer um possível papel no desenvolvimento do fenótipo da doença.

Palavras-chaves: Anemia Falciforme; Haplótipos; Genótipo; Hemoglobina fetal; Fenótipo.

ABSTRACT

SICKLE CELL ANEMIA IN SALVADOR-BAHIA: PHENOTYPE AND MOLECULAR CHARACTERIZATION AND SEARCH OF SPECIFIC GENE SEQUENCES POTENTIALLY IMPORTANT TO GAMMA GENES EXPRESSION OF THE FETAL HEMOGLOBIN. **ELISÂNGELA VITÓRIA ADORNO**. Sickle cell hemoglobin (HbS) results of a single nucleotide change (GAG → GTG) in the sixth codon of the β-globin gene, where valine replaces glutamic acid in the β-globin chain. The sickle cell anemia or the HbS homozygous frequently present heterogeneous clinical manifestation, strongly associated with fetal hemoglobin (HbF) levels. The present study investigated phenopypic characteristics and molecular markers in sickle cell anemia patients from Salvador-BA, identifying the presence of gene sequences potencially important to γ gene expression. The hemoglobin profile and HbF level were determined by high performance liquid chromatography (HPLC). Information about the clinical phenotype was obtained by patient record. The $\alpha_2{}^{3.7Kb}$- thalassemia was investigated by polymerase chain reaction (PCR) and β^S – globin gene haplotypes were investigated by PCR and restriction fragment length polymorphism (RFLP) techniques. The Gγ and Aγ-globin genes promoter and the HS2-LCR regions were amplified asymmetrically and sequencing in an ABI Prism 3100 prism DNA Sequencer. The statistical analyses were conducted by EPI-INFO software version 6.04 and statistical significance was established at $p \leq 0.05$. A total of 131 patients were analyzed, of whom 125 had the β^S genotypes identified; have been found 64 (51.2%) were CAR/Ben; 36 (28.8%) Ben/Ben; 18 (14.4%) CAR/CAR; 2 (1.6%) CAR/Aty; 2 (1.6%) Ben/Cam; 1 (0.8%) CAR/Cam; 1 (0.8%) CAR/Arab-India and 1 (0.8%) Sen/Aty. The $\alpha_2{}^{3.7Kb}$- thalassemia was studied in 110 patients, with 30 (27.3%) heterozygous and two (1.8%) homozygous. The use of blood transfusion therapy was higher (p=0.009) in patients with HbF≤10.0%. Patients with different α-genes genotypes presented differences for Hb (p=0.018); PCV (p=0.019); MCV (p=0.0004) and MCH (p=0.039). HbF levels were higher for Ben/Ben than CAR/CAR (p=0.007) and CAR/Ben (p=0.013). The HS2-LCR sequences analyzed demonstrated G→A change in a –10.677 position in patients Ben haplotype carries with high HbF level, suggesting a possible association among this polymorphism and the γ-globin gene expression and HbF synthesis. The analyses of Gγ gene promoter region showed a T→C substitution in a –157 position, which suggests a common sequence characteristic among patients studied. We also described a 4 bp deletion in –222 to –225 position of the Gγ globin gene promoter region and related with Cam haplotype. Our data showed new polymorphisms located at HS2–LCR and Gγ globin gene promoter regions, justifying further studies associating HbF levels, biologic markers and related mechanisms in order to clarify whether they could interfere in phenotypic characteristics of the disease.

Key Words: Sickle cell anemia; Haplotypes; Genotype; Fetal hemoglobin; Phenotype.

1.1 A molécula de hemoglobina

A hemoglobina (Hb) é uma proteína com peso molecular aproximado de 64 kilodáltons (KD), representando 95% das proteínas dos eritrócitos, cuja função é absorver, transportar e distribuir o oxigênio para os diversos tecidos do organismo. A molécula de hemoglobina possui quatro subunidades, formadas pela porção protéica (globina) e o grupo heme, que é composto pelo complexo ferro-protoporfirina IX. O átomo de ferro constitui o sítio de ligação do oxigênio, sendo que cada molécula de hemoglobina é capaz de combinar-se com quatro moléculas de oxigênio (BUNN & FORGET, 1986; WEATHERALL & PROVAN, 2000). As cadeias de globina podem ser de seis tipos, a α (alfa), β (beta), γ (gama), δ (delta), ε (epsilon), e ζ (zeta), que se agrupam normalmente, duas a duas, de acordo com o tipo de hemoglobina (BUNN & FORGET, 1986; PERUTZ, 2001).

Os genes da globina estão localizados no grupo α, em uma extensão de 40 kilobases (Kb), no braço curto do cromossomo 16 e no grupo β, em uma extensão de 60 Kb, no braço curto do cromossomo 11, cuja organização segue a mesma ordem em que são expressos; o conjunto destes genes é o resultado da duplicação de um gene primordial que existiu há aproximadamente 500 milhões de anos, sendo provavelmente originado de uma diferenciação posterior ao longo do processo evolutivo (STEINBERG & BENZ JUNIOR, 1995; HARDISON, 2001). O grupo de genes α é constituído pelos genes α^1 e α^2, localizados na região 3' e que diferem apenas no segundo íntron; os pseudogenes ψ ($\Psi\,\alpha^1$ e $\Psi\,\alpha^2$); o gene ζ^2 e o seu pseudogene ($\Psi\zeta^1$), além do gene θ^1 (Figura. 1.1 A). Estes pseudogenes, apesar de possuírem estrutura similar aos seus genes correspondentes, não

expressam cadeias polipeptídicas e o gene θ, até o presente momento, não está associado à expressão de hemoglobina funcional (STEINBERG & BENZ JUNIOR, 1995; WEATHERAL & PROVAN, 2000).

O grupo de genes β é formado por seis genes, o gene ε, os dois genes γ (γ^A e γ^G), um pseudogene β (ψβ) e os genes δ e β (figura. 1.1 B). As cadeias polipeptídicas decorrentes da expressão dos genes γ, devido a mecanismos de duplicação e conversão gênica, diferem no aminoácido localizado na posição 136, podendo ser uma glicina (γ^G) ou alanina (γ^A). Além disso, a cadeia γ^A possui um sítio polimórfico na posição 75, que pode apresentar uma isoleucina ($\gamma_I{}^A$) ou treonina ($\gamma_T{}^A$) (STAMATOYANNOPOULOS & NIENHUIS, 1994; WEATHERAL & PROVAN, 2000; NAGEL & STEINBERG, 2001).

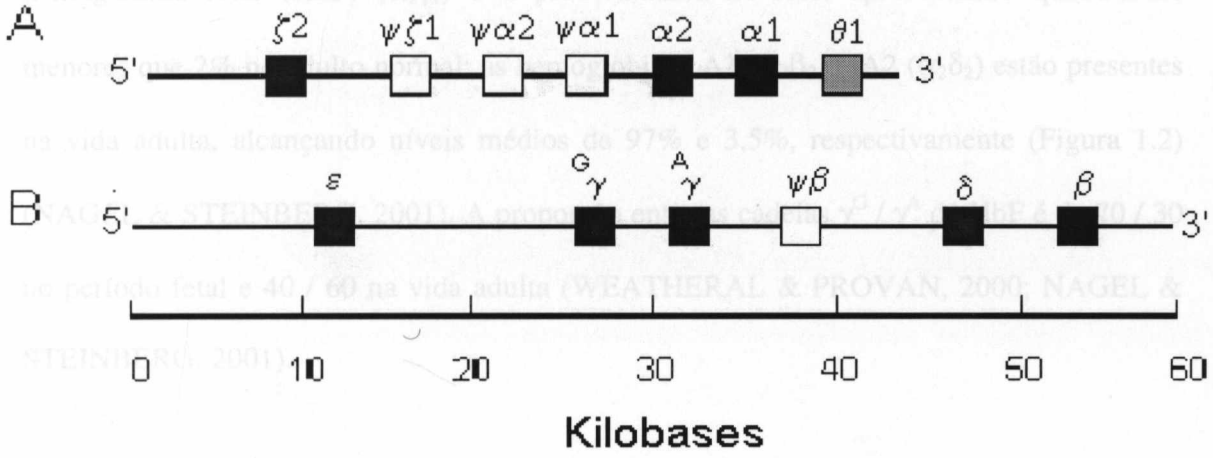

Figura 1.1 Representação dos loci dos genes das globinas α e não-α humana. Em A, o grupo de genes da globina α, localizado no cromossomo 16p13.3; em B, o grupo de genes da globina β, localizado no cromossomo 11p15.5. Adaptado de WEATHERALL & PROVAN, 2000.

A regulação da expressão dos genes da globina envolve mecanismos seqüenciais de ativação e inativação, ainda não completamente esclarecidos, tais como interações de fatores transcricionais em estágios específicos; a proximidade de seqüências regulatórias denominadas de região controladora do locus (LCR) no grupo de genes β e de sítios hipersensíveis à DNase I (HS-40) no grupo de genes α; seqüências *cis* que atuam positiva ou negativamente na regulação da transcrição e, fatores *trans* eritróides ou não-eritróides (BHERINGER et al., 1990; STEINBERG, 1995; NAGEL & STEINBERG, 2001; STAMATOYANNOPOULOS, 2005).

Diferentes hemoglobinas foram identificadas durante o processo de desenvolvimento ontogênico humano, sendo que as hemoglobinas Gower 1 ($\zeta_2\varepsilon_2$), Gower 2 ($\alpha_2\varepsilon_2$) e Portland ($\gamma_2\zeta_2$) são encontradas somente nos primeiros estágios da embriogênese; a hemoglobina fetal (HbF) ($\alpha_2\gamma_2$) é a predominante no feto, apresentando quantidades menores que 2% no adulto normal; as hemoglobinas A1 ($\alpha_2\beta_2$) e A2 ($\alpha_2\delta_2$) estão presentes na vida adulta, alcançando níveis médios de 97% e 3,5%, respectivamente (Figura 1.2) (NAGEL & STEINBERG, 2001). A proporção entre as cadeias γ^G / γ^A da HbF é de 70 / 30 no período fetal e 40 / 60 na vida adulta (WEATHERAL & PROVAN, 2000; NAGEL & STEINBERG, 2001).

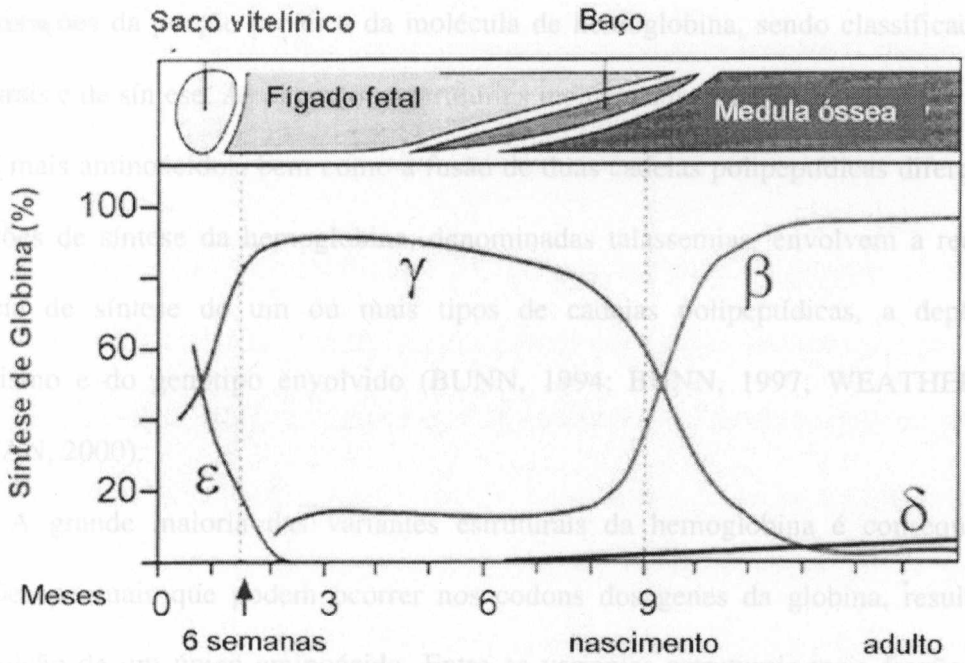

Figura 1.2. Representação esquemática da expressão dos genes da globina e principais sítios de produção durante os diferentes períodos do desenvolvimento ontogênico humano. Adaptado de STEINBERG, 2001.

1.2 Hemoglobinopatias

As hemoglobinopatias constituem um grupo de doenças genéticas, caracterizadas por alterações da porção protéica da molécula de hemoglobina, sendo classificadas como estruturais e de síntese. As alterações estruturais incluem substituição, deleção e inserção de um ou mais aminoácidos, bem como a fusão de duas cadeias polipeptídicas diferentes e as alterações de síntese da hemoglobina, denominadas talassemias, envolvem a redução ou ausência de síntese de um ou mais tipos de cadeias polipeptídicas, a depender do mecanismo e do genótipo envolvido (BUNN, 1994; BUNN, 1997; WEATHERALL & PROVAN, 2000).

A grande maioria das variantes estruturais da hemoglobina é conseqüência de mutações pontuais que podem ocorrer nos codons dos genes da globina, resultando na substituição de um único aminoácido. Entre as variantes estruturais mais freqüentemente descritas, estão as hemoglobinas S, C, D e E (NAGEL et al., 1979; CHARACHE, 1990).

A hemoglobina S (HbS) é decorrente de uma mutação pontual (G**A**G→G**T**G) no sexto códon do gene da globina β, conduzindo à substituição do ácido glutâmico pela valina na sexta posição da cadeia polipeptídica (STEINBERG, 1995). A HbS pode polimerizar-se em tensões diminuídas de oxigênio, formando estruturas filamentosas (polímeros de HbS deosxigenada) que se depositam nas hemácias, modificando sua forma e tornando-as falciformes. O fenômeno de falcização pode ser revertido quando níveis elevados de oxigênio são novamente atingidos, sendo que falcizações sucessivas alteram a estrutura da membrana da hemácia, favorecendo a formação de células irreversivelmente falcizadas (SMITH et al., 1981; ANTONARAKIS et al., 1984; EMBURY, 1995; CHANG et al., 1997).

Os indivíduos heterozigotos para a hemoglobina S (AS) possuem hemácias com aproximadamente 20 a 45% da hemoglobina variante e são assintomáticos; os homozigotos (SS) possuem a anemia falciforme, com hemácias contendo 80% ou mais de HbS, sendo portadores de anemia hemolítica grave, acompanhada por manifestações clínicas variáveis (SMITH et al., 1981; EMBURY, 1995; CHANG et al., 1997; WEATHERALL & PROVAN, 2000).

A talassemia α é uma alteração que envolve a diminuição ou ausência da síntese das cadeias α da hemoglobina, podendo ser decorrente de deleções ou mutações pontuais nos genes α^1 e / ou α^2. A talassemia α^2 ou α^+ é representada pela perda de um dos genes α em pelo menos um dos cromossomos (−α/αα) e a talassemia α^1 ou α^0 é caracterizada pela perda de dois genes α no mesmo cromossomo (−−/αα) (ADAMS et al., 1994; FOGLIETTA et al., 1996). O grau elevado de similaridade presente nos genes α favorece a ocorrência de eventos de recombinação gênica (*crossing-over* desigual) durante a meiose, resultando em deleção do gene α em um cromossomo (- α) e triplicação no outro (ααα). A talassemia α^2 é a mais freqüente, apresentando dois tipos de deleções, a de 3,7Kb e de 4,2Kb (DODÉ et al., 1993).

A talassemia α possui incidência elevada entre os povos da Ásia, Oceania, Oriente Médio e Mediterrâneo, bem como em todo o continente africano (ADAMS et al., 1994; FOGLIETTA et al., 1996). A deleção de 3,7Kb é o tipo de talassemia α mais freqüente no Brasil, sendo que SONATI et al. (1991) registraram 20 a 25% na população negróide do sudeste; na Bahia, COUTO et al. (2003) descreveram 23% dessa talassemia entre gestantes portadoras do perfil de hemoglobinas AC e AA; ALBUQUERQUE (2002) encontrou 19,3% de talassemia $\alpha_2^{3,7Kb}$ e 0,51% de $\alpha_2^{anti\text{-}3,7Kb}$ também em gestantes baianas.

1.3 Epidemiologia da HbS

A hemoglobina S possui freqüência elevada na África, principalmente na região Centro-Ocidental, Atlântico-Ocidental e Sul. O gene β^S apresenta freqüência entre 0,12 a 0,14 no Congo e Zaire e de 0,08 a 0,10 no Senegal. A hemoglobina S também é encontrada em países do Mediterrâneo, incluindo a Itália e Grécia, bem como na Arábia Saudita, Kuait e Índia. Nos Estados Unidos da América e América Latina, aproximadamente 8% da população negróide é portadora da HbS, estimando-se o nascimento de 1 / 625 crianças com anemia falciforme nos Estados Unidos (WANG & LUKENS, 1998; COSTA, 2001).

O gene β^S apresenta distribuição heterogênea entre os diferentes estados brasileiros, variando de acordo com a região estudada. RAMALHO (1986) descreveu a freqüência de 6,6% indivíduos heterozigotos para HbS (AS) na população negróide do Estado de São Paulo (Sudeste do Brasil); mais recentemente, BRANDELISE et al. (2004) descreveram a prevalência de 0,02% para a doença falciforme (SS e SC) durante o programa de triagem neonatal realizado em Campinas, envolvendo 281.884 recém-nascidos.

Em Porto Alegre, região Sul do Brasil, DAUDT et al. (2002) encontraram 1,2% do gene S entre recém-nascidos; em Caxias do Sul, foi descrita a freqüência de 0,09% para os heterozigotos AS entre doadores de sangue de descendência italiana (LISOT & SILLA, 2004). No estado de Minas Gerais, o programa de triagem neonatal detectou um caso de doença falciforme para cada 1591 nascimentos (SERJEANT, 2000).

Na região Nordeste, BANDEIRA et al. (1999) encontraram a freqüência de 5,1% para os heterozigotos AS e a prevalência de 0,2% para a doença falciforme no estado de

Pernambuco; em Natal, Rio Grande do Norte, ARAÚJO et al. (2004) encontraram 1,5% de recém-nascidos heterozigotos AS e 0,05% de portadores da anemia falciforme. O estado da Bahia apresenta a maior freqüência brasileira para a HbS, tendo sido encontrado 7,4% do genótipo AS em 1.200 crianças em idade escolar (AZEVEDO et al., 1980), variando de acordo com o grupo populacional estudado; recentemente, ADORNO et al. (2005) descreveram a freqüência de 9,8% de heterozigotos AS, 0,9% heterozigotos duplos SC e 0,2% de portadores da anemia falciforme (SS) em recém-nascidos de uma maternidade pública da cidade de Salvador-BA.

1.4 Manifestações Clínicas na Anemia Falciforme

Os portadores da anemia falciforme possuem um quadro clínico heterogêneo, com retardo no crescimento e desenvolvimento, além de alterações em diversos órgãos, que são provenientes da hemólise contínua e dos fenômenos de vaso-oclusão ocorridos durante o curso da doença (BUNN & FORGET, 1986; WEATHERALL & PROVAN, 2000).

A grande quantidade de HbS presente nas hemácias do portador da anemia falciforme, leva ao aumento da densidade celular, favorecendo a polimerização intracelular, que diminui a maleabilidade da hemácia e proporciona a obstrução de vasos sangüíneos de pequeno e médio calibre (WANG & LUKENS, 1998). Os fenômenos vaso-oclusivos acontecem principalmente em órgãos onde o fluxo sangüíneo é lento e a tensão de oxigênio e o pH são mais baixos, tais como o rim, baço e medula óssea. Vários fatores estão associados à crise vaso-oclusiva, incluindo a polimerização da HbS; desidratação celular; aumento da rigidez do eritrócito e da viscosidade sangüínea; ativação e adesão das células endoteliais e de plaquetas; desequilíbrio do tônus vascular com elevação dos níveis de endotelina e redução do óxido nítrico (WANG & LUKENS, 1998; STUART & NAGEL, 2004). Assim, os eventos de adesão entre hemácias falcizadas e leucócitos ao endotélio vascular desempenham um papel importante no fenômeno da vaso-oclusão, onde níveis plasmáticos de molécula de adesão à célula vascular (VCAM) encontram-se aumentados, assim como os níveis de molécula de adesão intracelular solúvel (sICAM-1) (CONRAN et al., 2004). Além disso, polimorfismos envolvendo glicoproteínas de membrana de plaqueta têm sido descritos como fatores de risco para doença vascular em portadores da anemia falciforme (CASTRO et al., 2004).

Os eventos de dor aguda se desenvolvem de forma bastante heterogênea e correspondem às manifestações clínicas mais comuns da anemia falciforme. Os episódios de dor e inchaço dos pés e das mãos (dactilite) são mais freqüentes nos dois primeiros anos de vida; após esse período, a interrupção do fluxo sangüíneo nas extremidades de ossos grandes e estruturas periarticulares favorecem o desenvolvimento de crises de dor, principalmente na tíbia, úmero e fêmur, podendo ocorrer crises de dor abdominal, frequentemente atribuídas a infartos pequenos de mesentério. (WANG & LUKENS, 1998; COSTA, 2001).

A crise de seqüestro esplênico é mais freqüente nos dois primeiros anos de vida, sendo caracterizada pelo aumento do baço, diminuição da concentração de hemoglobina (≥2 g/dL em relação aos níveis basais) sempre acompanhada de reticulocitose, podendo acarretar em colapso circulatório que pode conduzir ao óbito por anemia e choque hipovolêmico (STUART & NAGEL, 2004).

As infecções são a maior causa de morbidade e mortalidade de crianças portadoras de anemia falciforme, sendo o *Streptococcus pneumoniae* o principal agente etiológico. O risco de infecção é maior nos primeiros anos de vida, especialmente para a meningite bacteriana causada por pneumococos; entretanto, antes da realização da triagem neonatal e da profilaxia com antibióticos e vacinas, a infecção era a principal causa de morte entre pacientes menores de vinte anos de idade (COSTA, 2001; OHENE-FREMPONG & STEINBERG, 2001; GARY, 2003).

A fisiopatologia que envolve a susceptibilidade elevada à infecção em indivíduos com anemia falciforme, ainda não se encontra totalmente compreendida, mas sabe-se que a asplenia funcional, que ocorre progressivamente, dificulta a opsonização de bactérias

encapsuladas, favorecendo a ocorrência de infecções por agentes como, *S. pneumoniae*, *Haemophilus influenzae*, *Neissearia meningitidis*, *Escherichia coli*, *Enterobacter* sp, *Klebsiella* sp, *Salmonella* sp.e *Staphylococcus aureus* (WANG & LUKENS, 1998; COSTA, 2001; DI NUZZO & FONSECA, 2004). No entanto, acredita-se que outros fatores, além da disfunção esplênica, participem deste processo. Atualmente, tem sido sugerido que deficiências na via alternativa do complemento, de opsoninas séricas e alterações envolvendo a função de leucócitos contribuam para a ocorrência de infecções freqüentes nesses pacientes (WANG & LUKENS, 1998; COSTA, 2001; OHENE-FREMPONG & STEINBERG, 2001; GARY, 2003).

A síndrome torácica aguda é uma causa freqüente de mortalidade entre adultos jovens portadores de anemia falciforme, sendo caracterizada por febre, dor no peito, tosse, leucocitose e infiltrado pulmonar. Entre os fatores de risco associados à sua ocorrência, encontram-se a faixa etária, com uma freqüência elevada na idade pediátrica, os níveis diminuídos de HbF, os valores persistentemente elevados de leucócitos e de concentração média de hemoglobina. As causas que levam ao desenvolvimento da síndrome torácica aguda parecem ser multifatoriais, incluindo infecção pulmonar, infarto, embolia pulmonar, trombose localizada na microvasculatura, lesão vascular e inflamação (WANG & LUKENS, 1998; COSTA, 2001; OHENE-FREMPONG & STEINBERG, 2001; STUART & NAGEL, 2004).

A úlcera de perna também tem uma prevalência elevada entre adultos jovens, sendo encontrada na freqüência de 5 a 10% (COSTA, 2001). Diversas condições parecem contribuir para o desenvolvimento de úlceras de perna, tais como o clima tropical, a dinâmica circulatória, anemia e infecção local. A fisiopatologia desta manifestação clínica não está completamente esclarecida, tendo sido sugerido que a rigidez elevada das

hemácias contendo HbS, dificulta a circulação sangüínea através dos capilares da derme. Além disso, os níveis elevados de HbF parecem proteger os indivíduos portadores de anemia falciforme contra a ocorrência de úlcera de perna (OHENE-FREMPONG & STEINBERG, 2001; STUART & NAGEL, 2004).

O portador da anemia falciforme pode desenvolver alterações oftalmológicas resultantes de lesões nas artérias oculares, que podem ocasionar microaneurismas e vascularização colateral. Outra manifestação clínica que acomete com freqüência elevada estes indivíduos é a necrose asséptica de cabeça de fêmur, ocorrendo em 10% dos pacientes adultos que apresentam episódios dolorosos constantes e níveis elevados de hemoglobina (WANG & LUKENS, 1998; COSTA, 2001).

O priapismo é decorrente da interrupção do fluxo sangüíneo nos corpos cavernosos e esponjoso devido à presença de hemácias falcizadas, sendo uma complicação que ocorre com relativa freqüência entre homens jovens. Geralmente, o priapismo ocorre após o estado de ereção normal e está associado à desidratação e hipoventilação, resultando em acidose metabólica (WANG & LUKENS, 1998; COSTA, 2001; OHENE-FREMPONG & STEINBERG, 2001). As manifestações clínicas do priapismo podem ocorrer de forma aguda, com ereção dolorosa por várias horas, ou de forma crônica, com eventos de ereção reversíveis, que persistem por várias semanas (COSTA, 2001).

A anemia hemolítica crônica presente na anemia falciforme pode contribuir para o aparecimento de crise aplástica temporária. Atualmente, a aplasia tem sido associada à causa infecciosa, principalmente ao parvovírus B19, como conseqüência direta da citotoxidade aos precursores eritróides, principalmente às unidades formadoras de colônia eritróide (CFU-E) (WANG & LUKENS, 1998; BORSATO et al., 2000; STUART & NAGEL, 2004).

O acidente vascular cerebral (AVC) é uma complicação grave que pode afetar indivíduos com anemia falciforme e está associado à mortalidade elevada, principalmente, em crianças entre 2 e 5 anos de idade, com redução de sua incidência entre 10 e 19 anos (OHENE-FREMPONG & STEINBERG, 2001; STUART & NAGEL, 2004). Alguns fatores de risco estão relacionados à ocorrência de AVC, como concentrações diminuídas de hemoglobina e de HbF, contagem elevada de leucócitos, pressão sangüínea sistólica elevada, ocorrência prévia de acidente isquêmico transitório e síndrome torácica aguda (BUCHANAN et al., 2004; STUART & NAGEL, 2004).

1.5 Fatores Genéticos Moduladores da Anemia Falciforme

Apesar dos indivíduos portadores desta anemia possuírem a mesma mutação, vários fatores são descritos por alterarem o quadro clínico da doença. Entre estes, estão a presença de variações nos níveis da HbF, tipo de haplótipo ligado ao grupo de genes da globina β, mutações nas regiões promotoras dos genes γG e γA, presença de variações no segundo sítio hipersensível à DNase I (HS2) da região controladora do locus da globina β (LCR) e presença de talassemia α (BUNN, 1994; CHANG et al., 1997; STEINBERG, 2001).

- Hemoglobina Fetal

A hemoglobina fetal inibe a polimerização da HbS, com formação de um híbrido assimétrico, composto por cadeias polipepitídicas das duas hemoglobinas ($\alpha_2\beta^S\gamma$), sendo

que o mesmo interage com os sítios envolvidos na estabilização dos polímeros formados pela HbS desoxigenada (BHAUMIK, 1994; STUART & NAGEL, 2004). No entanto, BHAUMIK (1994) demonstrou que o híbrido formado entre a HbS/HbF, quando constituído pelas cadeias γG, possui mais estabilidade que o formado com a cadeia γA. A presença da alanina na posição 136 da cadeia γ parece interferir com a interação entre as cadeias γ e β^S, diminuindo o efeito na prevenção da polimerização da HbS.

A hemoglobina fetal é um dos mais conhecidos moduladores do fenótipo clínico da anemia falciforme. Os níveis elevados da HbF estão comumente associados a gravidade clínica diminuída, redução das crises de dor, transfusões e hospitalizações, além da diminuição da mortalidade entre crianças e adultos. Atualmente, diversos agentes citotóxicos (hidroxiuréia e 5-azacitidina), fatores de crescimento hematopoético (eritropoetina) e ácidos graxos de cadeia curta (butirato e derivados) têm sido utilizados no tratamento da anemia falciforme, uma vez que podem estimular a síntese da HbF em decorrência de mecanismos diversos (CHARACHE, 1990; STEINBERG, 2001; STUART & NAGEL, 2004).

- Haplótipos ligados ao grupo de genes da globina β^S

Os haplótipos ligados ao grupo de genes da globina β são definidos como a associação não randômica da combinação de vários sítios de clivagem para endonucleases de restrição localizados ao longo do grupamento de genes β (Figura 1.3A). Estes haplótipos

estão presentes em um número limitado de combinações na população e não estão relacionados a doenças (STEINBERG, 1995).

Os haplótipos ligados ao grupo de genes da globina β^S têm sido classificados em cinco tipos diferentes, de acordo com a sua origem e área geográfica onde predominam. O haplótipo Benin (Ben) tem sido associado à África Ocidental; o Bantu ou República Centro Africana (CAR) à África Oriental e Centro-Sul; o Senegal (Sen) à África Atlântico Ocidental; o Índia-Arábia Saudita (Saudi) à Índia e Península Arábica Oriental e o Camarões (Cam) à Costa Ocidental Africana (Figura 1.3B) (PAGNIER et al., 1984; NAGEL, 1984; SUTTON et al., 1989). Estes haplótipos também estão relacionados a um quadro clínico e níveis de HbF variados, sendo que o hapótipo Sen está associado a níveis elevados de HbF (> 15%) e curso clínico menos grave da doença; o Ben a níveis medianos de HbF (5 a 15%) e curso clínico intermediário e o CAR a níveis diminuídos de HbF (< 5%) e quadro clínico mais grave. Os portadores do haplótipo Saudi apresentam níveis elevados de HbF e curso clínico heterogêneo (NAGEL,1984; POWARS, 1991; RAHGOZAR et al. , 2000).

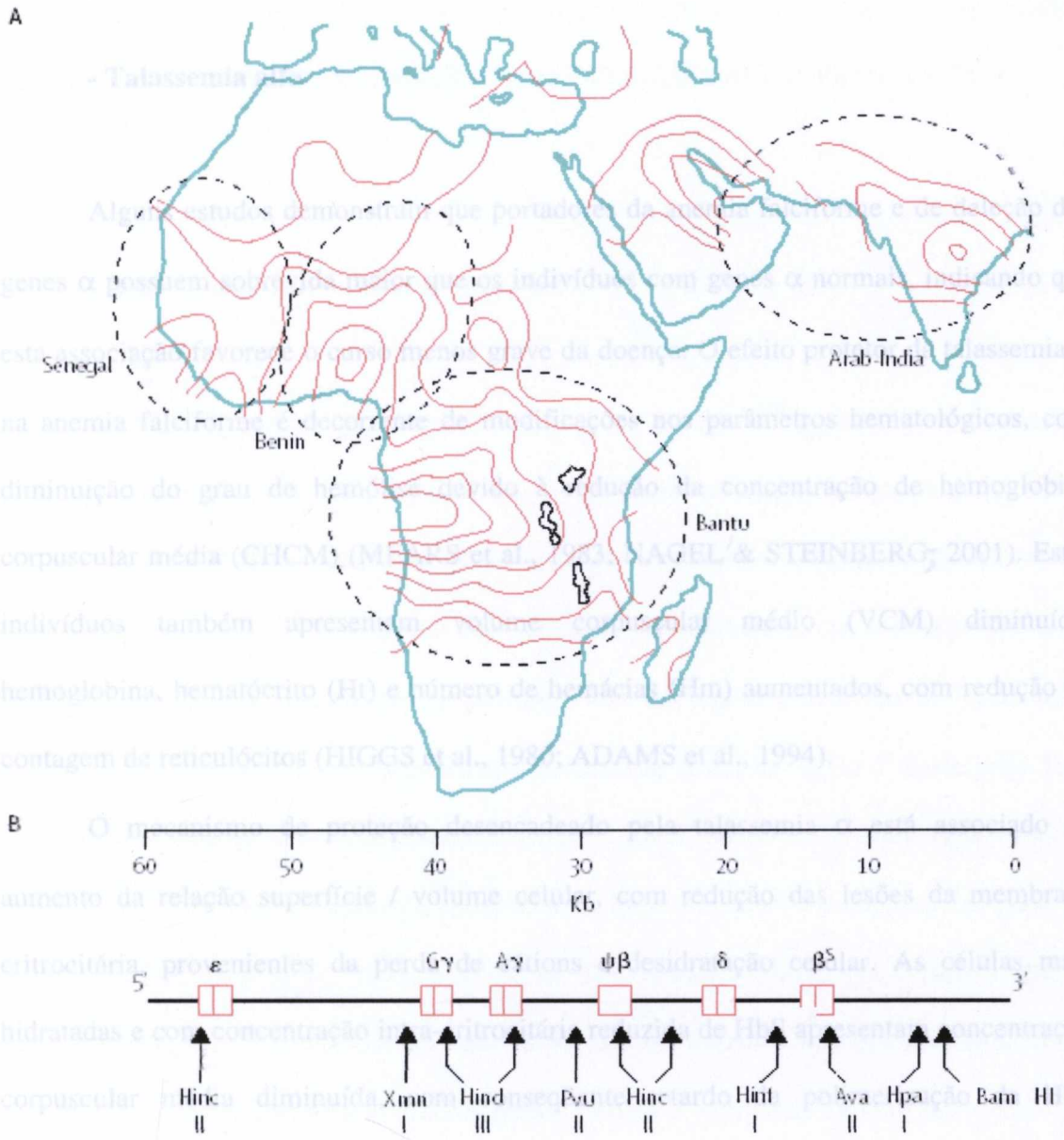

Figura 1.3 A-Distribuição geográfica dos haplótipos ligados ao gene da globina β^S na África e regiões do Oriente Médio. B- Seqüência de polimorfismos genéticos localizados no cromossomo 11, com o padrão de clivagem para diferentes endonucleases de restrição. Adaptado de STUART & NAGEL, 2004.

- Talassemia alfa

Alguns estudos demonstram que portadores da anemia falciforme e de deleção dos genes α possuem sobrevida maior que os indivíduos com genes α normais, indicando que esta associação favorece o curso menos grave da doença. O efeito protetor da talassemia α na anemia falciforme é decorrente de modificações nos parâmetros hematológicos, com diminuição do grau de hemólise devido à redução da concentração de hemoglobina corpuscular média (CHCM) (MEARS et al., 1983; NAGEL & STEINBERG, 2001). Estes indivíduos também apresentam volume corpuscular médio (VCM) diminuído, hemoglobina, hematócrito (Ht) e número de hemácias (Hm) aumentados, com redução da contagem de reticulócitos (HIGGS et al., 1980; ADAMS et al., 1994).

O mecanismo de proteção desencadeado pela talassemia α está associado ao aumento da relação superfície / volume celular, com redução das lesões da membrana eritrocitária, provenientes da perda de cátions e desidratação celular. As células mais hidratadas e com concentração intra-eritrocitária reduzida de HbS apresentam concentração corpuscular média diminuída, com conseqüente retardo da polimerização da HbS desoxigenada (STEINBERG & EMBURY, 1986; WEATHERALL, 1995).

A presença da talassemia α^2, além de prolongar a sobrevida dos portadores da anemia falciforme, também reduz a ocorrência de úlceras crônicas na região maleolar de membros inferiores; no entanto, como conseqüência da redução da hemólise e aumento do hematócrito, pode haver elevação da viscosidade sangüínea, com aumento das manifestações vaso-oclusivas. Assim, a gravidade e a freqüência das crises de dores ósseas

não são reduzidas, podendo ocorrer complicações clínicas, como a necrose óssea e retinopatias (STEINBERG & EMBURY, 1986; WEATHERALL & PROVAN, 2000).

- Regiões promotoras dos genes gama G (γG) e gama A (γA)

Os haplótipos ligados ao grupo de genes da globina β^S podem refletir variações polimórficas em elementos *cis* regulatórios, os quais podem alterar a ligação de proteínas que ativam ou inibem a transcrição de um gene, modificando o balanço recíproco entre a expressão dos genes β^S e γ. Uma das variações de elementos *cis* é a ocorrência do polimorfismo C→ T, localizado na posição −158 da região promotora do gene γG e associado à presença do sítio para a enzima de restrição XmnI na região 5' deste gene. Este polimorfismo está presente nos haplótipos Sen e Saudi, sendo fortemente relacionado a níveis elevados de HbF e expressão aumentada do gene γG, quando comparado ao gene γA (OFORI-ACQUACH et al., 1999; NAGEL & STEINBERG, 2001; STEINBERG, 2001). Recentemente, OFORI-ACQUAH et al. (2004) demonstraram a influência dominante de polimorfismos na região promotora dos genes γ sobre a expressão da HbF em indivíduos portadores da anemia falciforme.

- Região controladora do locus da globina β (LCR)

A região controladora do locus da globina β compreende uma série de cinco sítios hipersensíveis a ação da DNase I (HS-1 a HS-5) presentes entre 6 a 18Kb na posição 5' antes do gene ε e que, provavelmente, desempenham um papel importante na expressão específica de tecidos e de genes do grupo da globina β (Figura 1.4) (STEINBERG, 1995). Cada um destes sítios contém diferentes combinações de domínios conservados, onde ocorre a ligação de proteínas que influenciam o processo da transcrição. Alterações na seqüência destes domínios podem conduzir a ligações que modificam a expressão destes genes. Entre estes sítios, o HS-2 é o que possui evidência maior de estar associado aos níveis elevados de HbF descritos em portadores da anemia falciforme (STEINBERG, 1995; STEINBERG, 2001; NAGEL & STEINBERG, 2001).

Os fatores transcricionais (*trans*) podem ser de origem eritróide ou não eritróide e são importantes na transcrição dos genes da globina, tais como os fatores GATA-1, NF-E2, Sp-1, YY-1 e Ap-1 que interagem com o LCR e a seqüências *cis* presentes nas regiões promotoras dos genes (YANG & PACE, 2001; OFORI-ACQUAH et al., 2001; STAMATOYANNOPOULOS, 2005). Mais recentemente, tem sido investigada a participação de outros fatores que podem influenciar os níveis de HbF; estes elementos foram denominados de QTL (*locus quantitativo de características*) e estão localizados nos cromossomos 6, 8 e X (GARNER et al., 2002; WYSYNSKI et al., 2004).

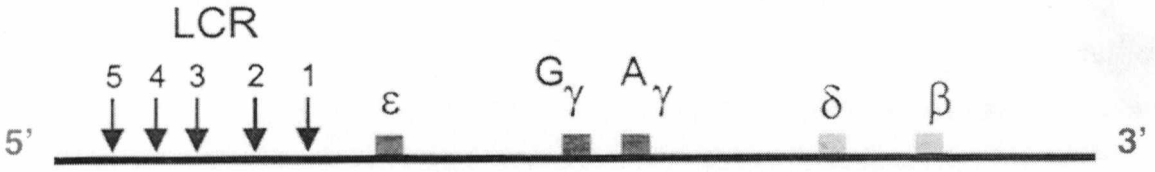

Figura 1.4 Representação esquemática dos sítios hipersensíveis à DNase I e sua localização na região controladora do locus da globina β (LCR) no cromossomo 11. Adaptado de NAGEL & STEINBERG, 2001.

2.1 Objetivo Geral

Realizar a caracterização fenotípica e de marcadores moleculares em um grupo de pacientes portadores da anemia falciforme da cidade de Salvador-BA, visando identificar seqüências gênicas potencialmente importantes para a expressão dos genes da globina gama.

2.2 Objetivos Específicos

- Realizar a caracterização molecular dos haplótipos ligados ao grupo de genes da globina β^S em portadores da anemia falciforme;
- Realizar a caracterização molecular da talassemia $\alpha_2^{3,7Kb}$;
- Correlacionar o dados clínicos e hematológicos com o tipo de haplótipo, presença da talassemia $\alpha_2^{3,7Kb}$ e níveis de HbF;
- Realizar a caracterização molecular das seqüências gênicas localizadas na região promotora dos genes γG e γA e no segundo sítio hipersensível à ação da DNase I da região controladora do locus da globina β (HS2-LCR) em pacientes portadores de diferentes haplótipos.

A diversidade fenotípica da anemia falciforme tem sido associada à presença dos vários haplótipos ligados ao grupo de genes da globina β^S que podem estar relacionados a um quadro clínico intermediário. Estes halótipos têm sido relacionados aos níveis de HbF e à origem geográfica da mutação (POWARS, 1991; EL HAZMI et al., 1999; NAGEL & STEINBERG, 2001). O haplótipo Ben tem sido relacionado a níveis médios de HbF e a um quadro clínico intermediário, enquanto o CAR tem sido correlacionado a níveis mais baixos de HbF e a um quadro clínico mais grave.

No Brasil, os haplótipos CAR e Ben são os mais prevalentes, sendo descrita a freqüência 66,2% para o CAR e 23% para o Ben, em um estudo envolvendo a população negróide do sudeste brasileiro (ZAGO et al., 1992) e de 66,7% para o CAR e 30,0% para o Ben na região Norte do país (PANTE-DE-SOUZA, et al., 1998). GONÇALVES et al. (1994) registraram o predomínio do haplótipo CAR em uma população de portadores da anemia falciforme de São Paulo, tendo sido confirmado por COSTA et al. (1994) que estudaram os haplótipos ligados ao grupo de genes da globina β^S em indivíduos portadores da anemia falciforme dos estados de São Paulo e Bahia, demonstrando diferenças entre as duas regiões, sendo que São Paulo apresentou freqüência elevada do haplótipo CAR, diferente do estado da Bahia, que apresentou freqüências similares para ambos; FIGUEIREDO et al. (1996), estudando pacientes com anemia falciforme de São Paulo, sugeriram que indivíduos portadores do haplótipo CAR poderiam estar associados a um fenótipo mais grave.

Estudos recentes, desenvolvidos na população da cidade do Salvador, contribuíram para demonstrar a necessidade da caracterização molecular e ampliação dos conhecimentos

relacionados à anemia falciforme na população do estado da Bahia. LYRA et al. (2005) em estudo realizado em portadores de anemia falciforme de São Paulo e de Salvador, demonstraram a freqüência mais levada para o haplotipo Ben em Salvador (48,0%) e para o haplotipo CAR em São Paulo (55,0%), com predomínio do genótipo CAR/Ben nas duas regiões; a freqüência da talassemia $\alpha_2^{3,7Kb}$ também foi similar nas duas populações. Entretanto, os portadores de anemia falciforme de São Paulo foram aqueles que apresentaram freqüência mais elevada de internamento hospitalar por eventos de vaso-oclusão, quando comparados aos indivíduos de Salvador-Bahia.

GONÇALVES et al. (2003) ao estudarem 80 pacientes portadores de anemia falciforme, identificaram um comportamento heterogêneo quanto à caracterização molecular dos haplótipos ligados ao grupo de genes da globina β^S e dos níveis de HbF apresentados pelos pacientes. Nesse estudo, os autores analisaram os dados hematológicos, o perfil de hemoglobinas e os haplótipos ligados ao grupo de genes da globina β^S, identificando um total de 77 (48,1%) cromossomos contendo o haplótipo CAR; 73 (45,6%) o Ben; um (0,63%) o Sen e em nove (5,63%) um haplótipo caracterizado como o Atípico (Atp), que provavelmente foi gerado por uma variedade de mecanismos genéticos, envolvendo os haplótipos Ben, Sen ou CAR, como sugerido por ZAGO et al. (2000). Dezessete (21,3%) pacientes apresentaram o genótipo CAR/CAR; 17 (21,3%) o Ben/Ben; 37 (46,3%) o CAR/Ben; um (1,25%) o Ben/Sen; um (1,25%) o Ben/Atp; seis (7,5%) o CAR/Atp e um (1,25%) o Atp/Atp. Os pacientes com genótipo Ben/Ben apresentaram a média mais elevada de HbF (9,9% ± 3,5), seguidos pelos portadores dos genótipos CAR/Ben (8,2% ± 4,6) e pelos CAR/CAR (7,5%±4.,3). Diante da identificação do haplótipo Sen, descrito no referido trabalho, levantou-se a hipótese do Estado da Bahia ter

recebido um fluxo gênico da África Atlântico Ocidental, como ocorreu em outros estados brasileiros, durante o período do tráfico de escravos. O fato de apenas um único haplótipo Sen ter sido encontrado, poderia ser o reflexo do curso clínico menos grave da doença nesta população, fato que reduz os cuidados médicos especiais necessários, como descrito em literatura (NAGEL & STEINBERG, 2001) ou ser decorrente da origem recente desta mutação em nossa população.

Outro achado importante do trabalho de GONCALVES et al. (2003), foi o encontro de níveis elevados da HbF em indivíduos CAR/CAR e CAR/BEN, o que confirma a grande miscigenação racial ocorrida nesta população e a possível existência de seqüências gênicas de importância na expressão dos genes γ e conseqüentemente na síntese da HbF. Resultados semelhantes foram descritos por FIGUEIREDO & STEINBERG (1996), que estudaram 85 indivíduos brasileiros portadores de anemia falciforme e encontraram os haplótipos CAR e Ben associados a níveis elevados de HbF. Com o objetivo de identificar estas seqüências, foi proposta a realização do estudo da região controladora do locus da globina β, bem como de seqüências presentes nos genes da globina γ, com importância potencial no controle da expressão destes genes e na interação com elementos transcricionais.

Outro trabalho que corroborou para o desenvolvimento de um estudo que possibilitasse a caracterização do perfil clínico e molecular dos pacientes com anemia falciforme na Bahia, foi realizado por ADORNO et al. (2005), no qual foi confirmada a freqüência elevada para o gene da HbS e para a talassemia $\alpha_2^{3,7\ Kb}$. Neste trabalho, investigou-se a presença de hemoglobinopatias em 590 recém-nascidos da Maternidade Pública Tsylla Balbino da cidade do Salvador. Os resultados demonstraram 57 (9,8%) recém-nascidos portadores da HbS em heterozigose (FAS); 36 (6,5%) portadores da HbC

em heterozigose (FAC); um (0,2%) com anemia falciforme (FS) e cinco (0,9%) com doença SC (FSC). A talassemia $\alpha_2^{-3,7\ kb}$ foi identificada em 114 neonatos (22,2%), dos quais 101 (19,7%) foram heterozigotos e 13 (2,5%) foram homozigotos, com diferenças estatisticamente significativas para os valores hematológicos, quando comparados aos dos recém-nascidos portadores de genes α normais.

De acordo com o exposto e com os resultados previamente alcançados, julgamos de grande importância o estudo fenotípico de portadores da anemia falciforme em Salvador – Bahia e sua associação com marcadores moleculares, visando a identificação de seqüências que possam potencialmente interferir na expressão dos genes γ e, conseqüentemente, na síntese da HbF.

1. The β-globin gene cluster haplotypes in sickle cell anemia patients from northeast Brazil: a clinical and molecular view.

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SHORT COMMUNICATION

The β-Globin Gene Cluster Haplotypes in Sickle Cell Anemia Patients from Northeast Brazil: A Clinical and Molecular View

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ABSTRACT

The β^S-globin haplotypes were studied in 78 sickle cell Brazilian patients from Bahia, Northeast Brazil, that has a large population of African origin. Hemoglobin (Hb) profiles were developed by high-performance liquid chromatography (HPLC), and β^S-globin gene haplotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques. We identified 44 (55.0%) patients with the CAR/Ben (Central African Republic/Benin) genotype, 16 (20.0%) Ben/Ben, 13 (16.2%) CAR/CAR and seven (8.8%) with other genotypes. Analyses

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of the phenotypes showed clinical differences related only to Hb F levels and blood transfusion therapy; the presence of $-\alpha^{-3.7}$-thalassemia (thal) demonstrated statistical significance when associated with hematocrit ($p = 0.044$), MCV ($p = 0.0007$), MCH ($p = 0.012$) and spleen sequestration events. The haplotype diversity found in the present study can be justified by information about the origin of the slave traffic period in Bahia during the 19th century. The specific characteristics described among the Bahian sickle cell patients could be confirmed by increasing the number of patients with specific genotypes and further studies of genetic markers.

Key Words: β-Globin haplotypes; Sickle cell anemia; Northeast Brazil; Slave trade.

The β^S-globin gene haplotypes are named according to the geographic areas where they predominate and are useful in the definition of African population origins (1). They have been classified as five different types: the Benin (Ben) has been associated with Midwestern Africa; the Bantu or Central African Republic (CAR) with South-Central and Eastern Africa; the Senegal (Sen) with Atlantic West Africa, the Arab–Indian type with the Indian subcontinent and Eastern Arabian Peninsula, and the Cameroon (Cam) along the west coast of Africa (1,2). The Senegal haplotypes have been associated with high Hb F levels and a less severe clinical course, the Benin haplotype with an intermediate clinical course and Hb F levels, and the Central African Republic haplotype with a low Hb F level and a more severe clinical course (2,3). The Arab–Indian haplotype presents the highest Hb F levels with a heterogeneous clinical course (4).

Brazil is the largest country of South America and has a population with a high rate of racial admixture with a strong compound of African genes that were introduced by the slave trade (5). The country has a high prevalence of hemoglobin (Hb) disorders, with the South presenting a frequency of 6.6% sickle cell trait (Hb AS) in a Black population (6), and the Bahia state, located in Northeast Brazil, with a frequency of 7.5% to 15.7%, when different groups of this population were studied (7). In the present study, we investigated the β^S-globin gene haplotypes in 80 sickle cell anemia patients from the Blood Center of Bahia State, in order to confirm the African origin of this population.

The Hb profile was obtained by high-performance liquid chromatography (HPLC) (VARIANT II™; Bio-Rad Laboratories, Hercules, CA, USA) and the DNA was isolated from peripheral blood leukocytes by the GFX™ Genomic Blood DNA Purification KIT (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The β^S-globin gene haplotypes were investigated by polymerase chain reaction (PCR) and the haplotype polymorphic sites identified by restriction fragment length polymorphism analysis (RFLP) as previously described (8).

Among the 160 β^S chromosomes analyzed, 78 (48.8%) were characterized as the Benin type, 74 (46.2%) Central African Republic, three (1.9%) Cameroon, one (0.6%) Arab–Indian, one (0.6%) Senegal and three (1.9%) were characterized as other haplotypes. The genotype characterization showed 44 (55.0%) CAR/Ben, 16 (20.0%) Ben/Ben, 13 (16.2%) CAR/CAR and seven (8.8%) classified as other genotypes. Statistical significance for use of blood transfusion therapy occurred more frequently in

patients with Hb F levels below 10.0% ($p = 0.0015$); statistical differences were observed for hematocrit ($p = 0.044$), MCV ($p = 0.0007$), MCH ($p = 0.012$) and the spleen sequestration events ($p = 0.032$) among $-\alpha^{-3.7}$-thal carriers, according to other studies (9,10).

In this study, the Benin haplotype frequency (48.8%) was slightly higher than the Central African Republic haplotype (46.2%), and the prevalence of the CAR/Ben genotype (54.0%) was consistent with the results described by Gonçalves et al. (11) who studied the β^S-globin gene haplotypes among sickle cell disease patients from Salvador-Bahia, Brazil. The slave trade in Brazil was extensively reported by Curtin (12) who described Brazil as the largest, single importer into the Americas. The information from the British and Brazilian government offices described uncertainty about the Brazilian imports during the 1820s and 1830s, with a total of 8000 slaves of unknown origin imported into Bahia in the 19th century between 1817 and 1843, justifying the presence of the Cameroon, Senegal and Arab–Indian haplotypes found in the present study (12).

These results are different from those observed among other American countries, confirming the diversity of the African influence in Bahia. The United States of America and Jamaica received slaves from Midwestern Africa during the British Atlantic slave trade, where the Benin haplotype is more prevalent. However, in Mexico (Costa Chica region), Colombia, Central-Western regions of Venezuela, Cuban and Puerto Rican regions, there is a predominance of the Central African Republic haplotype, suggesting a different African origin for these populations (13–15).

The results described in Bahia are different from other regions of Brazil, such as the southeast and north, where there is a predominance of the CAR β^S-globin gene haplotypes of 66.2% and 66.7%, respectively, demonstrating the heterogeneity of the African slave trade brought to the country (16,17). These results indicate a contribution of Africans from Congo, Mozambique and Angola, where the CAR haplotype is predominant, that received ships from Bahia with tobacco and returned with slaves; this slave trade was intensified between 1815 and 1824 (18,19). Nevertheless, there is evidence that the northeastern region of Brazil, mainly Bahia State, received Africans from Central West Africa until the middle of the 19th century (Bight of Benin and Bight of Biafra), justifying the frequencies of CAR and Benin haplotypes found in this population (19).

These findings may contribute to the investigation of slave trade routes in Brazil and African origins of the Bahian population that seems to be quite different to other Brazilian states and other world populations An increase in the number of samples analyzed will probably confirm a phenotypical difference among the sickle cell patients from Bahia and other patient groups worldwide.

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CLINICAL AND MOLECULAR CHARACTERISTICS OF SICKLE CELL ANEMIA IN BAHIA, BRAZIL

ABSTRACT

One hundred and twenty five sickle cell anemia patients from Northeastern Brazil, were studied; 64 (51.2%) were CAR/Ben; 36 (28.8%) Ben/Ben; 18 (14.4%) CAR/CAR; two (1.6%) CAR/Aty; two (1.6%) Ben/Cam; one (0.8%) CAR/Cam; one (0.8%) Car/Arab-India and one (0.8%) Sen/Aty. The α_2 $^{3.7Kb}$- thalassemia was studied in 110 patients, with 30 (27.3%) heterozygous and two (1.8%) homozygous. The PCV values were lower (p=0.045) and use of blood transfusion therapy was higher (p=0.009) in patients with HbF≤10.0%. Patients with α_2 $^{3.7Kb}$- thalassemia had high Hb (0.018) and PCV (p=0.019) and low MCV (0.0004) and MCH (0.039). HbF levels were higher to Ben/Ben than CAR/CAR (p=0.007) and CAR/Ben (p=0.013). HbF levels seems to be an important marker of sickle cell anemia phenotype in Northeastern Brazilians and further studies will identify the role of others prognostic factors, once that sickle cell anemia is a healthy problem in Bahia, Brazil.

Key Words: Sickle cell anemia; β^S-globin gene haplotype; Fetal hemoglobin; α_2 $^{3.7Kb}$ – thalassemia; Phenotype

INTRODUCTION

Sickle cell hemoglobin (HbS) results from a single nucleotide change (GAG → GTG) in the sixth codon of the β-globin gene, where valine replaces glutamic acid in the β-globin chain (Bunn & Forget 1986). The sickle cell anemia or the HbS homozygous state has severe chronic hemolytic anemia, presenting a heterogeneous phenotype, which may include painful episodes, splenic sequestration and less commonly stroke, priapism, aplastic crisis or other chronic complications such as cholithiasis, hepatobiliary disease, retinopathy, delayed growth and sexual development (Nagel & Ranney 1990; Nagel & Sieinberg 2001). Several factors have been described to modify the disease clinical picture, such as $\alpha^{3.7kb}{}_2$ – thalassemia; fetal hemoglobin (HbF) levels and β^S - globin gene haplotypes (Nagel & Ranney 1990; Steinberg 2001). The β^S - globin gene haplotypes have been associated with five different genotypes, according to several restriction enzymes site presence at β - globin cluster, the geographical origin and HbF levels. The Benin (Ben) type is commonly described in Midwestern Africa; the Bantu or Central African Republic (CAR) in South-Central and Eastern Africa; the Senegal (Sen) in Atlantic West Africa; the Arab-India type in Indian subcontinent and Eastern Arabian penisula and the Cameroon (Cam) along the West Coast of Africa (Nagel & Ranney 1990; Nagel & Sieinberg 2001). The Sen haplotype has been associated with a high HbF levels and a less severe phenotype; the Ben haplotype with an intermediate HbF levels and mild clinical course and the CAR haplotype with low HbF level and a severe phenotype (Nagel & Ranney 1990; Steinberg 2001). The Arab-India haplotype has the highest HbF levels, with heterogeneous clinical manisfestation (Rahgozar *et al.* 2000). Brazil is the biggest country of South America and has a high rate of racial admixture in its population and a strong compound of African genes, introduced by the slave trade (Azevêdo 1980). This country has a high prevalence of hemoglobin disorders, with the Southern region presenting a sickle cell trait (AS) frequency of 6.6% among the black population (Ramalho *et al.* 1976) and the Bahia state, located at the Northeast of Brazil with a frequency of 7.5% to 15.7%, among different Bahian population groups (Azevêdo *et al.* 1980). The other common group of inherited hemoglobin disorders and a high worldwide distribution are the thalassemias syndromes, characterized by globin chain synthesis reduction or absence. The α_2 - thalassemia followed by a 3.7 Kb deletion has been described in 20 to 25% of the Brazilian black population (Sonati *et al.1991*). The $\alpha_2{}^{3.7\ Kb}$ - thalassemia presence in sickle cell anemia has been associated with an inhibitory effect on intracellular polymer formation, providing less hemolysis, lower mean corpuscular volume (MCV) and a high hemoglobin concentration; on the other hand, there is no reduction of the vaso-occlusive events (Steinberg & Embury 1986). At the present study we investigated the β^S -globin gene haplotypes in a group of sickle cell anemia patients from Bahia, a Northeastern Brazilian State, associating with $\alpha_2{}^{3.7Kb}$ - thalassemia, β^S globin gene haplotypes, fetal hemoglobin levels, hematological data and phenotypic characteristics.

MATERIALS AND METHODS

A total of 125 sickle cell patients (SS) from the Blood Center of Bahia (HEMOBA) were studied. Sixty three were females and 62 males with ages ranging from 01 to 55 years (19.1 ± 12.94). Approval was obtained from the Institutional Ethical Committee of the Gonçalo Moniz Research Center of FIOCRUZ – Bahia and an informed consent was signed for each patient or official responsible. Information about the clinical picture was obtained by patient record.

Hematological analyses were carried out by electronic cell counter (Coulter T-890, Coulter Corporation, FL, USA). The hemoglobin profiles were investigated by High Performance Liquid Chromatography (HPLC – VARIANT I / BIO-RAD, CA, USA) and the DNA was isolated from peripheral blood leukocytes by the GFX™ Genomic Blood DNA Purification KIT (Amersham Pharmacia Biotech, NJ, USA). The $\alpha_2{}^{3.7Kb}$- thalassemia was investigated by Polymerase Chain

Reaction (PCR) and β^S – globin gene haplotypes were investigated by PCR and Restriction Fragment Length Polymorphism (RFLP) techniques, using specific restriction endonucleases (XmnI, HindIII, HincII, HinfI, HpaI) (Sutton *et al.* 1989; Dodé *et al.* 1998).
The statistical analyses were conducted in the EPI-INFO software version 6.04 and statistical significance was established at $P \leq 0.05$.

RESULTS

One hundred and twenty-five sickle cell anemia Brazilian patients (SS) were included in this study. The median hemoglobin concentration (Hb) was 7.6 g/dl (± 1.66); median packed cell volume (PCV) 0.26 (±0.5); median cell volume (MCV) 94.3 fl (± 13.23); median cell hemoglobin (MCH) 29.0 pg (± 4.85); median cell hemoglobin concentration (MCHC) 31.2 g/dl (± 5.54) and median fetal hemoglobin levels (HbF) was 9.80 % (± 6.21). Among 250 β^S chromosomes analyzed, 138 (55.2%) were Ben; 104 (41.6%) CAR; three (1.2%) Cam; three (1.2%) Atypical (Aty); one (0.4%) Arab-India and one (0.4%) Sen. Sixty four patients (51.2%) were CAR/Ben; 36 (28.8%) Ben/Ben; 18 (14.4%) CAR/CAR; two (1.6%) CAR/Aty; two (1.6%) Ben/Cam; one (0.8%) Car/Cam; one (0.8%) CAR/Arab-India and one (0.8%) Sen/Aty.
There were 83 (66.4%) hospitalizations among the total of patients, of which 29 (23.2%) had four or more events. Seventy one (56.8%) received blood therapy, of which 34 (27.2%) received four or more. Forty eight patients (38.4%) had infections, mainly pneumonia and leg ulcers. Among 34 (27.2%) patients presenting clinical complications, nine (7.2%) had spleen sequestration, four (3.2%) cerebrovascular accident (CVA), two (1.6%) priapism, three (2.4%) retinopathy, four (3.2%) acute chest syndrome, one (0.8%) aplastic crisis, one (0.8%) presented an association of spleen sequestration, cerebrovascular accident and heart failure and one (0.8%) had association of spleen sequestration, cerebrovascular accident and breathing failure. Sixty-six patients (52.8%) had painful episodes, of which 32 (25.6%) had more than four events.
Table 1 shows analyses of phenotype among Bahian sickle cell anemia patients with HbF levels of ≤ 10.0% >. The hematological analyses demonstrated higher PCV value for patients with HbF>10.0% (p=0.045). The means of HbF levels were 12.4% (± 6.46) for Ben/Ben genotype; 9.2% (± 5.66) for CAR/Ben and 7.5% (± 4.05) for CAR/CAR.
The HbF levels and phenotypes characteristics analyses among CAR/Ben; CAR/CAR and Ben/Ben genotypes are shown in table 2.The genotypes and phenotypes characteristics are shown in table 3. Three CAR/Ben and two CAR/CAR patients presented CVA events. The Sen/Aty patient had 0.8% of HbF; the two CAR/Aty had 7.1% and 9.6%; the CAR/Arab-India had 26.4%; the CAR/Cam had 5.0%; the two Ben/Cam patients had 17.10% and 27.4%. The Sen/Aty (56 years) did not develop clinical complications or used blood therapy; outside this, all above mentioned patients had hospitalizations and used blood transfusion therapy; the CAR/Aty and Ben/Cam patients presented painful episodes; one Ben/Cam patient (HbF=27.40) had spleen sequestration and CAR/Cam developed CVA.

The analyses of age and different genotypes, demonstrated 64 (51.2%) patients ≤18 years old and 61 (48.8%) >18 years old. Among the Ben/Ben patients, the mean of HbF was higher between ≤18 years old patients group (p=0.0003), with mean of age of 7.6 years old (± 6.3). The leg ulcers presence was higher between the age > 18 years (p=0.049), with mean of age of 30.0 years (± 10.7); the patients with CAR/Ben genotype and high frequency of pneumonia episodes had the age ≤18 years (p=0.02), with mean of 10.2 years (± 5.8). Statistical difference was not found between the CAR/CAR genotype.
Among the patients with age ≤18 years, the mean of HbF was higher for Ben/Ben (16.0% ± 6.0) than CAR/Ben (10.6% ± 6.0) and than CAR/CAR (7.5% ± 4.0) patients (p=0.0013). The $\alpha_2^{3.7Kb}$-thalassemia was investigated among 110 patients, 30 (27.3%) were heterozygous and two (1.8%)

homozygous. Alpha $_2$ $^{3.7Kb}$- thalassemia in heterozygous state was found among three (2.7%) CAR/CAR patients; 18 (16.4%) CAR/Ben; eight (7.3%) Ben/Ben and one (0.9%) CAR/Arab-India genotype. The two (1.8%) homozygous for α_2 $^{3.7Kb}$- thalassemia were CAR/Ben. Table 4 shows hematological data and phenotypic characteristics analyses between different α - genes genotypes. The CAR/Ben patients with α_2 $^{3.7Kb}$- thalassemia had lower MCV (p=0.0001) and MCH (p=0.002) values and lower frequency of painful episodes (p=0.012). The analyses of Ben/Ben and CAR/CAR patients did not show statistical differences.

DISCUSSION

The β^S - globin gene haplotypes are named according to the African geographic origin, considering the African slave trade occurred in specifics population (Nagel & Ranney 1990).

In this report, the Ben haplotype frequency (55.2%) was higher than CAR haplotype (41.6%) and the CAR/Ben genotype (51.2%) predominance was consistent with the results described by Gonçalves *et al.* (2003) when studied β^S - globin gene haplotypes among sickle cell disease patients from Salvador (Bahia-Brazil). Furthermore, associations between Aty with CAR (1.6%) and Sen haplotypes (0.8%); Cam with Ben (1.6%), and CAR (0.8%); the Arab-India with CAR (0.8%) haplotype were found. Ours results are different from those observed among others American countries, confirming the African gene heterogeneity composition of Brazilian pupulation. The United States of America (USA) and Jamaica received slaves from Midwestern Africa during the British Atlantic slave trade, where the Ben haplotype is more predominant. However, in Mexico, Colombia, Central-Western regions of the Venezuela, Cuba and Puerto Rican regions, there is a CAR haplotype predominance, suggesting a specific African gene origin in these populations (Magaña *et al.* 2002; Lugo *et al.* 2003).

The β^S - globin gene haplotypes distribution described in the Bahia population was different from other Brazilian regions, such as the Southeastern and Northern, where there is a predominance of 62.2% and 66.7% of CAR β^S – globin gene haplotypes, respectively, demonstrating the African slave trade diversity in Bahia State, throughout the Eighteenth and Nineteenth centuries (Verger 1968; Costa *et al.*1994; Goncalves *et al.* 1994; Figueiredo *et al.* 1996; Pante-de-Souza *et al.* 1999). At the present study, we report for the first time the Cam and Saudi Arabia-India haplotypes in Bahia; this finding brings contributions for the slave trade routes investigation in Brazil, confirming the specific African origin of Bahia population, already described in previous reports (Goncalves *et al.* 2003; Adorno *et al.* 2004).

The literature shows that the phenotype diversity exhibited by the sickle cell disease patients appears to be affected by HbF levels, once that patients with higher HbF levels present less hemolysis, high hemoglobin concentration, an intermediary clinical picture and high survival (Powars & Hiti 1993). However, there are controversies in Lebanon, where 50 sickle cell disease patients were studied, and the highest HbF levels were associated with the most severe phenotype, suggesting that HbF is an important but not the only factor that affect the sickle cell anemia clinical manifestation (Inati *et al.* 2003). Our data showed that patients with higher HbF levels had higher PCV value and received less blood therapy, suggesting a less severe phenotype, in accordance with other studies (Steinberg 2001; Inati *et al.* 2003). However, we have not found association of HbF levels with other clinical data, like hospitalization, pneumonia events, leg ulcers, spleen sequestration, CVA or painful episodes.

Preview reports (Powars & Hiti 1993; Inati *et al.* 2003) have discussed the β^S-globin gene haplotypes and its role as a marker for the phenotypic heterogeneity in sickle cell anemia; HbF levels have been frequently associated to phenotype variations. Generally, patients with CAR haplotype present the lowest HbF levels, often below 5% and carriers of Ben haplotype have intermediate HbF levels, from 5 to 15% (Steinberg 2001; Inati *et al.* 2003).

The CAR/Ben patients of this study demonstrated intermediary HbF levels, the Ben/Ben genotype had high HbF levels, presenting statistical differences in comparison with the other genotypes and CAR/CAR genotype presented HbF levels above 5%. These results are different from other countries and Brazilian States (Costa *et al.* 1994; Goncalves *et al.*1994; Figueiredo *et al.* 1996; Nagel & Steinberg 2001), although our data are consistent with Goncalves et al. (2003), who described β^S-globin gene haplotypes in Salvador-Bahia and found sickle cell anemia patients CAR haplotype carrier and high HbF level, in concordance with other world-wide and Brazilian study works which found a similar association (Mouele *et al.* 1999; Inati *et al.* 2003; Figueiredo & Steinberg 1996). The presence of high HbF levels found in CAR/CAR genotype could be explained by sequence variation in regulatory regions of 5'HS2 and flanking region of the γ gene expression, as previously discussed by Lanclos et al. (1991) and Nagel & Steinberg (2001b), suggesting that several factors are affecting simultaneously the HbF synthesis, probably having an important role in the globin gene transcription regulation. However, there is controversy on whether polymorphism of the same elements contributes to variation of the HbF level among patients with sickle cell anemia (Lanclos *et al.* 1991; Steinberg 2001; Ofori-Acquah *et al.*2001). Furthermore, the other elements (transacting) also appear to influence the HbF regulation, such as quantitative trait loci (QTL) on chromosomes 6, 8 and X-chromosome (Chang *et al.* 1997; Garner *et al.* 2002). More recently, Wyszynski *et al.* (2004) found evidence for an association of single nucleotide polymorphisms (SNPs) bordering the QTL located at 6q22.3-q23.2 with increases in HbF levels in patients with sickle cell anemia.

In this report, it was only found association with β^S – globin gene haplotypes and HbF levels and these results are in accordance with previous reports describing CAR and Ben patients with high HbF levels without ameliorating the clinical symptoms of the sickle cell anemia (Inati *et al.* 2003). Other reports suggest that CAR haplotype is associated with a highest incidence of organ damage and, generally, patients with Ben haplotype have less severe disease than CAR haplotype (Mouele *et al.* 1999; Inati *et al.* 2003). However, at the present report, the CAR/CAR genotype did not change the clinical disease course and the CAR/Arab-India and CAR/Aty had the high HbF levels and developed a mild clinical picture, although all events of CVA were associated with the presence of CAR haplotype, such as previously discussed by Sarnaik & Ballas (2001), who suggested that the presence of CAR/Ben, Aty or CAR/CAR haplotypes seem to be at a higher risk for CVA than other haplotypes. The Sen/Aty patient described here did not present any clinical symptoms, suggesting that the Sen haplotype presence may be associated with a mild clinical manifestation, as observed by Diop *et al.* (1999). However, the low HbF levels confirm once more that other factors can affect the association of β^S-globin gene haplotypes, HbF level and phenotype. These results did not agree with other reports, which demonstrated Sen haplotype and the Arabian-India haplotypes strongly associated with the highest HbF synthesis and a high γ gene expression, when compared with the A γ gene, associating this event with the presence of the Xmn I restriction site, located at 5' to the Gγ – globin gene (Lanclos *et al.* 1999; Steinberg 2001).

The presence of $\alpha_2^{3.7Kb}$- thalassemia in sickle cell anemia patients contributed to an increase of Hb, PCV and VCM and MCH reduction, according to previous reports (Adams *et al.*1994; Diop *et al.*1999). Furthermore, the co-existence of $\alpha_2^{3.7Kb}$ – thalassemia and CAR/CAR genotype decreased the occurrence of painful episodes, also observed by Mukherjee et al. (1997), although discordant with Billett *et al.* (1995), who associates α– thalassemia with increase of painful crises. Also consistent with previous report, the HbF levels did not present significant difference among different α-genotypes (Figueiredo *et al.* 1996). The results of the present study confirm the high diversity of β^S – gene globin haplotypes and phenotypic heterogeneity among Brazilian sickle cell anemia patients. Further studies need to be conducted in order to identify other modifying factors that can interfere with the gene expression and phenotype.

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Table 1. Gender, phenotypic characteristics and HbF levels among sickle cell anemia patients from Salvador-Bahia-Brazil

	HbF> 10.0%	HbF≤ 10.0%	P value
Gender			
Male, n (%)	28 (45.2%)	34 (54.8%)	0.92†
Female, n (%)	28 (44.4%)	35 (55.6%)	
Hospitalizations, n / total (%)	39/56 (69.6%)	44/69 (63.8%)	0.62 †
Blood transfusion, n / total (%)	24/56 (42.9%)	47/69 (68.1%)	**0.008** †
Pneumonia, n / total (%)	14/56 (25.0%)	11/69 (15.9%)	0.30†
Leg ulcers, n / total (%)	06/56 (10.7%)	15/69 (21.7%)	0.16 †
Spleen sequestration, n / total (%)	09/56 (16.1%)	06/69 (8.7%)	0.32 †
Cerebrovascular accident, n / total (%)	02/56 (3.6%)	04/69 (5.8%)	0.69 ‡
Painful episodes, n / total (%)	31/56 (55.4%)	35/69 (50.7%)	0.80 †

* Kruskal-Wallis H **ANOVA † X^2 Test ‡ Fisher Exact Test

Table 2. Age, phenotypic characteristics and haplotypes among sickle cell anemia patients from Salvador - Bahia - Brazil

Genotpypes Groups	HbF Level (N)	Age (years), median	Hospitalization, n/total (%)	Blood Therapy, n/total (%)	Pneumonia, n/total (%)	Leg ulcers, n /total (%)	Spleen sequestration, n/total (%)	Cerebrovascular accident, n/total (%)	Painful episodes, n/total (%)
CAR/Ben	Hb F>10.0 (26)	14.5	18/26 (69.2)	10/26 (38.5)	07/26 (26.9)	01/26 (3.8)	04/26 (15.4)	02/26 (7.7)	13/26 (50.0)
	Hb F≤10.0 (38)	21.0	27/38 (71.0)	25/38 (65.8)	05/38 (13.2)	07/38 (18.4)	05/38 (13.2)	01/38 (2.6)	22/38 (57.9)
	P Value	**0.047***	0.90†	**0.047**†	0.20‡	0.13‡	1.00‡	0.56‡	0.71†
CAR/CAR	Hb F> 10.0 (04)	18.0	03/04 (75.0)	03/04 (75.0)	01/04 (25.0)	01/04 (25.0)	-------	-------	03/04 (75.0)
	Hb F≤10.0 (14)	16.0	08/14 (57.1)	08/14 (57.1)	02/14 (14.3)	03/14 (21.4)	01/14 (7.1)	02/14 (14.3)	06/14 (42.9)
	P Value	0.95*	1.00‡	1.00‡	1.00‡	1.00‡	1.00‡	1.00‡	0.58‡
Ben/Ben	Hb F>10.0 (23)	7.0	16/23 (69.6)	10/23 (43.5)	05/23 (21.7)	04/23 (17.4)	04/23 (17.4)	--------	12/23 (52.2)
	Hb F≤10.0 (13)	23.0	06/13 (46.2)	11/13 (84.6)	03/13 (23.1)	05/13 (38.5)	--------	--------	05/13 (38.5)
	P Value	**0.004****	0.30‡	**0.04**‡	1.00 ‡	0.23 ‡	0.27 ‡	--------	0.81 †

* Kruskal-Wallis H **ANOVA † X^2 Test ‡ Fisher Exact Test

Table 3. β^S-Globin gene haplotypes and phenotypic characteristics among sickle cell anemia patients from Salvador - Bahia - Brazil

Genotpypes Groups (N)	Hb F % Mean ± SD	Hospitalization, n/total (%)	Blood Transfusion, n/total (%)	Pneumonia, n/total (%)	Leg ulcers, n /total (%)	Spleen sequestration, n/total (%)	Cerebrovascular accident, n/total (%)	Painful episodes, n/total (%)
CAR/CAR (18) Ben/ Ben (36)	7.5± 4.05 12.4±6.46	11/18 (61.1%) 22/36 (61.1%)	11/18 (61.1%) 21/36 (58.3%)	03/18 (16.7%) 08/36 (22.2%)	04/18 (22.2%) 09/36 (25.0%)	01/18 (5.6%) 04/36 (11.1%)	2/18 (11.1%) 0/36	09/18 (50.0%) 17/36 (47.2%)
P Value	**0.007***	0.77†	0.92†	0.73‡	1.00‡	0.65‡	0.11‡	0.76†
CAR/CAR (18) CAR/Ben (64)	7.5± 4.05 9.2±5.66	11/18 (61.1%) 45/64 (70.3%)	11/18 (61.1%) 35/64 (54.7%)	03/18 (16.7%) 12/64 (18.8%)	04/18 (22.2%) 08/64 (12.5%)	01/18 (5.6%) 09/64 (14.1%)	2/18 (11.1%) 03/64 (4.5%)	09/18 (50.0%) 35/64 (54.7%)
P Value	0.32**	0.65†	0.83†	1.00‡	0.45 ‡	0.45‡	0.30‡	0.93†
CAR/Ben (64) Ben/Ben (36)	9.2±5.66 12.4±6.46	45/64 (70.3%) 22/36 (61.1%)	35/64 (54.7%) 21/36 (58.3%)	12/64 (18.8%) 08/36 (22.2%)	08/64 (12.5%) 09/36 (25.0%)	09/64 (14.1%)0 4/36 (11.1%)	03/64 (4.5%) 0/36	35/64 (54.7%) 17/36 (47.2%)
P Value	**0.013** **	0.47 †	0.89†	0.88†	0.19†	0.77 ‡	0.55‡	0.71 †

* Kruskal-Wallis H ** ANOVA † X^2 Test ‡ Fisher Exact Test

Table 4. Gender, hematological data, phenotypic characteristics and α - genes genotype among sickle cell anemia patients from Salvador - Bahia - Brazil

	αα / αα	α_2 $^{3.7Kb}$ - thalassemia	P value
Gender Male, n (%) Female, n (%)	 44 (77.2%) 34 (64.2%)	 13 (22.8%) 19 (35.8%)	 0.20 †
Mean value and standard deviation of HbF (%))	9.9 ± 6.25	10.7 ± 6.97	0.56**
Mean value and standard deviation of Hb (g/dl)	7.3 ± 1.58	8.3 ± 1.34	**0.018** **
Mean value and standard deviation of PCV	0.24± 0.46	0.27 ± 0.43	**0.019** **
Mean value and standard deviation of MCV (fl)	96.8± 9.90	86.1 ± 9.56	**0.0004** **
Mean value and standard deviation of MCH (pg)	29.4± 4.40	26.6 ± 4.60	**0.039** **
Hospitalization, n / total (%)	50/78 (64.1%)	22/32 (68.8%)	0.81 †
Blood Transfusion, n / total (%)	44/78 (56.4%)	15/32 (46.9%)	0.48 †
Pneumonia, n / total (%)	20/78 (25.6%)	03/32 (9.4%)	0.10 ‡
Leg ulcers, n / total (%)	13/78 (16.7%)	04/32 (12.5%)	0.77‡
Spleen sequestration, n / total (%)	06/78 (7.7%)	06/32 (18.8%)	0.10 ‡
Cerebrovascular accident, n / total (%)	04/78 (5.1%)	01/32 (3.1%)	1.00 ‡
Painful episodes, n / total (%)	38/78 (48.7%)	20/32 (62.5%)	0.20 †

* Kruskal-Wallis H ** ANOVA † X^2 Test ‡ Fisher Exact Test

3. Sequence Variation of Gγ and Aγ genes Promoter and Hypersensitive Site 2 (HS2) of the β-Globin Locus Control Region in Sickle Cell anemia Patients with Fetal Hemoglobin Levels and β^S Haplotypes Diversity.

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Short Title: Sequence variation in sickle cell anemia patients, fetal hemoglobin and β^S Haplotypes

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ABSTRACT

Sickle cell anemia patients from Bahia, Brazil were studied, associating the phenotype, HbF levels, β^S-globin gene haplotypes and sequences variations of Gγ and Aγ genes promoter and hypersensitive site 2 of the β-globin locus control regions (HS2–LCR). The patients presented a high diversity of phenotype characteristics and HbF levels. The highest HbF levels were associated with less severe phenotype, a decrease of use of blood transfusion therapy (p=0.01) and leg ulcer (p=0.03). The HS2-LCR sequence analyses demonstrated a G→A change in a –10.677 position in patients with Ben haplotype and high HbF level, suggesting a possible association of this polymorphism with gamma genes expression. The analyses of Gγ gene promoter region showed a T→C substitution in a –157 position, suggesting a common sequence characteristic among of the Northeastern Brazilian sickle cell anemia patients. It was also described a 4 bp deletion in –222 to –225 position of the Gγ globin gene promoter region and related with Cam haplotype. The other analyses confirmed previous polymorphisms described by Lanclos *et al.* (1991). The data in the present study show new polymorphisms located at HS2–LCR and Gγ globin gene promoter regions, justifying further studies associating HbF levels, molecular markers and biologic mechanisms.

Key Words: Fetal hemoglobin; Sickle cell anemia; β^S-globin gene haplotypes; Phenotype, Locus Control Region; Hypersensitive site.

SEQUENCE VARIATION OF Gγ AND Aγ GENES PROMOTER AND HYPERSENSITIVE SITE 2 (HS2) OF THE β-GLOBIN LOCUS CONTROL REGIONS IN SICKLE CELL ANEMIA PATIENTS WITH FETAL HEMOGLOBIN LEVELS AND β^S-GLOBIN GENE HAPLOTYPES DIVERSITY

INTRODUCTION

Sickle cell hemoglobin (HbS) is characterized by a single nucleotide change (GAG → GTG) in the sixth codon of the β-globin gene, where valine replaces glutamic acid in the β-globin chain [1]. The clinical manifestations of sickle cell anemia have a wide spectrum of severity and have been described to differ accord to fetal hemoglobin (HbF) levels [2], which contribute to decrease the HbS red blood cell content, interfering in a polymerization process by the formation of asymmetrical hybrid forms ($\alpha_2\gamma\beta^S$) [3,4]. A high HbF level has been associated with a decrease of clinical severity with less painful events, blood therapy use and hospitalizations [5,6]. The amount of HbF also has been associated with β^S - globin gene haplotypes, usually recognized by a set of polymorphic restriction enzymes profile on the β-globin gene cluster region [7,8]. The major β^S - globin gene haplotypes have been named Benin (Ben), Bantu or Central African Republic (CAR), Senegal (Sen), Arab-India and Cameroon (Cam), according to the geographical origin and ethnic group in which they are frequently described [7,9].

On this basis, several agents such as 5-azacytidine and hydroxyurea, have been used in sickle cell anemia in order to rise HbF levels and decrease the severity of clinical manifestations [8,10,11]. However, hemoglobin gene switching is a process of sequential globin gene activation and inactivation, involving complex interactions of stage-specific transcription factors, chromosomal gene order, globin locus control region (LCR) gene proximity, a region characterized by a series of five DNase I-hypersensitive sites (5'-HS1-HS5), and erythroid-specific and ubiquitous trans-acting factors, interacting with the promoters regions of the β-globin gene cluster [9,12]. Of particular importance is the 5'-HS2-LCR enhancer that contains a core 46bp binding sequence for the NF-E2 and AP-1 proteins, flanked by multiple *cis*-acting sequences that modulate the enhancer activity, containing two TA repeat sequences and single nucleotide polymorphism (SNP) in the *cis*-acting sequences [13,14]. Some mutations in the γ-globin gene promoter regions are associated with increase of HbF synthesis, such as the polymorphism in the −158 position (C→T) at Gγ-globin gene promoter regions. This is related to increase the Gγ-globin chain synthesis and HbF levels described in Sen haplotype, as well as associated with a non-deletion hereditary persistence of fetal hemoglobin (HPFH) phenotype [8,15]. In this report, were associated the HbF levels, hematological data, phenotypic characteristics and sequence variation of Gγ and Aγ -globin genes promoter and HS2-LCR regions in a group of sickle cell anemia patients from the Northeast of Brazil.

MATERIALS AND METHODS

Patients

A total of 131 sickle cell anemia Brazilian patients (SS) from the Blood Center of Bahia (HEMOBA) were studied. Sixty-five were females and 66 males with ages varying from 01 to 73 years. Information about the clinical picture was obtained by patient record.

The study was approved by the Institutional Ethical Committee of the Gonçalo Moniz Research Center of FIOCRUZ – Bahia and an informed consent was signed for each patient or official responsible.

Hematological and Hemoglobin Analyses

Hematological analyses were carried out by electronic cell counter (Coulter T-890, Coulter Corporation, FL, USA). The hemoglobin profile and HbF level were investigated by High Performance Liquid Chromatography (HPLC – VARIANT I / BIO-RAD, CA, USA).

β^S - globin gene haplotypes

DNA was isolated from peripheral blood leukocytes by the GFX™ Genomic Blood DNA Purification KIT (Amersham Pharmacia Biotech, NJ, USA) and β^S – globin gene haplotypes were investigated by PCR and Restriction Fragment Length Polymorphism (RFLP) techniques. PCR products were digested by XmnI, HindIII, HincII, HinfI and HpaI restriction enzymes [16].

Asymmetric amplification and nucleotide sequencing

The Gγ and Aγ-globin genes promoter and the HS2-LCR regions were amplified asymmetrically and sequencing in an ABI Prism 3100 prism DNA Sequencer (Applied Biosystems, CA, USA) using Kit BigDye 03 ™Terminator Sequencing Standard (Applied Biosystems). The multiple sequence alignments of the Gγ (688 bp), Aγ (675 bp) genes promoter and LCR-HS2 (740 bp) regions were compared with GenBank database [17,18,19].

Statistical analysis

The statistical analyses were conducted using the EPI-INFO software, version 6.04. Statistical significance was established at $P \leq 0.05$.

RESULTS

One hundred and thirty one sickle cell anemia Brazilian patients (SS) were included in this study. Among the patients, 84 (64.1%) had hospitalizations; 74 (56.5%) received blood transfusion therapy and 50 (38.2%) had infections, mainly pneumonia and leg ulcers; 70 patients (53.4%) had painful episodes, 15 (11.4%) had spleen sequestration and six (4.6%) cerebrovascular accident (CVA).

The median age was 19.5 years (± 13.6); 66 (50.4%) patients were ≤ 18 years old and 65 (49.6%) were > 18 years. Among the patients ≤ 18 years old, the use of blood therapy and the presence of leg ulcer were lowest (p=0.02; p=0.0004, respectively) and HbF levels, number of the spleen sequestration and pneumonia events were higher (p=0.0006; p=0.03; p=0.0004, respectively) than patients > 18 years old.

The mean of HbF was 16.6% (± 6.7) between patients ≤ 5.0 years old; 10.9% (± 5.7) in patients > 5.0 and ≤ 10.0 years old; 8.9% (± 5.3) in patients >10.0 and ≤ 15.0 years old and 7.7% (± 4.5) in patients > 15 years old (p<0.00001). However, there was no HbF level variation between patients age > 15.0 years old.

The patients were classified in four groups according to HbF level. Group I was composed by 35 (26.7%) patients with HbF ≤ 5.0%; group II had 39 (29.8%) patients with HbF > 5.0% and ≤ 10.0%; group III had 32 (24.4%) patients with HbF > 10.0% ≤ 15.0% and group IV had 25 (19.1%) patients with HbF >15.0% [20].

Table 1 shows age, gender and hematological data in sickle cell anemia patients and table 2 shows the phenotypic characteristics distribution among the different sickle cell anemia patients groups.

The β^S- globin gene haplotypes analyses were developed in 125 patients and found 64 (51.2%) patients with genotype CAR/Ben; 36 (28.8%) Ben/Ben; 18 (14.4%) CAR/CAR; two

(1.6%) CAR/Aty; two (1.6%) Ben/Cam; one (0.8%) Car/Cam; one (0.8%) CAR/Arab-India and one (0.8%) Sen/Aty. The HbF level means was 9.2% (± 5.7) to CAR/Ben patients; 12.4% (± 6.5) to Ben/Ben; 7.5% (± 4.0) to CAR/CAR; 8.4% (± 1.8) to CAR/Aty; 22.2% (± 7.3) to Ben/Cam; 5.0% to CAR/Cam; 26.4% to CAR/Arab-India and 0.8% to Sen/Aty patient. Ten patients were selected who were carriers of β^S- globin gene haplotypes and HbF levels diversity, emphasizing previous reporters difference [6,21] and developed sequence analyses of Gγ and Aγ-globin genes promoter and the HS2-LCR regions (table 3).

DISCUSSION

The HbF is a known inhibitor of the HbS polymerization and is associated with decrease of clinical severity in sickle cell anemia patients [3,6]. Previous reports show a phenotypic heterogeneity of the sickle cell anemia patients and its associations with HbF levels; generally, patients with high HbF levels present less hemolysis, high hemoglobin concentration, an intermediary clinical picture and high survival rate [21]. Salzano (1985) [22] studied 409 SS Brazilian patients from the Southeast and observed a positive correlation between HbF level, Hb concentration and PCV, suggesting that patients with high HbF levels probably had less symptoms related to anemia. The present report found statistical correlation between HbF level, PCV and MCV, in accordance with Falusi & Kulozik (1990) [23], who studied sickle cell anemia patients from Nigeria. The data from this study is partially consistent with Borba *et al.* (2003) [24] who studied sickle cell anemia Brazilian patients undergoing hydroxyrea treatment and did not find any association between raise of HbF levels and Hb concentration, although demonstrating a significant correlation between MCV and HbF levels. The findings of this study also suggested an association among high HbF levels and low frequency leg ulcers, as discussed by Buchanan *et al.* (2004) [25]. In this report it is suggested that patients with high HbF levels have mild phenotypic characteristics, needing less blood therapy in accordance to previous reports that described a strong association between high HbF level and a better phenotype [5,8].

The HbF levels decreased with age, presenting the highest levels between patients ≤ 5 years old and the lowest in the age > 15.0 years old. These results did not agree with previous reports, that described a low HbF levels from childhood through adolescence and high levels among adult life [22,26].

Previous reports [20,21] have discussed the β^S-globin gene haplotypes and HbF levels and its role as a marker of the phenotypic heterogeneity in sickle cell anemia. Generally, patients with CAR haplotype have the lowest HbF levels, often below 5% and Ben haplotype carriers have intermediate HbF levels, from 5 to 15% [8,20,27]. This study found a high variation of HbF level between the studied sickle cell anemia patients group with uncommon association with β^S- globin gene haplotypes and description of a high HbF level in CAR haplotype carriers; low HbF levels in Ben carriers and two Ben/Cam patients with HbF levels higher than 15%.

Sawado *et al.* (2001) [28] suggested that β- globin gene locus has a constitutively open chromatin conformation before the terminal differentiation and have speculated that NF-E2 complex recruitment to LCR and active promoters may be a rate-limiting step in the activation of β-globin gene expression. This study hypothesized that sequences variation at Gγ and Aγ genes promoter and HS2-LCR regions may be an important factor in the establishment of the β^S- globin gene haplotypes, HbF levels and phenotypic characteristics diversity described among Northeastern Brazilian sickle cell anemia patients.

The presence of specific mutations have been associated with some β^S- globin gene haplotypes and an association between HbF level variability and HS2 enhancer polymorphism has been suggested [13,29,30,31]. Bordin *et al.* (2002) [32] analyzed 3'-HS site, located 20 kb downstream to the β-globin gene, that is involved in chromosomal organization, describing a TAA

insertion and suggested an association with Ben haplotype. A G→A substitution outside the core sequences of HS2-LCR (-10.677 position) was described in five sickle cell anemia patients with Ben haplotype and high HbF level; the CAR/Ben and Ben/Ben patients with a low HbF levels did not have this polymorphism; in the small group investigated, there are two CAR/CAR patients without this substitution and high HbF level. This finding suggests that this polymorphism site can play an important role on γ-globin gene transcription regulation in association only with Ben haplotype, probably by affecting interaction of binding site of specific *trans*-acting factor. The HS2-LCR polymorphisms report here is located at the proximity of a binding site of a GATA-1 and ubiquitous *trans*-acting factor and can constitute a motif associated to Ben chromosome. The other HS2-LCR polymorphisms described here have been related to specific haplotypes, in accordance to previous reports [13,14,33].

The analyses of Gγ and Aγ genes promoter regions confirm previous polymorphisms described by Lanclos *et al.* (1991) [15]. However, this study described a nucleotide substitution at –157 position (T→C) at Gγ gene promoter, which suggests a common characteristic of the Northeastern Brazilian sickle cell anemia patients, located at vicinity of another polymorphism (-158 C→T), associated with high γ-globin gene expression [15]. The Ben/Cam patients presented a 4bp deletion in -222 to -225 position of the Aγ-globin gene promoter region, that may be a *cis*-acting element that increases the expression of γ-globin gene when binding with a *trans*-acting factor, as described by Lu *et al.* (1996) [34]. Furthermore, the two patients had high HbF levels and presented another similar 4 bp deletion in –222 to –225 position of the Gγ-globin gene promoter region.

There are controversies about the role of HS2-LCR polymorphic sites and its contribution in the HbF levels variation in sickle cell anemia [8,15]. There are other elements that appear to have influence of the HbF regulation, such as a quantitative trait loci (QTL) on 6, 8 and X chromosomes [35,36]. More recently, Wyszynski *et al.* (2004) [37] found evidence for an association of single nucleotide polymorphisms (SNPs) bordering the QTL region located at 6q22.3-q23.2 and described an increase of HbF levels in sickle cell anemia patients. Ofori-Acquah *et al.* (2004) [38] suggested that, compared to HS2, polymorphism in the γ-globin gene promoter region exert a dominant influence on HbF level in sickle cell disease.

The present study confirms the phenotypic heterogeneity of Brazilian sickle cell anemia patients and further studies related to HbF level, Gγ and Aγ-globin genes and LCR regions and other biologic markers, need to be conducted, in order to clarify their real role in the phenotypic disease characteristics. The authors believe that new information about sequence variations, Gγ / Aγ ratio and β^S-globin gene haplotypes interactions comprehend an important key to the development of new drugs therapies, emphasizing the increase of γ gene expression. The sequence analyses of this study described a new polymorphic site on LCR-HS2 and Gγ-globin gene promoter regions that needs further investigation in order to establish a possible influence on γ-globin gene expression.

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Table 1. Fetal hemoglobin, age, gender and hematological characteristics among sickle cell anemia patients from Salvador - Bahia - Brazil

HbF Groups	HbF, % (Mean ± SD)	Age, Years (Median ± SD)	Gender F female M-male	Hb, g/dL (Mean ± SD)	PCV, decimal fraction (Mean ± SD)	MCV, fL (Mean ± SD)	MCH, pg (Mean ± SD)	MCHC, g/dL (Mean ± SD)
Group I (n=35)	3.1 ±1.3	21 ± 10.9	F 15 M 20	7.6 ± 1.7	24.8 ± 0.6	88.9 ± 9.2	27.6 ± 4.7	30.9 ± 3.4
Group II (n=39)	7.4±1.7	21 ± 11.8	F 21 M 18	7.1 ± 1.7	23.0 ± 0.5	98.4 ± 9.1	30.4 ± 3.8	30.9 ± 2.8
Group III (n=32)	12.2±1.5	21 ± 16.9	F 20 M 12	7.9 ± 1.4	25.4 ± 0.4	95.8 ± 12.8	30.2 ± 4.9	31.5 ± 2.3
Group IV (n=25)	19.7±4.0	4 ± 8.1	F 9 M 16	8.6 ±1.3	28.3 ± 0.4	89.2 ± 9.7	27.2 ± 4.9	30.4 ± 2.2
p-Value	< **0.00001***	< **0.00001***	0.18 †	0.11 **	**0.04 ** **	**0.02 ** **	0.09 **	0.81 **

* Kruskal-Wallis H ** ANOVA † X^2 Test

Analyses by Fisher test demonstrated difference between group II / IV to Hb (p=0.02), PCV (p=0.005), MCV (p=0.02), MCH (p=0.04); group I / II to MCV (p=0.004) and III / IV to PCV(p=0.04)

Table 2. Fetal hemoglobin and phenotypic characteristics among sickle cell anemia patients from Salvador - Bahia - Brazil

HbF Groups	Hospitalization, n/total (%)	Blood Transfusion, n/total (%)	Pneumonia, n/total (%)	Leg ulcers, n /total (%)	Spleen sequestration, n/total (%)	Cerebrovascular accident, n/total (%)	Painful episodes, n/total (%)
Group I (n=35)	21 / 35 (60.0)	23 / 35 (65.7)	06 / 35 (17.1)	10 / 35 (28.6)	02 / 35 (5.7)	03 / 35 (5.7)	20 / 35 (57.1)
Group II (n=39)	24 / 39 (61.5)	26 / 39 (66.7)	05 / 39 (12.8)	06 / 39 (15.4)	04 / 39 (10.3)	01 / 39 (2.6)	19 / 39 (30.8)
Group III (n=32)	22 / 32 (56.2)	18 / 32 (56.2)	07 / 32 (21.9)	06 / 32 (18.8)	04 / 32 (12.5)	02 / 32 (6.25)	18 / 32 (56.2)
Group IV (n=25)	17 / 25 (68.0)	07 / 25 (28.0)	07 / 25 (28.0)	00 / 25	05 / 25 (20.0)	00 / 25	13 / 25 (52.0)
p-Value	0.84	**0.01** †	0.47 †	**0.03** †	0.39 †	0.39 †	0.92 †

* Kruskal-Wallis H ** ANOVA † X^2 Test

Analyses by Fisher test demonstrated difference between group I / IV to use of blood transfusion (p=0.008) and leg ulcers (p=0.003); group II / IV to use of blood transfusion (p=0.006) and III / IV to leg ulcers (p=0.03).

Table 3. Summary of the difference among HbF levels, Haplotypes and Polymorphic *cis*-Acting sequences found among sickle cell anemia patients from Salvador - Bahia - Brazil

A

			HS 2				
N^0	HbF	β-^{S} Haplotype	-10,924	-10,905	-10,677	-10,623 -10,570	-10,390
01	27,4	Cam/Ben	T/G	A/G	***G/A**	8(TA)N12(TA) / 8(TA)nTG7(TA)	A/T
02	17,1	Cam/Ben	T/G	A/G	***G/A**	8(TA)N12(TA) / 8(TA)nTG7(TA)	A/T
03	9,6	CAR/Aty	T/T	A/A	G/G	8(TA)N11(TA) / 8(TA)N10(TA)	A/T
04	1,2	CAR/Ben	T/G	A/G	G/G	8(TA)N11(TA) / 8(TA)nTG7(TA)	A/T
05	1,3	Ben/Ben	G/G	G/G	G/G	8 (TA)nTG7(TA) / 8(TA)nTG7(TA)	T/T
06	10,1	CAR/Ben	T/G	A/G	***G/A**	8(TA)N11(TA) / 8(TA)nTG7(TA)	A/T
07	17,6	CAR/Ben	T/G	A/G	***G/A**	8(TA)N11(TA) / 8(TA)nTG7(TA)	A/T
08	8,7	CAR/CAR	T/T	A/A	G/G	8(TA)N11(TA) / 8(TA)N11(TA)	A/A
09	14,9	CAR/CAR	T/T	A/A	G/G	8(TA)N11(TA) / 8(TA)N11(TA)	A/A
10	11,5	Ben/Ben	G/G	G/G	***G/A**	8(TA)nTG7(TA) / 8(TA)nTG7(TA)	T/T

B

			Gγ						Aγ			
N^0	HbF	β-^{S} Haplotype	-533 - 532	-403 - 390	-369	-309	-225 - 222	-157	-369	-271	-225 - 222	+25
01	27,4	Cam/Ben	I / I	N/N	G/C	G/A	***N/d**	***C/C**	G/G	C/C	N/d	G/G
02	17,1	Cam/Ben	I / I	N/N	G/C	G/A	***N/d**	***C/C**	G/G	C/C	N/d	G/G
03	9,6	CAR/Aty	I / I	N/D	G/C	G/A	N/N	***C/C**	G/G	C/T	N/N	G/G
04	1,2	CAR/Ben	I / I	N/D	G/C	G/A	N/N	***C/C**	G/G	C/T	N/N	G/G
05	1,3	Ben/Ben	I / I	N/N	G/G	G/G	N/N	***C/C**	G/G	C/C	N/N	G/G
06	10,1	CAR/Ben	I / I	N/D	G/C	G/A	N/N	***C/C**	G/G	C/T	N/N	G/G
07	17,6	CAR/Ben	I / I	N/D	G/C	G/A	N/N	***C/C**	G/G	C/T	N/N	G/G
08	8,7	CAR/CAR	I / I	D/D	C/C	A/A	N/N	***C/C**	G/G	T/T	N/N	G/G
09	14,9	CAR/CAR	I / I	D/D	C/C	A/A	N/N	***C/C**	G/G	T/T	N/N	G/G
10	11,5	Ben/Ben	I / I	N/N	G/G	G/G	N/N	***C/C**	G/G	C/C	N/N	G/G

3A. In the HS 2, N = CACATATACG and n= CACATATACGTG.

3B. In the Gγ gene promoter region, I= change AGA to AAG in the -533 and -532; N = reference sequence; D = 6 bp deletion between -400 and -395 e d = 4 bp deletion between -222 and -225; in the Aγ gene promoter region, N = reference sequence, d = 4 bp deletion between -222 and -225.

*Sequence variations described only in patients investigated at the present work.

A anemia falciforme apresenta distribuição mundial ampla, com incidência elevada entre países Africanos, onde alcança a freqüência gênica de 0,12 a 0,14 na África Centro-Ocidental e de 0,08 a 0,10 no Senegal e África Atlântico-Ocidental, ocorrendo em freqüência menor entre os países do Mediterrâneo, principalmente Grécia, Itália e Israel, assim como na Arábia Saudita e Índia (NAGEL & STEINBERG, 2001; COSTA, 2001). Nos Estados Unidos da América, estima-se que um a cada 625 nascimentos seja portador da anemia falciforme; na América do Sul, a anemia falciforme é considerada um problema de saúde pública entre descendentes de africanos, uma vez que a HbS foi historicamente trazida para esta região durante a imigração de africanos (WANG & LUKENS, 1998; NAGEL & STEINBERG, 2001).

ALVARES FILHO et al. (1995) descreveram a distribuição geográfica da HbS no Brasil, encontrando freqüências menores entre as populações da região Sul e Sudeste (2,71% e 2,35%, respectivamente) e maiores nas regiões Norte e Nordeste (5,93% e 6,13%, respectivamente), sendo os estados do Pará, Bahia e Piauí, os que apresentaram prevalência maior da anemia falciforme (0,09%; 0,06% e 0,05%, respectivamente); além disso, foram observadas variações na freqüência da HbS em uma mesma região, com diferenças marcantes na região Nordeste. Estes resultados confirmaram as observações realizadas por AZEVÊDO (1973) que descreveu a heterogeneidade da população brasileira, com base na participação ativa da imigração ocorrida, principalmente durante o período de 1850 a 1950, que contribuiu com a vinda de mais de cinco milhões de indivíduos de origem africana e européia.

Cumpre ressaltar que um dos fatores mais marcantes na história do Brasil foi a grande influência africana, fazendo-se presente de forma mais ou menos intensa, a

depender da região do país e da diversidade étnica dos imigrantes, confirmando as observações realizadas por KRIEGER et al. (1965), que descreveram um grau de panmixia de raças em aproximadamente 97% na população do Nordeste brasileiro. Esta miscigenação racial também foi documentada por AZEVÊDO et al. (1981), em um estudo realizado na Ilha de Itaparica, situada na Baía de Todos os Santos a 12Km de Salvador, onde ficou evidenciando o crescimento da população de mulatos, acompanhado pelo decréscimo dos brancos, reduzidos a um terço no período de 20 anos.

No presente estudo foram investigados os níveis de HbF, a presença de talassemia $\alpha_2^{-3,7Kb}$, os tipos de haplótipos ligados ao grupo de genes da globina β^S e variações na seqüência de nucleotídeos das regiões promotoras dos genes γG e γA e do sítio HS2-LCR, considerados como fatores genéticos moduladores da anemia falciforme.

Na primeira fase deste estudo, 160 cromossomos β^S foram analisados, tendo sido encontrada uma freqüência ligeiramente maior do haplótipo Ben (48,8%), quando comparada ao haplótipo CAR (46,2%). Além disso, foram encontrados 1,9% do haplótipo Cam, 0,6% do Índia-Arábia Saudita e 0,6% do Sen, com freqüência de 54,0% para o genótipo CAR/Ben (ADORNO et al., 2004). Estes dados confirmam os resultados obtidos por GONÇALVES et al. (2003), que estudaram haplótipos ligados ao grupo de gene da globina β^S em pacientes com anemia falciforme de Salvador-BA, descrevendo 48,1% de freqüência para o haplótipo CAR e 45,6% para o Ben, com predominância do genótipo CAR/Ben (46,3%). Além disso, os dados encontrados no presente trabalho confirmam as informações referentes à imigração de Africanos para a região Nordeste brasileira, principalmente para a Bahia, que recebeu indivíduos vindos da região Centro-Ocidental da África (Baía de Benin e Baía de Biafra) até a metade do século XIX, justificando as

freqüências equivalentes dos haplótipos CAR e Ben na região (VERGER, 1968; FLORENTINO, 1997).

Entretanto, quando comparados a outras regiões brasileiras, os resultados do nosso estudo apresentam diferenças quanto ao tipo de haplótipo predominante. ZAGO et al. (1992) estudaram 37 indivíduos negróides portadores do gene β^S da região de São Paulo, descrevendo predominância do haplótipo CAR (66,2%) em relação ao Ben (23,0%); o mesmo foi observado por GONCALVES et al. (1994), quando estudaram pacientes com anemia falciforme de São Paulo e por LYRA et al., (2005), que descreveram o predomínio do haplótipo Ben em Salvador e do haplótipo CAR em São Paulo, em um estudo comparativo entre os portadores de anemia falciforme das duas regiões. Na região Amazônica, norte do país, em um estudo desenvolvido em comunidades descendentes de africanos portadoras do gene β^S, foram descritas as freqüências de 60% para o haplótipo CAR; 30% para o Sen e 10% para o Ben; em Belém foi descrita a freqüência de 86% para o haplótipo CAR; 9% para o Ben e 4% para o Sen entre pacientes portadores da anemia falciforme (PANTE-DE-SOUSA et al., 1998; 1999). Estes resultados confirmam a heterogeneidade étnica existente no Brasil, decorrente principalmente da imigração intensa de africanos para o país. Ressaltamos que diferente do nordeste, a região Norte recebeu uma contribuição maior de africanos originários do Congo, Moçambique e Angola, onde existe o predomínio do haplótipo CAR, bem como de regiões da África Atlântico-Ocidental, onde predomina o haplótipo Sen (FLORENTINO, 1997).

Os dados relativos aos haplótipos do grupo de genes da globina β^S descritos neste trabalho também foram diferentes daqueles descritos em outros países da América; os Estados Unidos e a Jamaica receberam imigrantes da África Centro-Ocidental, onde o

haplótipo Ben é mais freqüente; no entanto, o México, Colômbia, algumas regiões da Venezuela e Porto-Rico apresentam freqüência maior do haplótipo CAR, sugerindo diferentes origens africanas para estas populações (ARENDS et al., 2000; CUELLAR-AMBROSI et al., 2000; VIVENES DE LUGO et al. 2003; MAGAÑA et al., 2003). Além disso, o encontro dos haplótipos Cam, Saudi e Sen neste estudo, poderia ser justificado pela inexistência de dados consistentes a respeito do tráfico de escravos no período de 1820 a 1830, onde um total de 8.000 africanos de origem desconhecida foi trazido para a Bahia (CURTIN, 1969). Desta forma, os dados referentes ao estudo dos haplótipos ligados ao grupo de genes da globina β^S poderão contribuir para a investigação da rota realizada pelos africanos, além de fornecer esclarecimentos relativos a este grupo de imigrantes no estado da Bahia.

A hemoglobina fetal é um dos moduladores genéticos da anemia falciforme mais extensivamente estudado, o qual parece afetar o curso clínico da doença. Inicialmente, achava-se que somente os níveis elevados de HbF fossem capazes de influenciar o fenótipo da anemia falciforme (POWARS et al. 1984; POWARS et al. 1994). Estudo posterior demonstrou que qualquer aumento nos níveis de HbF poderia interferir clinicamente ou até terapeuticamente na doença (PLATT et al., 1991). RAHGOZAR et al. (2000) descreveram que pacientes SS assintomáticos do Iran apresentavam níveis elevados de HbF. Entretanto, INATI et al. (2003), estudando 50 pacientes com doença falciforme do Líbano, encontraram associação entre níveis aumentados de HbF e o fenótipo grave.

Em nosso estudo avaliamos os níveis de HbF em 131 portadores de anemia falciforme e observamos que os indivíduos com níveis mais elevados desenvolviam menos freqüentemente úlceras de membros inferiores e tinham idade $\leq$ a 18 anos, como discutido

por BUCHANAN et al. (2004). Entretanto, estes dados são diferentes daqueles obtidos por SALZANO (1985) em estudo desenvolvido em pacientes do Rio de Janeiro, que demonstrou uma correlação positiva entre os níveis de HbF e a ocorrência de úlceras de membros inferiores.

Os dados relacionados ao uso diminuído de terapia transfusional em portador de anemia falciforme com HbF aumentada e idade ≤ 18 anos são consistentes com estudos anteriores (ODENHEIMER et al., 1987; MOUELE et al., 1999; STEINBERG, 2001). No entanto, a análise cuidadosa destes resultados, evidencia a necessidade de avaliar um número maior de pacientes, com a finalidade de confirmar a presença de um curso clínico menos grave da doença na população pediátrica ou a menor utilização desta modalidade de terapia no serviço ambulatorial onde os pacientes foram selecionados.

Todavia, nós não encontramos nenhuma outra associação entre níveis elevados de HbF e a redução de eventos clínicos no grupo de pacientes investigados, apesar de outros trabalhos terem descrito associações com a redução dos episódios de crise de dor e do número de hospitalizações (ODENHEIMER et al., 1987; PLATT et al., 1991). Desta forma, nossos resultados sugerem que a HbF é um fator importante, mas não o único a afetar o fenótipo dos portadores da anemia falciforme em Salvador-BA.

A análise realizada entre HbF e os dados hematológicos, demonstrou correlação positiva para os valores de Ht e VCM, indicando níveis maiores entre os pacientes portadores de níveis elevados de HbF. Além disso, foi observada uma tendência para o aumento da concentração de Hb e valor de HCM entre os indivíduos com níveis mais elevados de HbF, embora sem significância estatística, confirmando os achados de trabalhos anteriores (SALZANO,1985; FALUSI et al., 1990). Quando analisados o gênero

e os níveis de HbF, não foram encontradas diferenças, como descrito por STEINBERG et al. (1995). Entretanto, CHANG et al. (1997) e STEINBERG (2001) sugeriram que as mulheres apresentam valores aumentados de HbF em relação aos homens, devido a influência do cromossomo Xp22.2.

Os níveis de HbF reduziram com a idade, apresentando valores elevados entre os pacientes com idade ≤ a 5 anos, diminuindo entre os > de 15 anos de idade. Estes achados diferem dos descritos por SALZANO (1985) e MOUELE et al. (1999), quando sugeriram que os valores de HbF tendem a diminuir até a adolescência, voltando a aumentar na vida adulta.

No presente estudo foi encontrada uma prevalência elevada de pneumonia e seqüestro esplênico entre os portadores de anemia falciforme com idade ≤ a 18,0 anos, independente do nível de HbF apresentado, concordando com estudos prévios (KOKO et al., 1998; OVERTURF, 2003; DI NUZZO & FONSECA, 2004) e confirmando a ausência de correlação entre os níveis de HbF e a ocorrência destes eventos. Além disso, 6,1% dos indivíduos com idade > que 18,0 anos apresentaram pneumonia, sugerindo que os riscos inerentes a esta patologia persistem na idade adulta, confirmando a necessidade de prevenção contínua, como sugerido por LESPRIT & LESPRIT (2004).

A análise da talassemia $\alpha_2^{3,7kb}$ foi realizada em 110 pacientes, tendo sido encontrado 27,3% de portadores em heterozigose e 1,8% em homozigose, resultados superiores aos descritos por FIGUEIREDO et al. (1996) que encontraram a freqüência de 17,6% de heterozigose e 1,2% de homozigose para a talassemia $\alpha_2^{3,7kb}$ em portadores de anemia falciforme de São Paulo. Os dados encontrados no presente estudo também confirmam os

resultados encontrados por LYRA et al., (2005), que descreveram a freqüência de 28,2% para a talassemia $\alpha_2^{3,7kb}$ entre indivíduos portadores de anemia falciforme de Salvador-BA.

A talassemia alfa é considerada um dos fatores genéticos associado à melhoria do fenótipo do portador da anemia falciforme, com redução dos eventos de hemólise, aumento da concentração de Hb e do Ht, diminuição do valor do VCM, da contagem de reticulócitos e redução de úlceras de membros inferiores; entretanto, o aumento da viscosidade sangüínea pode conduzir mais freqüentemente à ocorrência de eventos vaso-oclusivos e crises de dor (NAGEL & STEINBERG, 2001).

Neste estudo encontramos correlação entre a presença de talassemia alfa e os valores hematológicos, com redução do HCM e do VCM, elevação da concentração de Hb e do valor de Ht; embora sem significância estatística, os eventos de crises de dor e seqüestro esplênico foram maiores entre os portadores de talassemia, ao contrário da ocorrência de AVC e úlceras de membros inferiores, em concordância com trabalhos anteriores (FIGUEIREDO et al., 1996; MUKHERJEE et al., 1997).

Diversos autores têm discutido sobre a influência dos haplótipos ligados ao grupo de genes da globina β^S como marcador da heterogeneidade fenotípica da anemia falciforme (POWARS & HITI, 1993; INATI et al., 2003). O haplótipo CAR tem sido freqüentemente associado a níveis diminuídos de HbF (< 5%) e a um fenótipo mais grave; o haplótipo Ben a níveis intermediários desta hemoglobina (5% a 15%) e a uma clínica menos grave que o CAR (POWARS, 1991; STEINBERG, 2001).

Os nossos resultados demonstraram que os portadores de anemia falciforme com o genótipo CAR/Ben apresentavam níveis de HbF intermediários e os Ben/Ben níveis maiores, quando comparados aos indivíduos CAR/CAR. Cumpre ressaltar que em todos os

tipos de haplótipos descritos, os níveis de HbF encontravam-se acima dos valores descritos em outros países e em outras regiões do Brasil (COSTA et al., 1994; FIGUEIREDO et al., 1996; NAGEL & STEINBERG, 2001), confirmando os dados obtidos por GONCALVES et al. (2003), que estudaram pacientes com anemia falciforme de Salvador e identificaram comportamento heterogêneo entre o tipo de haplótipo e sua correlação com os níveis de HbF. Dados similares foram descritos por MOUELE et al. (1999) que encontraram níveis elevados de HbF entre indivíduos CAR/CAR do Congo.

Os indivíduos portadores dos genótipos CAR/Ben e Ben/Ben descritos no presente estudo que apresentaram níveis de HbF mais elevados, possuíam menor idade e utilizaram menos terapia transfusional; entretanto, o uso de terapia transfusional nos indivíduos CAR/CAR foi independente da idade e do nível de HbF, podendo indicar um quadro clínico mais grave entre os portadores deste haplótipo. Além disso, todos os eventos de AVC ocorridos na população estudada estiveram associados à presença do haplótipo CAR, como previamente discutido por SARNAIK & BALLAS (2001).

Também foi observado no presente estudo, que os pacientes com um mesmo tipo de haplótipo apresentavam uma variação significativa nos níveis de HbF, principalmente entre os pacientes CAR/Ben e Ben/Ben, sendo que quatro indivíduos entre os 18 CAR/CAR, apresentaram HbF > 10.0%, confirmando a hipótese de que outros fatores não associados ao gene da globina β^S podem estar relacionados aos níveis de HbF nesta população (INATI et al., 2003).

O seqüenciamento do DNA das regiões promotoras dos genes γG e γA e do LCR-HS2 foi realizado em dez pacientes selecionados de acordo com o tipo de genótipo para o gene da globina β^S e o nível de HbF. Diversas variações na seqüência de nucleotídeos da

região promotora dos genes γG e γA têm sido associadas ao tipo de haplótipo; os pacientes com haplótipo CAR apresentam alteração na posição –271 da região promotora do gene γA (C→T), além da deleção de seis pares de bases (CTTTAA) na posição –395 a - 400 da região promotora do gene γG, descrita em aproximadamente 40% dos portadores deste mesmo haplótipo (STEINBERG, 2001). O haplótipo Ben está associado à troca de seqüências na posição –309 (C→G) e -369 (C→G) da região promotora do gene γG; o haplótipo Cam apresenta a deleção de quatro pares de bases (AGCA) localizada entre as posições –222 a - 225 na região promotora do gene γA (LANCLOS et al., 1991; ONER et al.; 1992; LU & STEINBERG, 1996).

No presente estudo, os polimorfismos encontrados nestas posições confirmaram os achados prévios de LANCLOS et al. (1991) e ONER et al. (1992). Entretanto, descrevemos a deleção AGCA na posição –222 a - 225 na região promotora do gene γG em indivíduos com haplótipo Cam. Além disso, identificamos a substituição T→ C na posição -157 da região promotora do gene γG, presente em diferentes haplótipos, podendo significar uma característica própria da população estudada. Embora, OFORI-ACQUAH et al. (2004) tenham sugerido que a influência maior sobre os níveis de HbF em portadores de anemia falciforme deva estar mais associada a polimorfismos localizados em seqüências da região promotora dos genes γ que em alterações no HS2-LCR, não encontramos correlação entre a presença de polimorfismos específicos nas regiões promotoras dos genes γ, quando associamos ao tipo de haplótipo ligado ao grupo de genes da globina β^S e a diferenças nas concentrações da HbF.

A região controladora do locus da globina β (LCR) tem sido associada a alterações na expressão do gene γ, sendo primeiramente descrita por apresentar sítios hipersensíveis à

ação da DNase I (HS). Os segmentos de 1 a 5 (HS-1 a 5) da LCR contêm elementos *Cis* múltiplos, além de apresentar polimorfismos que atuam como sítios de ligação para fatores transcricionais, tais como NF-E2, AP-1, GATA-1, USF, YY1, também apresentam duas seqüências repetidas TA no HS2-LCR, onde se ligam os fatores HOX-B2 (ONER et al., 1992; OFORI-ACQUAH et al. 2001, STAMATOYANNOPOULOS, 2005).

Entretanto, a associação entre polimorfismos no HS2-LCR e níveis de HbF ainda é controversa; ADEKILE et al. (1992) sugeriram que os haplótipos ligados ao grupo de genes da globina β^S estão relacionados à seqüências específicas do HS2-LCR que contem determinantes genéticos que podem resultar no aumento da expressão do gene γ da globina. Trabalhos mais recentes têm indicado que embora os níveis de HbF estejam associados à atividade *enhancer* do HS2-LCR e a variações na seqüência de nucleotídeos desta região, experimentos envolvendo clonagem e expressão em genes repórteres, ainda não confirmaram a correlação entre o nível de HbF e o tipo de haplótipo ligado ao grupo de genes da globina β^S, sugerindo que outro fator pode estar associado às diferenças nos níveis de HbF exibidas pelos portadores da anemia falciforme (PLONCZYNSKI et al. 1997; OFFORI-ACQUAH et al., 1999; SAMAKOGLU et al., 1999; OFFORI-ACQUAH et al., 2001).

A análise do sítio HS2-LCR confirmou a existência de variações na seqüência de nucleotídeos desta região e sua associação ao tipo de haplótipo ligado ao grupo de genes da globina β^S. A substituição T→ G na posição -10.924 e de A→ G na posição -10.905 estão presentes no haplótipo Ben, sendo que esta última modificação cria um sítio de ligação para o fator transcricional Sp1. A alteração A→ T na posição –10.390 cria um sítio para o H4TF-1, que também está presente na região promotora do gene histona H4, interferindo

com a sua expressão; entretanto, a função deste sítio na expressão de genes eritróides ainda não está clara, uma vez que a remoção desta seqüência parece não interferir com a atividade *enhancer* do segmento remanescente (ONER et al., 1992; OFORI-ACQUAH et al. 1999). A análise das seqüências repetidas TA também se mostrou compatível com o haplótipo Ben (CACATATACG → CACATATACG**TG**) (ONER et al., 1992). Além destes polimorfismos previamente identificados, descrevemos a substituição G→A na posição –10.677 do HS2-LCR em portadores do haplótipo Ben; a mudança de nucleotídeos está localizada próximo aos sítios de ligação do fator *trans* GATA-1 e de fatores ubíquos, sugerindo uma possível associação entre o polimorfismo, a expressão dos genes γ e a síntese da HbF.

Recentemente, além dos genes γ e do LCR, alguns estudos têm demonstrado outros possíveis elementos importantes na regulação da síntese da HbF. GARNER et al. (2002) sugeriram que a interação entre o sítio XmnI localizado na região promotora do gene γG e o QTL no braço longo do cromossomo 8 (8q) pode influenciar na produção da HbF, uma vez que o QTL 8q parece codificar um fator regulatório ou uma subunidade, que pode agir como ativador transcricional capaz de se ligar no sítio XmnI, modulando desta forma, a expressão do gene γG e alterando a síntese da HbF. WISZINSKI et al. (2004) demonstraram que alguns polimorfismos próximos ao QTL no braço longo do cromossomo 6 (6q) também parecem estar associados ao aumento da concentração de HbF.

No presente estudo encontramos a substituição G→ A na posição –10.677 da HS2-LCR, que foi descrita apenas nos indivíduos com haplótipo Ben e níveis elevados de HbF; os indivíduos portadores deste haplótipo e níveis diminuídos de HbF não apresentaram este polimorfismo. Desta forma, estudos de clonagem e expressão gênica envolvendo os

polimorfismos novos descritos deverão ser realizados com o objetivo de compreender melhor os mecanismos moleculares envolvidos e um possível papel na modulação das manifestações clínicas apresentadas pelos portadores da anemia falciforme de Salvador-Bahia.

No presente estudo foi realizada a investigação de algumas características fenotípicas, de marcadores moleculares e de possíveis variações nas seqüências de nucleotídeos das regiões promotoras dos genes γ e do HS2-LCR em portadores da anemia falciforme de Salvador-BA.

Assim, o presente trabalho levou às seguintes conclusões:

1. Os portadores de anemia falciforme em idade pediátrica apresentaram níveis mais elevados de HbF, utilizaram menos terapia transfusional e desenvolveram menos freqüentemente úlceras de membros inferiores;
2. A determinação dos haplótipos ligados ao grupo de genes da globina β^S confirmou a presença de freqüência elevada do tipo Ben, seguido do haplótipo CAR, com predomínio do genótipo CAR/Ben. Foram identificados, pela primeira vez, os haplótipos Camarões (Cam) e Arábia Saudita-Índia (Saudi) e confirmada a presença do haplótipo Senegal (Sen) em portadores da anemia falciforme de Salvador-BA;
3. A talassemia $\alpha_2^{3.7Kb}$ foi identificada em 32 pacientes, sendo 30 (27.3%) heterozigotos e dois (1.8%) homozigotos;
4. A presença da talassemia $\alpha_2^{3,7Kb}$ modificou significativamente os valores hematológicos, aumentando os níveis de Hb e Ht e reduzindo os valores de VCM e HCM, porém não influenciou as características fenotípicas avaliadas;
5. Os portadores do genótipo Ben\Ben apresentaram níveis mais elevados de HbF quando comparados aos CAR/Ben e CAR/CAR;

6. Portadores dos genótipos CAR/Ben e Ben/Ben que possuíam menor idade, apresentaram níveis mais elevados de HbF e utilizaram menos terapia transfusional. Entretanto, o uso de terapia transfusional nos indivíduos CAR/CAR foi independente da idade e do nível de HbF, podendo indicar um quadro clínico mais grave entre os portadores deste haplótipo;
7. A análise de seqüências do HS2-LCR e das regiões promotoras dos genes γG e γA confirmou os polimorfismos descritos anteriormente e sua associação com tipos específicos de haplótipos ligados ao grupo de genes da globina β^S;
8. Foi descrita a substituição T→C na posição -157 da região promotora do gene γG, caracterizada como independente do tipo de haplótipo, sugerindo ser uma substituição associada à doença; também foi descrita a deleção AGCA entre as posições -222 a -225 do mesmo gene, provavelmente associada ao haplótipo Cam;
9. Foi descrita a substituição G→A na posição -10.677 do HS2-LCR, provavelmente associada ao haplótipo Ben e níveis elevados de HbF.

Desta forma, os resultados confirmam a heterogeneidade fenotípica e genotípica dos portadores da anemia falciforme de Salvador-BA, justificando a realização de estudos adicionais que investiguem o papel da HbF e de marcadores biológicos no desenvolvimento clínico da doença, bem como a possível correlação entre a variação nas seqüências gênicas encontradas na região promotora dos genes γ e no HS2-LCR e o fenótipo descrito nos pacientes que fizeram parte da casuística deste estudo.

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O presente estudo contribuiu com dados relevantes relacionados à diversidade fenotípica e genotípica presentes em portadores de anemia falciforme de Salvador-Bahia.

Como resultado do estudo descrevemos pela primeira vez o encontro dos haplótipos Camarões e Arábia Sadita-Índia em Salvador-Bahia, a substituição G→A na posição -10.677 do HS2-LCR, associada ao haplótipo Ben e níveis elevados de hemoglobina fetal em cinco pacientes portadores de anemia falciforme investigados, a deleção AGCA na posição -222 a -225 na região promotora do gene γG e a substituição T→C na posição -157 na região promotora do mesmo gene.

Baseados nestes achados julgamos necessária a investigação das seqüências de nucleotídeos nas regiões promotoras dos genes γG e γA e do sítio LCR-HS2, bem como de outras regiões consideradas de interesse na síntese da HbF em um número maior de pacientes. Também serão realizados ensaios de expressão, utilizando construções de plasmídeos que contenham as seqüências descritas e que possam potencialmente exercer alteração na expressão dos genes γ.

Seguindo a estratégia de clonagem e expressão gênicas, já foram realizados alguns experimentos utilizando células da linhagem K562, as quais têm sido empregadas como modelo para estudo da regulação da síntese da heglobina fetal/embriogênica, maturação eritróide e expressão gênica, incluindo estudo de fatores transcricionais importantes para a expressão de genes da globina γ (YOUNG et al., 1984; OFFORI-ACQUAH et al., 2001). A linhagem celular K562 é proveniente de pacientes portadores de leucemia mielóide crônica (LMC) em crise blástica, podendo diferenciar-se na linhagem eritróide por exposição a diversos agentes farmacológicos, tais como hemina, antibóticos, derivados de butiratos e retinóides (ANDERSSON et al., 1979; TOFFOLI et al., 1989; DEGOS et al., 1995).

Entretanto, GOLIAEI et al. (2004) descreveram o uso da hipertermia como indutor de células de eritroleucemia humana. Assim, nós avaliamos a utilização da hipertermia em células K562 transfectadas, como um possível modelo para demonstrar o efeito potencializador de construções contendo seqüências específicas de LCR-HS2.

A construção do plasmídeo foi realizada a partir da seqüência LCR-HS2 obtida de dois indivíduos portadores de anemia falciforme, um apresentando o genótipo CAR/Ben (P1) e o outro o Ben/Ben (P2). Após extração do DNA genômico e obtenção da região HS2-LCR pela técnica da reação da polimerase em cadeia (PCR), os produtos obtidos foram purificados pelo QIAquick Kit (Qiagen, Hilden, Germany) e clonados no vetor pCR2.1 TOPO (Invitrogen, Life Technologies) (Figura 1), para posteriormente serem transformados na bactéria Top 10 (Invitrogen), uma cepa de *Escherichia coli* quimicamente competente.

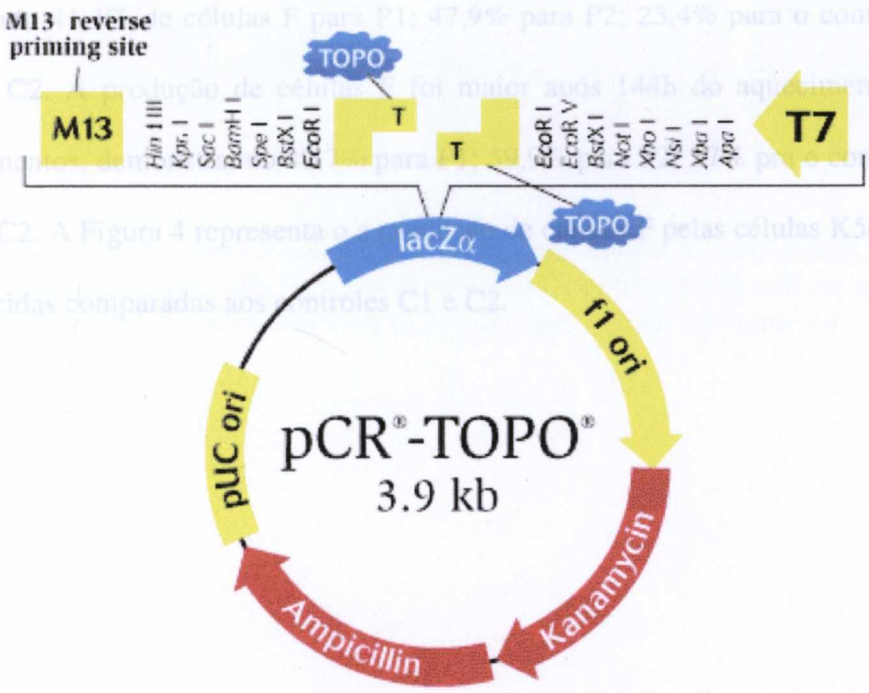

Figura 1. Esquema do Vetor pCR2.1TOPO utilizado para clonagem da região LCR-HS2.

A análise da expressão endógena dos genes γ foi realizada pela transfecção de células da linhagem K562 com os plasmídeos obtidos na clonagem, utilizando-se a técnica de lipofecção (Lipofectamine / Invitrogen-Life Technologies). Após 24h de incubação, as células transfectadas e as células controle não transfectadas (C1) foram submetidas à hipertermia (42°C) por 180min. Paralelamente, foi realizado um controle constituído de células K562 (C2) sem aquecimento (37°C, 5% de CO2).

A diferenciação eritróide das células K562 transfectadas e controles foi avaliada através de centrifugação em citospin e coloração das lâminas pelas técnicas de Kleihauer e Wright (DACIE & LEWIS, 1984), evidenciando a presença de células fetais (células F) (Figura 2) e precursores eritróides (Figura 3), respectivamente.

A estimativa da produção de células F foi obtida pela contagem de células F em função do total de células (células F + células não-F). Após 48h de aquecimento, foi observado 41,4% de células F para P1; 47,9% para P2; 23,4% para o controle C1 e 2,2% para o C2. A produção de células F foi maior após 144h do aquecimento em todos os experimentos, demonstrando 49,7% para P1; 59,9% para P2; 37% pra o controle C1 e 8,6% para o C2. A Figura 4 representa o a produção de células F pelas células K562 transfectadas e aquecidas comparadas aos controles C1 e C2.

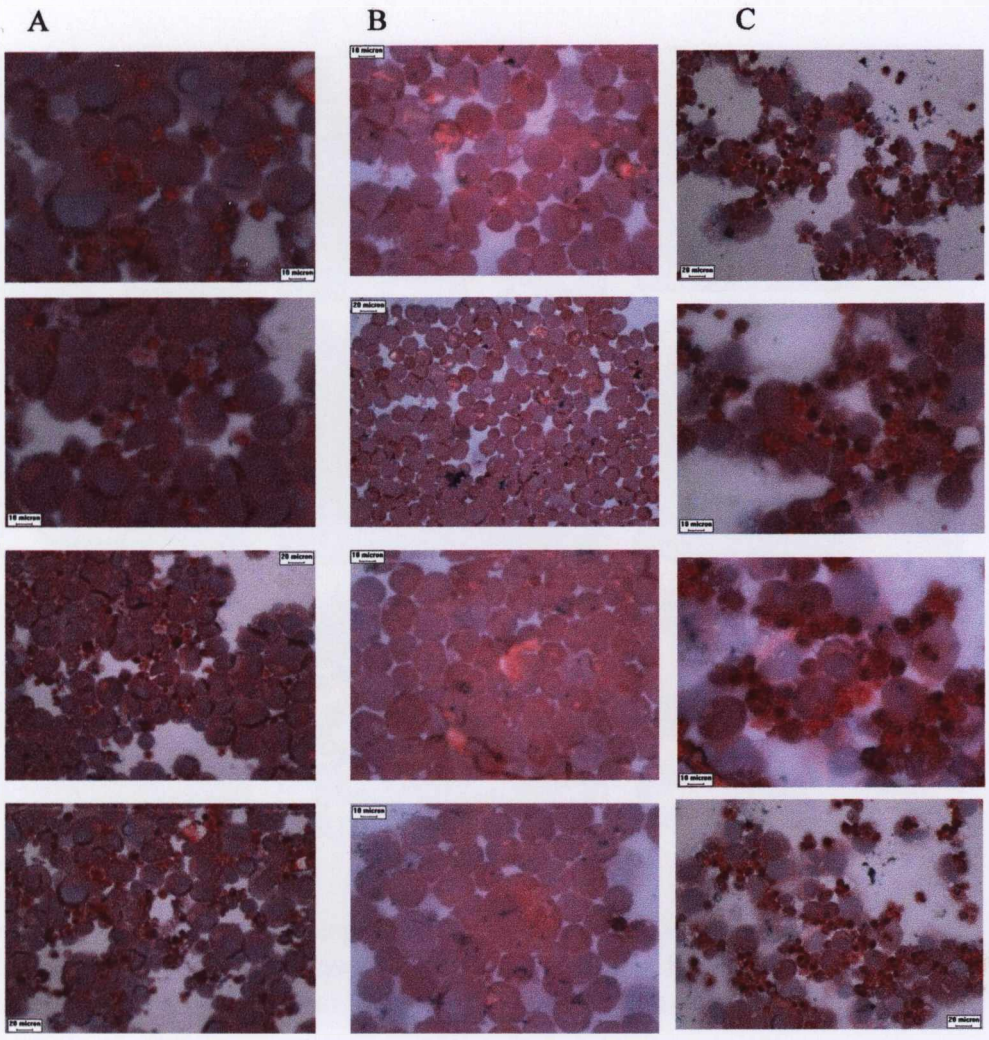

Figura 2. Fotografias evidenciando as células F após a coloração de Kleihauer. Em A, células K562 após 48h de aquecimento; em B, células K562 sem aquecimento; em C, células K562 transfectadas com LCR-HS2 após 48h de aquecimento.

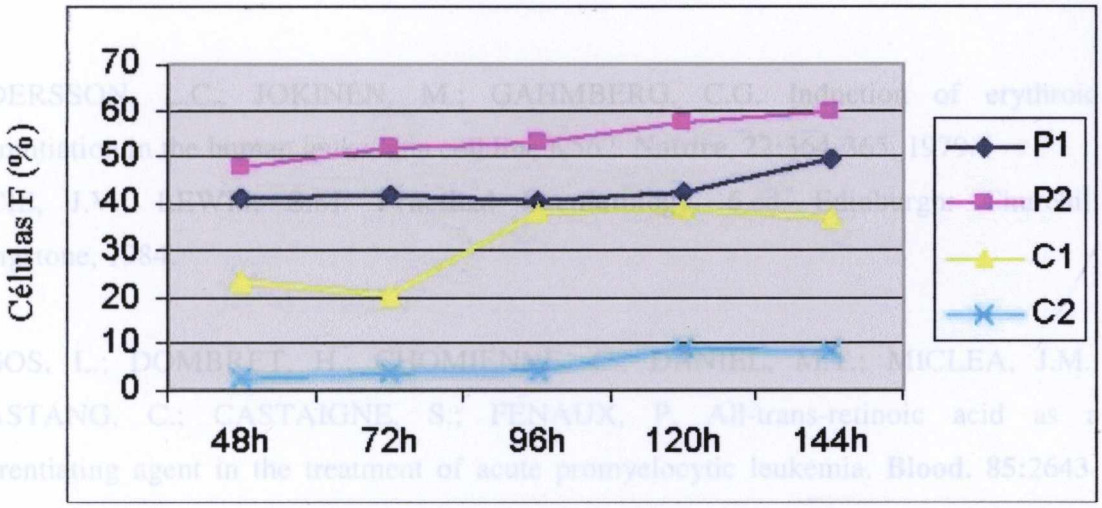

Figura 4. Efeito da hipertermia na produção de células F em culturas de células da linhagem K562 transfectadas. P1=LCR-HS2 do paciente com haplótipo CAR/Ben; P2=LCR-HS2 do paciente com haplótipo Ben/Ben; C1=controle de células K562 aquecidas e não transfectadas; C2=controle de células K562 não aquecidas e não transfectadas.

Desta forma, o modelo estabelecido mostrou-se adequado para utilização em estudos que envolvam a atividade de expressão gênica de regiões *enhancers* consideradas importantes na síntese da HbF.

Diante dos resultados descritos realizaremos ensaios de expressão gênica, utilizando diversas construções de plasmídeos que contenham o polimorfismo -10.677 (G→A) do LCR-HS2, bem como as alterações descritas para os polimorfismos na região promotora do genes γG. Com os resultados destes experimentos, será possível estabelecer possíveis mecanismos de ação destes polimorfismos, bem como a sua influência na síntese da HbF.

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HBF SYNTHESIS IN K562 CELL LINE TRANSIENTLY TRANSFECTED BY HS2-LCR SEQUENCE UNDER HYPERTHERMIA STRESS

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ABSTRACT

The sickle cell anemia has heterogeneous clinical manifestations and has been strongly associated with fetal hemoglobin levels, frequently related to the decrease of clinical severity. The K562 cell line has been used as a model of fetal/embryonic globin and erythroid maturation regulation and has been associated with differential expression of numerous genes, including the encoding of important transcription factors in the γ-globin genes expression. The present study tested the hypothesis that heat transient transfected K562 cell can be a good model to demonstrate the potential effects of specific LCR-HS2 constructs on HbF synthesis. It was demonstrated that the cell erythroid differentiation was higher among HS2-LCR transfected and heated K562 cell line than heated control cells with an enhancement of F cell production 144h after heat treatment. The viability of K562 and control transfected cells decreased after hyperthermia stress. The findings of this work confirmed the use of hyperthermia as a physical agent for erythroid differentiation on K562 cells, increasing the F cell production and related to cell growth reduction. It was also reported in the present study that the direct K562 cells transfection by naked plasmid DNA constructed with HS2-LCR enhancer sequence showed a high erythroid precursor growth and erythroid cells differentiation with F cell increase if transfected before a hyperthermia stress or stimulus.

1. INTRODUCTION

The sickle cell anemia has heterogeneous clinical manifestations. The phenotypic heterogeneity has been strongly associated with fetal hemoglobin ($\alpha_2\gamma_2$) levels, frequently related to reduce clinical severity (Charache 1990; Yang & Pace 2001; Stuart & Nagel 2004). Also, the fetal hemoglobin (HbF) levels have been associated with β^S - globin gene haplotypes, usually recognized by a set of polymorphic restriction enzymes sites on the β-globin gene cluster (Nagel & Ranney 1990; Steinberg 2001). The major β^S - globin gene haplotypes have been named Benin (Ben), Bantu or Central African Republic (CAR), Senegal (Sen), Arab-India and Cameroon (Cam), according to the geographical origin and ethnic group in which they were described (Nagel & Ranney 1990; Nagel & Steinberg 2001). The Sen haplotype has been associated with a high HbF levels and a less severe phenotype; the Ben haplotype with an intermediate HbF levels and mild clinical course and the CAR haplotype with low HbF level and a severe phenotype (Nagel & Ranney 1990; Steinberg 2001). The Arab-India haplotype has the highest HbF levels, with heterogeneous phenotype (Rahgozar *et al.* 2000).

The hemoglobin switching is a sequential process of globin gene activation and inactivation, involving complex interactions of stage-specific transcription factors, chromosomal gene order and gene proximity to the globin locus control region (LCR). This is characterized by a series of four DNase I-hypersensitive sites (HS1-HS5) and erythroid-specific and ubiquitous trans-acting factor, interacting with β-globin genes promoters (Tuan *et* al. 1992; Nagel & Steinberg 2001). Of particular importance is the HS2-LCR enhancer that contains a core 46bp binding sequence for the NF-E2 and AP-1 proteins, flanked by multiple *cis*-acting sequences that modulate the enhancer activity, containing two TA repeat sequences and single nucleotide polymorphism (SNP) in the *cis*-acting sequences (Oner *et al.* 1992; Plonczynski *et al.* 1997). However, association between LCR-HS2 polymorphisms and HbF levels is controversial; previous reports have suggested that the LCR HS-2 haplotype sequences are associated with the γ-globin gene expression, but the number of nucleotide changes in beta-globin gene cluster was not the primary cause of the HbF levels differences (Adekile AD *et al.* 1992; Offori-Acquah *et al.* 1999; Samakoglu *et al.* 1999; Offori-Acquah *et al.* 2001).

The K562 cell line has been used as a model of fetal/embryonic globin and erythroid maturation regulation and has been associated with differential expression of numerous genes, including encoding of important transcription factors in the γ-globin genes expression (Young K *et* al. 1984; Offori-Acquah *et al.* 2001). The K562 human leukemia cell line is originated from a patient with chronic myelogenous leukemia in terminal blast crisis (Anderson LC *et al.*1979). It can be differentiated into erythroid lineage by exposure to several pharmacological agents, such as hemin, antibiotics, retinoid and butyrate derivatives (Andersson *et al.*1979; Toffoli *et al.* 1989; Degos *et* al. 1995). Goliaei *et al.* (2004) described the use of hyperthermia, as a human erythroleukemic cell lines inductor.

Cellular therapy involving plasmid DNA has been described and justified by the use of a simple vector and also related to significant results in transgenic expression in several organs, obtained by the use of naked or complex plasmid DNA (Nishikawa *et al.*, 2005). Yang *et al.* (2005) studied the effects of the naked plasmid DNA encoding vascular endothelial growth (VEGF) on the survival of flap on rats and demonstrated that the therapy enhanced the survival of random pattern flaps by inducing angiogenesis.

Others reports have demonstrated the therapeutic use of lentviral vector in sickle cell anemia; Imren *et al.* (2004) indicated effective transduction of primitive human cord blood cells with a lentiviral vector therapeutic candidate. This was made by encoding an anti-sickling β-globin transgene, resulting in the long-term erythroid-specific production, reaching a β-globin protein levels therapeutically relevant. Hanawa *et al.* (2004) also showed the expression of a γ-globin lentiviral vector in a β-thalassemia murine model, describing a consistent therapeutic globin gene

expression, with animals transplanted by vector transduced stem cell exhibiting a high HbF synthesis. In this work, the hypothesis that heat transient transfected K562 cell line can be a good model to demonstrate the potential effects of specifics LCR-HS2 constructs on HbF synthesis was tested.

2. MATERIALS AND METHODS

2.1 *Patients selection*

Two sickle cell anemia patients (SS) from the Blood Center of Bahia (HEMOBA) in Brazil were studied. The patients had CAR/Ben (P1) and Ben/Ben (P2) β^S-globin gene genotype and low HbF levels (1.2% and 1.3%, respectively). The sequence analysis was developed in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, U.S.A.) using Kit BigDye 03 ^{TM}Terminator Sequencing Standard (Applied Biosystems), demonstrating characteristic sequence of HS2-LCR, previously related to β^S-globin gene haplotype (Oner *et al.*, 1992), without any additional mutations (table 1).

2.2 *Plasmid construct*

The LCR-HS2 was obtained by polymerase chain reaction (PCR) amplification from P1 and P2 patients. The PCR product was purified by QIAquick kit (Qiagen, Hilden, Germany) and cloned into pCR2.1TOPO (Invitrogen, California, USA) vector.

The plasmid DNA was transformed in a TOP10 chemically competent bacteria (Invitrogen, California, USA) and the correct insertion and sequences of the construct were confirmed by sequencing.

Control reactions were developed with K562 cells line under hyperthermia and without transfection (C1) and K562 cells line without hyperthermia and transfection (C2).

2.3 *Cell culture and transfection assay*

The K562 cells were maintained in RPMI-1640 culture medium (Invitrogen) supplemented with 10% heat inactived fetal calf serum (FCS) (Cultilab, Campinas, SP, BR) and 50 mg/L of Gentamicin (Invitrogen). Cells were incubated at 37 °C, 5.0 % of CO_2 and full humidity.

The constructs were incubated with cationic lipids (Lipofectamine; Invitrogen) for 15 min at room temperature before incubation with 10^7 cells in suspension for 3 h at 37°C and maintained overnight in RPMI + FCS at 37 °C and 5 % CO_2. The transfected and control K562 cells were heated at 42 °C for 180min (Goliaci *et al.* 2004). Parallel control was performed at 37 °C. The insert into K562 cells was confirmed by DNA extraction of 10^5 cells by EZ-DNA kit (Biological Industries, Israel) and sequencing.

2.3 *Erythroid differentiation and fetal hemoglobin synthesis evaluation*

Differentiation of heated and control K562 cells were searched after cytospin and stained by Kleihauer and Wright techniques (Dacie & Lewis1984), demonstrating fetal cells (F cell) and erythroids precursors, respectively. Fetal cells were quantified as percentages of the total cells. Cell viability was determined by Trypan blue dye exclusion test.

3. RESULTS

The cell erythroid differentiation was higher among transfected and heated K562 cell line than cell controls (figure 1).

After 48h heat treatment, the F cell production showed 41.4% to P1 and 47.9% to P2; the C1 control had 23.4% and C2 had 2.2%. Figure 2 shows F cell production between transfected and heated K562 cell line and cell controls.

The F cell production increased after 144h heat treatment in all experiments, demonstrating 49.7% to P1 and 59.9% to P2; C1 control had 37.0% and C2 had 8.6%. In this period, the F cell production by transfected and heated K562 cell line was 1.3 times (P1) and 1.6 times (P2) higher than K562 cell line only submitted to hyperthermia (C1). Figure 3 shows the F cell production by transfected and heated K562 cell line and cell controls.

The transfected and controls K562 cells viability were analyzed at several times (48h, 72h, 120h and 144h) after heat treatment, and indicated a progressive decrease of cell viability after the transfection assay and hyperthermia stress (figure 4).

4. DISCUSSION

The developmental regulation of globin gene expression and the response to pharmacological agents that elevate HbF, have been the subject of a previous report (Smith *et al.*, 2000) and the K562 cells, due to their potential of differential induction of embryonic and fetal hemoglobin synthesis, have been used for this purpose (Bhaumik, 1991). The present study investigated the possibility of the transient transfected K562 cell under hyperthermia stress be used as a model to demonstrate the potential effects of specific LCR-HS2 constructs on HbF synthesis.

The authors of this work confirmed the use of hyperthermia as physical agent for differentiation on K562 cells, increasing the F cell production and causing cell growth reduction, as described by Goliaei *et al.* (2004), which could be mediated by increasing the expression of heat shock proteins (HSP).

The presence of specific polymorphisms has been associated with some β^S- globin gene haplotypes and has suggested an association between HbF level variability and HS2 enhancer polymorphism (Dimovski *et al.* 1991; Öner *et al.* 1992). Sawado *et al.* (2001) have suggested that β- globin gene locus has a constitutively open chromatin conformation before the terminal differentiation, speculating that NF-E2 complex recruitment to LCR and active promoters may be a rate-limiting step in the activation of β- globin gene expression. There are controversies about the influence of LCR on HbF levels; previous reports have suggested that HbF level variability is associated with the HS2-LCR enhancer activity and with nucleotide sequences variation, although correlation between HbF level and common β^S- globin gene haplotypes is controversial (Adekile AD *et al.* 1992; Ploczynski *et al.* 1997; Ofori-Acquah *et al.* 1999, 2001). However, Ofori-Acquah *et al.* (2004) have demonstrated that γ-gene promoter polymorphisms can exert a higher influence on HbF level in sickle cell anemia patients than HS2-LCR polymorphisms; other study involving hereditary persistence of fetal hemoglobin (HPFH) have demonstrated the important role of the HS2-LCR on HbF synthesis in vitro (Takahashi *et al.* 2003).

The present investigation showed the increase of F cell among HS2-LCR K562 transfected cells from CAR/Ben and Ben/Ben sickle cell anemia patients. The transfected and heated cells have the highest number of F cell. In this work, two sickle cell anemia patients with low HbF levels and common HS2-LCR polymorphisms were selected in order to demonstrate that the single enhancer presence could be able to increase the endogenous HbF level in the K562 cell line.

The reactivation of HbF synthesis in sickle cell anemia represents a potential strategy for therapy; however, the drugs currently available have low efficacy and specificity and are associated with high toxicity. Further studies involving stable cellular genomic reporter assays containing construct plasmids can be used to develop novel inducers of HbF synthesis for therapy (Vadolas *et al.*, 2004). The findings of this study indicated that the direct K562 cells transfection by naked

plasmid DNA constructed with HS2-LCR enhancer sequence showed a high erythroid precursor growth and erythroid cells differentiation with F cell enhancement if transfected before the hyperthermia stress or stimulus. The present model can be utilized to further gene expression activity studies, in order to clarify the effects of specific enhancer and promoter region sequence changes in the fetal hemoglobin synthesis.

5. ACKNOWLEDGMENTS

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Table 1. Fetal hemoglobin levels, β^S-globin gene haplotypes and HS2-LCR sequence in two sickle cell anemia patients.

			HS 2				
	Hb F	**β-^{S} Haplotype**	**-10,924**	**-10,905**	**-10,677**	**-10,623 -10,570**	**-10,390**
P1	1,2	CAR/Ben	T/G	A/G	G/G	8(TA)N11(TA) / 8(TA)nTG7(TA)	A/T
P2	1,3	Ben/Ben	G/G	G/G	G/G	8 (TA)nTG7(TA) / 8(TA)nTG7(TA)	T/T

In the 5' HS 2, N = CACATATACG and n= CACATATACGTG.

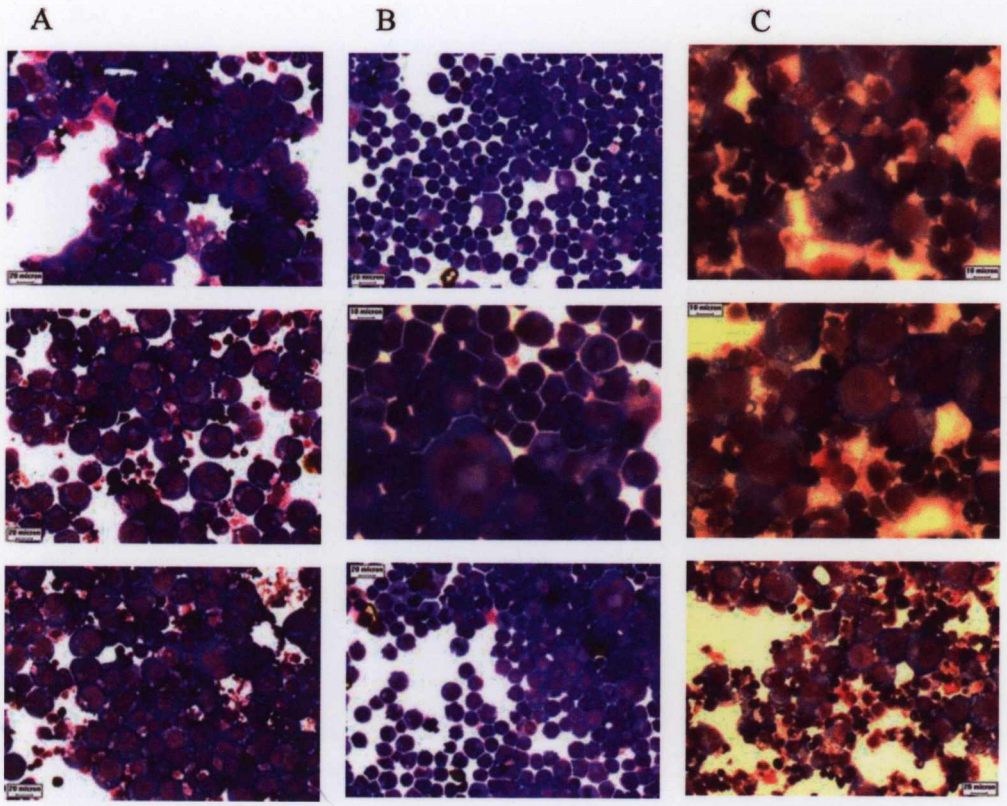

Figure 1 Wright stain to identify erythroids precursors; A - heated (48h) K562 cell line (C1); B- K562 cell line without transfection or heat (C2); C- transfected and heated (48h) K562 cell line (P2).

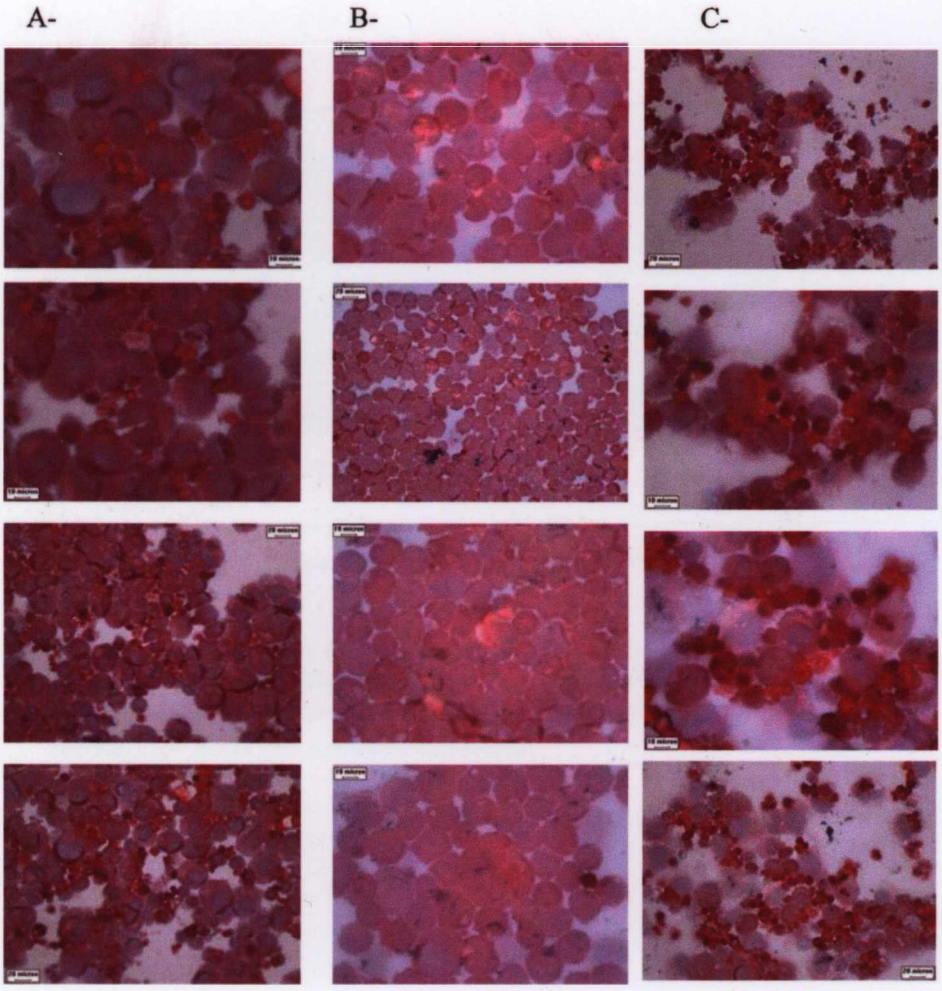

Figure 2. Kleihauer stain to identify F cells production; A - heated (48h) K562 cell line (C1); B- K562 cell line without transfection or heat (C2); C- transfected and heated (48h) K562 cell line (P2).

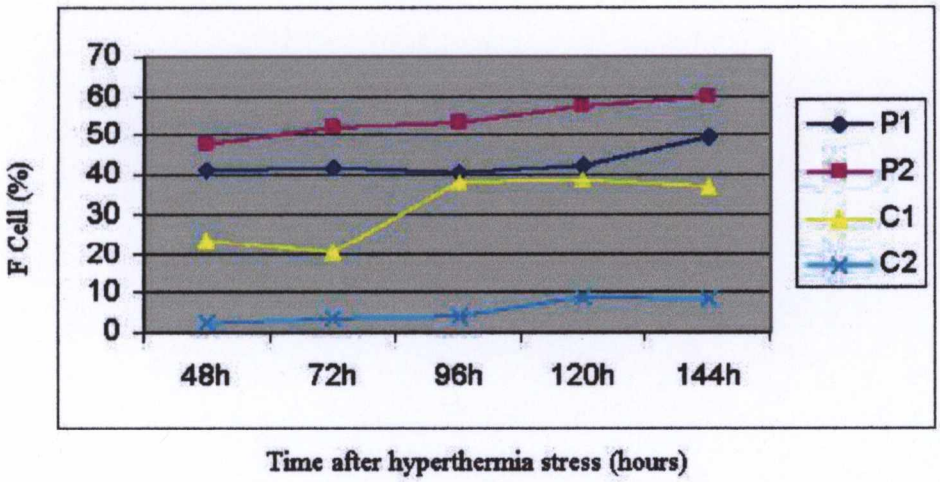

Figure 3. Hyperthermia effects on the Fetal cells production among transfected K562 cells line at different times after heat treatment. P1= LCR-HS2 of CAR/Ben haplotype; P2= LCR-HS2 of Ben/Ben haplotype; C1= K562 cell heat control; C2= K562 cell control without heat.

10.1 Eletroferograma do sequenciamento automático da região promotora do gene γG e do HS2-LCR

Eletroferograma do sequenciamento automático da região promotora do gene γG

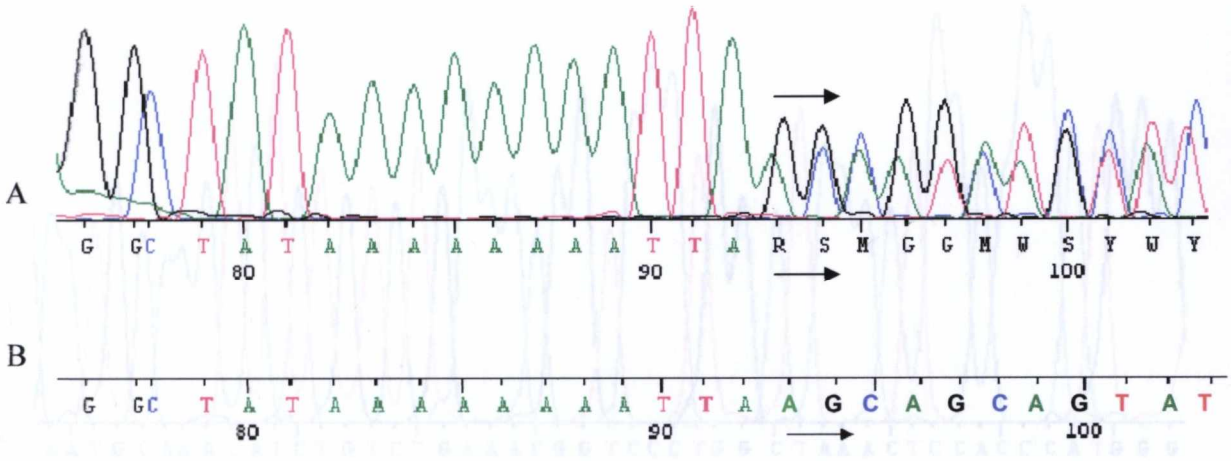

Figura 10.1 A - Deleção AGCA na posição –222 a –225 da região promotora do gene γG, descrita em dois pacientes portadores do genótipo Cam/Ben, que apresentaram níveis de HbF acima de 15%; B - Seqüência normal de nucleotídeos da região promotora do gene γG.

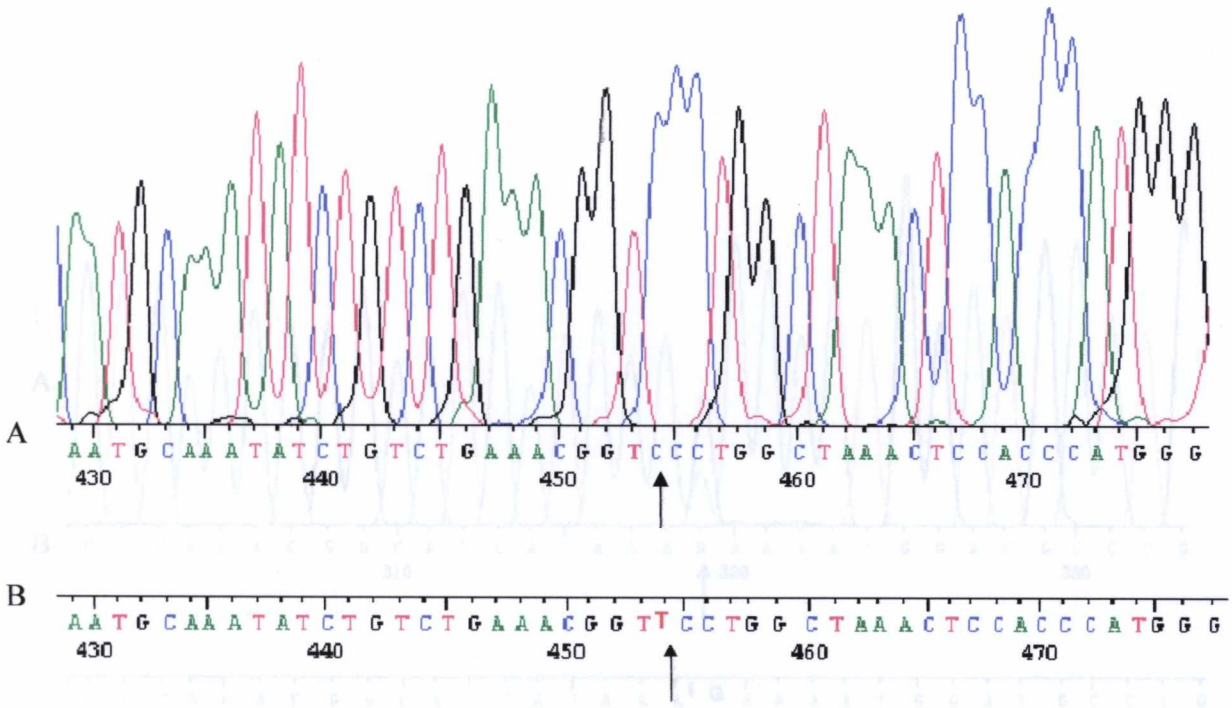

Figura 10.2 A - Substituição T→ C na posição – 157 da região promotora do gene γG, descrita em todos os pacientes analisados, independente do tipo de haplótipo ligado ao grupamento de genes da globina β^S e do nível de HbF; B - Seqüência normal de nucleotídeos da região promotora do gene γG.

Eletroferograma do sequenciamento automático do Segundo sítio hipersensível à ação da DNase I da região controladora do locus da globina β (HS2-LCR)

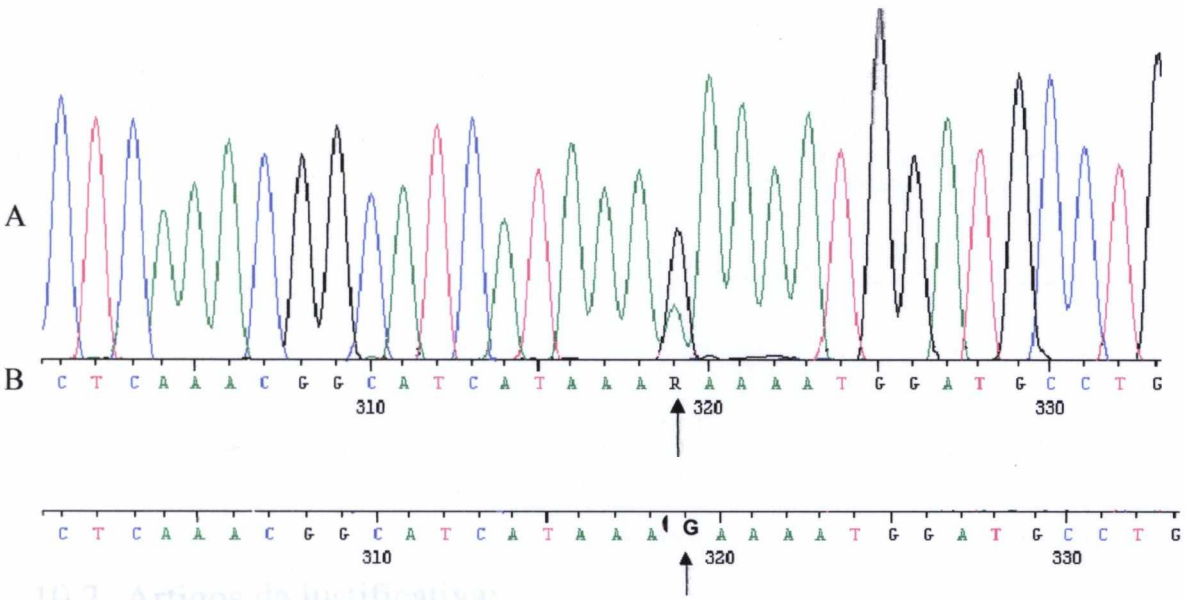

Figura 10.3 A – Substituição G→ A na posição – 10.677 do HS2-LCR descrita nos pacientes portadores do haplótipo Ben que apresentaram níveis de HbF elevados; B – Seqüência normal de nucleotídeos do HS2-LCR.

10.2 Artigos da justificativa:

10.2.1 Hemoglobinopathies in newborns from Salvador, Bahia, Northeast Brazil;

10.2.2 Beta S-haplotypes in sickle cell anemia patients from Salvador, Bahia, Northeastern Brazil.

Hemoglobinopathies in newborns from Salvador, Bahia, Northeast Brazil

Hemoglobinopatias em recém-nascidos de Salvador, Bahia, Nordeste do Brasil

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Abstract

Hemoglobinopathies are hereditary disorders of the hemoglobin molecule with a high prevalence worldwide. Brazil has a prevalence of 0.1 to 0.3% of newborns with sickle cell anemia and 20.0 to 25.0% of heterozygous α_2 thalassemia among African Brazilians. In the present study, we investigated the presence of variant hemoglobins and $\alpha_2^{3.7\,Kb}$ and $\alpha_2^{4.2\,Kb}$ thalassemia in newborns from Salvador, Bahia, Brazil. Samples of umbilical cord blood from a total of 590 newborns were analyzed, of which 57 (9.8%) were FAS; 36 (6.5%) FAC; one (0.2%) SF; and five (0.9%) FSC. One hundred fourteen (22.2%) newborns had $\alpha_2^{3.7\,Kb}$ thalassemia, of whom 101 (19.7%) were heterozygous and 13 (2.5%) homozygous, showing statistical significance for hematological data between newborns with normal α genes and $\alpha_2^{3.7\,Kb}$ thalassemia carriers. The $\alpha_2^{4.2\,Kb}$ thalassemia was not found. Frequencies found in the present study confirm that hemoglobinopathies are a public health problem in Brazil, emphasizing the need for neonatal screening and genetic counseling programs.

Hemoglobinopathies; Sickle Cell Anemia; Thalassemia; Newborn Infant

Introduction

Hemoglobinopathies are genetic globin gene disorders, characterized by the presence of variant hemoglobin and a decrease or absence of globin chain synthesis, known as thalassemia [1,2].

Hemoglobin S is the most common variant hemoglobin, and results from a single amino acid substitution of valine for glutamic acid at the sixth position of the β-globin chain; sickle cell anemia carriers are characterized by homozygosity of S hemoglobin. The hemoglobin S gene has a high frequency among Africans and African descendents, as well as in India, Greece, and the United States [3]. Primary studies in Brazil revealed a high prevalence of hemoglobin disorders. The sickle cell trait (AS) was reported in 6.6% of blacks in the State of São Paulo, in Southeast Brazil [4]. When the study was extended to the general population, frequencies of 2.7% of AS and 0.09% of sickle cell disease (0.07 % HbSS and 0.02% HbSC) were observed. These frequencies varied widely according to the degree of racial admixture in the different regions of the country. In the South of Brazil, a frequency of 1.2% of HbS gene was shown among newborns [5]. On the other hand, in the Northeast, frequencies of 5.1% of sickle cell trait (FAS) and 0.2% with sickle cell disease (FSC) were reported among newborns in the State of Pernambuco [6] and the State of Bahia, the frequency of AS genotype varies from 7.4%

to 15.7%, according to the population group studied [7].

Hemoglobin C (HbC) is a variant hemoglobin in which lysine replaces glutamic acid at the sixth amino acid position of the β-globin chain [2]. HbC has a prevalence of 3% among African-Americans and about 1-3% among Puerto Ricans [8,9]. In Brazil, HbC is the second most common variant hemoglobin and has been found around 2.2% to 5.2% when the heterozygous genotype (AC) has been considered in Bahia [7].

Thalassemia syndromes are found worldwide, especially α- and β-thalassemia [10,11]. In Southeast Brazil, a frequency of 1.3% of β-thalassemia trait and 0.1% of β-thalassemia major was reported for the general population [12], while α_2-thalassemia by a 3.7 kb DNA deletion ($\alpha_2^{3.7Kb}$-thalessemia) varied from 20.0% to 25.0% in black populations [13], and Borges et al. [14] found 49.9% of α-thalassemia in adult outpatients seen at the University of Campinas Hospital with microcytosis and hypochromia without anemia. In the Northeast, $\alpha_2^{3.7Kb}$-thalessemia was investigated in 106 pregnant women with AC and AA hemoglobin pattern, showing a 21.7% heterozygous and 0.9% homozygous rate for this alteration [15].

The Consensus Conference Panel convened by the National Institutes of Health (United States) in 1987 recommended newborn screening for hemoglobinopathies in order to decrease morbidity and mortality associated with sickle cell disease. *Streptococcus pneumoniae* is a common cause of death in sickle cell patients and the early diagnosis of sickle cell disease could alert clinicians to potential clinical complications of the disease and allow prompt clinical care and prophylactic therapy with vaccines and antibiotics [16,17].

In Brazil, not all States have consolidated neonatal screening programs for hemoglobinopathies, despite a ruling mandating them by the Ministry of Health in June 2001 (Portaria GM/MS 822 – Diário Oficial da União 2001; 7 jun). The sickle cell disease screening program in Minas Gerais State was considered a model for South America by Serjeant [18]. This program found 3.2% of screened babies with sickle cell trait, 1.3% heterozygous for HbC, and 0.08% with sickle cell disease. Other States have developed excellent newborn screening programs, such as Bahia, Pernambuco, Maranhão, the Federal District (Brasília), São Paulo, Rio de Janeiro, Espírito Santo, Santa Catarina, Paraná, Mato Grosso do Sul, and Rio Grande do Sul (Technical Advisory Group on Neonatal Screening, Ministry of Health, Brazil). In California (United States), a newborn hemoglobinopathy screening program evaluated two million newborns and found 492 with sickle cell disease, while 290 had hemoglobinopathy SS, 143 SC, and 47 Sβ+ thalassemia [19]. The screening programs' implementation is an important step for increasing survival, reducing hospitalization, and minimizing expenses associated with sickle cell disease [20,21]. Based on the high frequency of hemoglobinopathies in Salvador, Bahia, Brazil, we investigated the presence of variant hemoglobin and $\alpha_2^{3.7\ Kb}$ and $\alpha_2^{4.2\ Kb}$ thalassemias among newborns babies in order to identify potential associations between hemoglobin abnormalities and hematological characteristics.

Material and methods

Casuistic

From February to June 2000, a cross-sectional epidemiological study analyzed 590 neonates delivered by vaginal birth at the Tsylla Balbino Maternity Clinic located in Salvador, Bahia, Brazil. Information about newborns and gestational age was obtained from mothers by a questionnaire and patient records.

Newborns with gestational age less than thirty-eight weeks were considered premature [22]. Racial composition was determined by observation of facial characteristics and color of mammillae and scrotum [23].

Samples

Umbilical cord blood samples were collected by midwives under supervision of maternity clinic physician staff. After clamping, blood was drawn into a Vacutainer tube (Becton-Dickinson – Corkeysville, Maryland, United States) containing EDTA and transported to the Pathology and Molecular Biology Laboratory, Gonçalo Moniz Research Center, Oswaldo Cruz Foundation (FIOCRUZ). Hematological analysis was performed by automated cell counter (Coulter T-890 – Coulter Corporation, Florida, United States) and DNA was isolated from leukocytes (GFX™ Genomic Blood DNA Purification KIT – Amersham Pharmacia Biotech, United States).

Hemoglobin profile

Hemoglobin profile was analyzed by High Performance Liquid Chromatography – HPLC Variant Hemoglobin Testing System (Bio-Rad Laboratories – California, United States). HPLC analyses were interpreted by comparing peak retention times with those obtained for the AFCS control, utilizing the sickle cell kit for hemoglobin screening in newborns.

α^2-Thalassemia

The $-\alpha_2^{3.7Kb}$ and $-\alpha_2^{4.2}$ deletions were identified by polymerase chain reaction (PCR), using specific primers and reaction conditions as previously described [24].

Statistical analysis

The statistical analyses were conducted in the Epi Info software, version 6.04. Statistical significance was established at $P \leq 0.05$.

Ethical considerations

The study was approved by the Institutional Review Board/Ethics Committee of the Gonçalo Moniz Research Center, FIOCRUZ, after the newborn's parent or guardian had signed the informed consent form.

Results

The Tsylla Balbino Maternity Clinic is the largest public maternity ward in Salvador. During the study period, 2,958 children were born and 590 newborns (19.9% of total birth cohort) were analyzed, after statistical sample calculation. Of those analyzed, 63 (10.8%) had premature delivery and 523 (89.2%) had normal gestational age; 296 (51.3%) were female and 281 (48.7%) male. Racial composition was determined for 578 babies: 98 (17.0%) were classified as white, 309 (53.4%) as mulatto, and 171 (29.6%) as black. Mean weight was 3.152 kg (± 0.530).

Hematological data and hemoglobin profiles

Hemoglobin profiles were determined in 581 of the 590 newborns: 480 (82.6%) had the normal profile FA, and 101 (17.4%) presented variant hemoglobins, of which 57 (9.8%) were heterozygous for HbS (FAS), and 38 (6.5%) were heterozygous for HbC (FAC). One (0.2%) baby was homozygous for HbS (FS) and five (0.9%) were double heterozygous for HbS and HbC (FSC).

There was no statistical difference in the gestational ages of newborns with respect to normal and variant hemoglobin patterns. The racial distribution and hemoglobin types of 502 newborns are shown in Table 1. Of the 47 newborns found to have the FAS pattern, 24 (51.1%) were mulatto, 15 (31.9%) were black, and eight (17.0%) were white.

Newborns with normal and variant hemoglobin had statistically similar hematological characteristics, except for the FA and FAC groups, which were statistically different for PVC, RBC, and MCHC values, with p-values of 0.031, 0.003, and 0.0009, respectively (Table 2).

α_2 thalassemia analyses

Of the 590 newborns, 514 were analyzed for α_2 thalassemia: 400 (77.8%) had normal α-genes (αα/αα) and 101 (19.7%) were heterozygous (α/αα) and 13 (2.5%) homozygous (-α/α-) for the $\alpha_2^{3.7Kb}$ deletion. There was no statistical difference between presence of $\alpha_2^{3.7Kb}$ thalassemia and premature delivery.

Table 1

Ethnic characteristics and hemoglobin frequencies in newborns from the Tsylla Balbino Maternity Clinic. Salvador, Bahia, Brazil.

Racial group	Hemoglobin profile									
	FA		FAS		FAC		FSC		FS	
	n	%	n	%	n	%	n	%	n	%
White (n = 86)	76	18.2	8	17.0	2	6.0	–	–	–	–
Mulatto (n = 274)	227	54.4	24	51.1	19	57.6	3	75.0	–	–
Black (n = 142)	114	27.4	15	31.9	12	36.4	1	25.0	1	100.0
Total (n = 502)	417	100.0	47	100.0	33	100.0	4	100.0	1	100.0

Among the 114 newborns identified as $\alpha_2^{3.7Kb}$ thalassemia carriers, 21 (18.4%) were whites, 57 (50.0%) were mulattos, and 36 (31.6%) blacks. Table 3 shows the statistical analysis of hematological parameters of newborns with normal α-genes and $\alpha_2^{3.7Kb}$ thalassemia carriers.

Analysis of hemoglobin profiles and presence of $\alpha_2^{3.7Kb}$ thalassemia was performed on 451 of 590 newborns. Of the 377 newborns with FA pattern, 295 (78.2%) had normal α-genes, 72 (19.1%) were heterozygous, and ten (2.7%) homozygous.

Of the 43 newborns with FAS profile, 32 (74.4%) had normal α-genes, nine (20.9%) were heterozygous, and two (4.7%) were homozygous; of the 27 newborns with pattern FAC, 22 (81.5%) had normal α-genes and five (18.5%) were heterozygous; among the four newborns with FSC pattern, three (75.0%) had normal α-genes and one (25.0%) was heterozygous for $\alpha_2^{3.7Kb}$ thalassemia. The newborn with FS pattern had normal α-genes.

Table 4 shows the hematological data from newborns with normal and variant hemoglobin genotypes, as well as for $\alpha_2^{3.7Kb}$ thalassemia carriers.

There was no statistical difference in the hematological characteristics of newborns with

Table 2

Hematological data and hemoglobin profile among newborns from the Tsylla Balbino Maternity Clinic. Salvador, Bahia, Brazil.

Hemoglobin pattern	Hematological data (mean ± SD) PCV (%)	RBC (x10⁶mL)	Hb (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
FA (n = 480)	46.55 (±6.11)	4.31 (±0.56)	14.76 (±1.79)	108.42 (±6.20)	34.53 (±5.70)	31.82 (±2.68)
FAS (n = 57)	45.38 (±5.97)	4.19 (±0.53)	14.57 (±1.92)	108.40 (±6.42)	34.89 (±2.80)	32.17 (±1.57)
	p = 0.18*	p = 0.15*	p = 0.44 *	p = 0.99*	p = 0.38*	P = 0.66**
FA (n = 480)	46.55 (±6.11)	4.31 (±0.56)	14.76 (±1.79)	108.42 (±6.20)	34.53 (±5.70)	31.82 (±2.68)
FAC (n = 38)	43.37 (±7.73)	4.02 (±0.71)	14.44 (±1.72)	107.95 (±4.11)	36.78 (±6.84)	34.13 (±6.68)
	p = 0.031**	p = 0.003*	p = 0.27*	p = 0.25**	p = 0.23**	p = 0.0009**
FA (n = 480)	46.55 (±6.11)	4.31 (±0.56)	14.76 (±1.79)	108.42 (±6.20)	34.53 (±5.70)	31.82 (±2.68)
FSC (n = 5)	48.75 (±5.56)	4.45 (±0.47)	14.73 (±1.58)	109.55 (±5.45)	33.45 (±4.98)	30.55 (±4.20)
	p = 0.47*	p = 0.62*	p = 0.96*	p = 0.72*	p = 0.56*	p = 0.34*

* ANOVA; ** Kruskal-Wallis H.
PCV = Packed cell volume; RBC = Red blood cells; Hb = Hemoglobin; MCV = Mean cell volume; MCH = Mean cell hemoglobin; MCHC = Mean cell hemoglobin concentration.

Table 3

Hematological data and α genes status among newborns from the Tsylla Balbino Maternity Clinic. Salvador, Bahia, Brazil.

Hematological data (mean ± SD)	Normal α-genes (n = 400)	-$\alpha_2^{3.7Kb}$ thalassemia carriers (n = 114)	P-value
PCV (%)	46.98 (± 5.97)	44.90 (± 6.72)	0.002*
RBC (x 10⁶/mL)	4.26 (± 0.54)	4.41 (± 0.65)	0.002**
Hb (g/dL)	14.09 (± 1.69)	14.26 (± 1.72)	0.00009*
MCV (fL)	110.23 (± 4.43)	102.78 (± 6.25)	< 0.00001**
MCH (pg)	35.36 (± 3.78)	32.64 (± 3.49)	< 0.00001**
MCHC (g/dL)	32.10 (± 3.14)	31.76 (± 2.87)	0.30*

* ANOVA; ** Kruskal-Wallis H.
PCV = Packed cell volume; RBC = Red blood cells; Hb = Hemoglobin; MCV = Mean cell volume; MCH = Mean cell hemoglobin; MCHC = Mean cell hemoglobin concentration.

Table 4

Hematological data and hemoglobin profile among newborns with $\alpha_2^{3.7Kb}$ thalassemia from the Tsylla Balbino Maternity Clinic. Salvador, Bahia, Brazil.

Hemoglobin pattern	Hematological data (mean ± SD) PVC	RBC (x10⁶/mL)	Hb (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
FA (n = 82)	45.47 (± 5.96)	4.51 (±0.55)	14.38 (±1.47)	102.09 (±6.39)	32.08 (±2.67)	31.41 (±1.67)
FAS (n = 11)	41.99 (± 8.15)	4.04 (±0.82)	13.63 (±2.41)	104.41 (±4.18)	34.16 (±4.03)	32.66 (±3.16)
	p = 0.09*	p = 0.01*	p = 0.47**	p = 0.25*	p = 0.03*	p = 0.29**
FA (n = 82)	45.47 (±5.96)	4.51 (±0.55)	14.38 (±1.47)	102.09 (±6.37)	32.08 (±2.67)	31.41 (±1.67)
FAC (n = 5)	39.10 (±11.79)	3.75 (±10.9)	13.56 (±2.30)	103.92 (±4.01)	39.09 (±9.30)	36.66 (±10.15)
	p = 0.11**	p = 0.11**	p = 0.25*	p = 0.53*	p = 0.031**	p = 0.018**

* ANOVA, ** Kruskal-Wallis H

FA and FSC hemoglobin profile and thalassemia $\alpha_2^{3.7Kb}$ carriers.

Discussion

The frequencies of variant hemoglobin found in this study (Hb FAS 9.8%; Hb FAC 6.5%; FS 0.2%, and FSC 0.9%) are the highest described in Brazil. The high frequencies of variant hemoglobin are probably due to the high rate of racial admixture in the Bahian population, with a strong African gene component introduced by the African slave trade in Brazil. Furthermore, the Tsylla Balbino Maternity ward serves the majority of low-income women in Salvador, who are almost exclusively blacks or mulattos [23]. Among the newborns analyzed, only six (5 FSC and 1 FS) required special care, representing the symptomatic group that can develop severe clinical conditions and need early treatment. The 47 newborns identified as FAS displayed a racial distribution of 17.0% whites, 53.4% mulattos and 29.6% blacks. These variations of racial distribution of hemoglobin S among the newborns groups highlight the need for universal neonatal hemoglobinopathy screening in the Brazilian population. The comparison of hematological data among newborns with HbC and those with normal hemoglobin showed statistical significance for PVC, RBC, and MCHC due to increased blood viscosity, demonstrating the influence of HbC on hematological parameters [8,9].

The high frequencies of $\alpha_2^{3.7Kb}$ thalassemia (heterozygous 19.7%; homozygous 2.5%) are similar to those found in previous studies conducted in Brazilian populations [11]. The presence of $\alpha_2^{3.7Kb}$ thalassemia was not associated with premature delivery. However, van der Dijs et al. [25] speculated that the presence of α_2 thalassemia could decrease the capacity to extract oxygen from maternal circulation and could thus be a contributing factor to early delivery of newborn α_2 thalassemia carriers. Newborns with FAS and FAC pattern, such as the babies heterozygous for $\alpha_2^{3.7Kb}$ thalassemia, did not present symptoms of severe anemia. However, they still need genetic counseling [26].

We did not identify any newborns with $\alpha_2^{4.2Kb}$ thalassemia in our study, suggesting that this type of thalassemia is rare or absent in Bahia. The frequency of $\alpha_2^{3.7Kb}$ thalassemia among FAS newborns (20.9% of which were heterozygous and 4.7% of which were homozygous) could be beneficial for these S hemoglobin carriers and for the sickle cell anemia patients in Bahia. The α_2 thalassemia is considered a beneficial factor, since this condition is associated with less severe clinical manifestations among sickle cell disease carriers [8,9]. It decreases MCHC and the rate of hemolysis, resulting in higher Hb, PCV, and RBC values. Therefore, some complications such as leg ulcers, renal pathology, and strokes become less frequent. However, the frequency of other complications such as osteonecrosis and retinopathy may be increased [27].

The comparison of hematological data among newborns with α-normal genes and those with $\alpha_2^{3.7Kb}$ thalassemia showed statistical differences in all hematological parameters analyzed, except for MCHC. The hematological analysis of newborns with FA and FAS hemoglobin groups and $\alpha_2^{3.7Kb}$ thalassemia carriers showed statistically significant differences in RBC and MCH; the comparison of the FA and FAC groups and $\alpha_2^{3.7Kb}$ thalassemia carriers

showed significant differences in MCH and MCHC. The hematological differences found between newborns with normal α-genes and $\alpha_2^{3.7Kb}$ thalassemia carriers could be intensified by the presence of variant hemoglobins. Furthermore, differentiation within the Bahian pediatric population needs to be developed in order to determine whether the presence of $\alpha_2^{3.7Kb}$ thalassemia could be responsible for intensifying the hematological pattern of anemia, especially in child with iron deficiency, avoiding erroneous therapy.

In Bahia, the presence of hemoglobinopathies is a public health problem, and the early diagnosis of variant hemoglobin carriers provides an opportunity for counseling and early clinical follow-up of the child, both of which contribute to reduce child morbidity and mortality rates.

Resumo

Hemoglobinopatias são alterações hereditárias na molécula de hemoglobina com prevalência mundial elevada. O Brasil apresenta prevalência de 0,1 a 0,3% para recém-nascidos com anemia falciforme e freqüência de 20,0 a 25,0% para a ocorrência de heterozigotos da talassemia α_2 entre indivíduos afro-descendentes. O presente estudo investigou a presença de hemoglobinas variantes e talassemia $\alpha_2^{3.7Kb}$ e $\alpha_2^{4.2Kb}$ em recém-nascidos de Salvador, Bahia, Brasil. Analisamos o sangue do cordão umbilical de 590 recém-nascidos, sendo 57 (9,8%) com padrão FAS; 36 (6,5%) FAC; um (0,2%) SF e cinco (0,9%) FSC. Cento e catorze (22,2%) apresentaram talassemia $\alpha_2^{3.7Kb}$, dos quais 101 (19,7%) foram heterozigotos e 13 (2,5%) homozigotos, mostrando significância estatística para os dados hematológicos entre recém-nascidos com genes α normais e portadores de talassemia $\alpha_2^{3.7Kb}$. A talassemia $\alpha_2^{4.2Kb}$ não foi encontrada. As freqüências descritas neste trabalho confirmam que as hemoglobinopatias são um problema de Saúde Pública no Brasil, enfatizando a importância dos programas de triagem neonatal e aconselhamento genético.

Hemoglobinopatias; Anemia Falciforme; Talassemia; Recém-Nascido

Contributors

E. V. Adorno participated in the blood sample collection in the hospital, hemoglobin analysis, DNA extraction, molecular analysis, and drafting of the manuscript. F. D. Couto, J. F. Menezes and J. P. Moura Neto participated in the blood sample collection in the hospital, hemoglobin analysis, and DNA extraction. M. Rego participated in the statistical analysis. M. G. Reis participated in the interpretation of the results and drafting and revision of the manuscript. M. S. Gonçalves supervised the laboratory analysis, interpretation of the results, and drafting and revision of the manuscript.

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ß^S-Haplotypes in sickle cell anemia patients from Salvador, Bahia, Northeastern Brazil

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Abstract

ß^S-Globin haplotypes were studied in 80 (160 ß^S chromosomes) sickle cell disease patients from Salvador, Brazil, a city with a large population of African origin resulting from the slave trade from Western Africa, mainly from the Bay of Benin. Hematological and hemoglobin analyses were carried out by standard methods. The ß^S-haplotypes were determined by PCR and dot-blot techniques. A total of 77 (48.1%) chromosomes were characterized as Central African Republic (CAR) haplotype, 73 (45.6%) as Benin (BEN), 1 (0.63%) as Senegal (SEN), and 9 (5.63%) as atypical (Atp). Genotype was CAR/CAR in 17 (21.3%) patients, BEN/BEN in 17 (21.3%), CAR/BEN in 37 (46.3%), BEN/SEN in 1 (1.25%), BEN/Atp in 1 (1.25%), CAR/Atp in 6 (7.5%), and Atp/Atp in 1 (1.25%). Hemoglobin concentrations and hematocrit values did not differ among genotype groups but were significantly higher in 25 patients presenting percent fetal hemoglobin (%HbF) ≥10% (P = 0.002 and 0.003, respectively). The median HbF concentration was 7.54 ± 4.342% for the CAR/CAR genotype, 9.88 ± 3.558% for the BEN/BEN genotype, 8.146 ± 4.631% for the CAR/BEN genotype, and 4.180 ± 2.250% for the CAR/Atp genotype (P = 0.02), although 1 CAR/CAR individual presented an HbF concentration as high as 15%. In view of the ethnic and geographical origin of this population, we did not expect a Hardy-Weinberg equilibrium for CAR/CAR and BEN/BEN homozygous haplotypes and a high proportion of heterozygous CAR/BEN haplotypes since the State of Bahia historically received more slaves from Western Africa than from Central Africa.

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- Beta(S)-haplotypes
- Fetal hemoglobin
- Sickle cell anemia
- S hemoglobin
- Brazilian population

Introduction

Sickle cell hemoglobin (HbS) is the result of a single nucleotide change (GAG→GTG) in the ß-globin gene, where valine replaces glutamic acid (ß$^{6\ Glu\rightarrow val}$) at the sixth amino acid position of the ß-globin chain (1). Sickle cell anemia is caused by homozygosity of the ß^S-gene and has a worldwide distribution. The disease progresses as severe chronic hemolytic anemia, presenting a heterogenous clinical course according to patient back-

ground and geographic region of origin (2). Milder clinical symptoms have been described in patients presenting α-2 thalassemia and high levels of fetal hemoglobin (HbF), related to the presence of specific haplotypes (3,4). $ß^S$-Haplotypes are of different ethnic and geographic origins: the Benin type (BEN) originated in Midwestern Africa, the Bantu (CAR) type in South-Central and Eastern Africa, the Senegal (SEN) type in Atlantic West Africa, the Saudi Arabia-India type on the Indian subcontinent and the eastern Arabian peninsula, and the Cameroon type along the west coast of Africa (5).

Sickle cell disease affects millions worldwide, and occurs in one of every 500 African-American births, and in one of every 1000 to 4000 Hispanic-American births. In Brazil, the largest country in South America, the sickle cell trait is found at frequencies ranging from 6.9 to 15.4% of individuals of African descent (6). High immigration influxes have produced a population of significant cultural, social, and ethnic heterogeneity. Salvador is the capital of Bahia, a state in the Northeast region of Brazil, with 2.7 million people (7). The population has a high racial admixture with 85% of the African component (8). Historical data describing the slave trade in Bahia indicate the presence of slaves from central Africa (predominantly CAR haplotype) and from Western Africa (BEN haplotype), with a predominance of the latter. However, haplotype characterization has reported conflicting frequencies of CAR (9) and BEN (10) haplotypes.

The historian Verger (11) described the Nagô-Iorubá influence in Bahia State brought by Africans from the Benin Gulf region. In contrast, the rest of Brazil received large influxes of slaves from Congo and Angola (primarily CAR haplotype). In addition to a period of illicit slave trafficking, Bahia had four official periods of slave trading: a Guinea cycle during the XVI century, an Angola and Congo cycle during the XVII century, a Coast of Mine cycle during the XVIII century, and a Bay of Benin cycle between 1770 and 1850. Florentino (12) emphasizes that Bahia, beginning in 1815, was the only Brazilian state that restricted slave traffic through Ecuador, a fact that explains the correlation between genotype frequencies found in Bahia and Western Africa, principally the Bay of Benin region.

The unusual ethnic composition of Salvador, which was a transfer point during the African slave trade, represents an excellent opportunity to study the $ß^S$-haplotypes and to investigate the clinical picture of sickle cell anemia patients and the anthropological origins of the $ß^S$-gene in this Brazilian population.

Material and Methods

A total of 80 sickle cell disease patients (40 males and 40 females) were studied. Informed consent was obtained from all individuals or responsible person prior to enrollment and the study protocol was submitted to and approved by the FIOCRUZ Ethics Committee. Patients were recruited from both the Center for Hematological Studies (Fundação Hemocentro da Bahia, HEMOBA) and the University Hospital, Federal University of Bahia (Hospital Universitário Professor Edgar Santos, Universidade Federal da Bahia). Mean patient age was 13.17 ± 9.71 years (range: 1.6-51.5 years).

Hematological analyses were carried out using an electronic cell counter (Coulter Count T890). Hemoglobin type was determined by electrophoresis on cellulose acetate strips at pH 8.4, and the presence of HbS was confirmed by sickling and solubility tests, and by electrophoresis on agar-citrate at pH 5.3 (13). HbF was measured by alkali denaturation (13). DNA was isolated from peripheral blood leukocytes (14). $ß^S$-Haplotypes were established by PCR and by dot-blot methods that characterize DNA polymorphisms of the 5' flanking region and the

second intervening sequence (IVS-II) of the γ-globin genes (15,16) (Figure 1).

The EPI Info (version 6.04) and Statistical Package for the Social Sciences (SPSS, version 6.1) programs were used for statistical analyses. The effects of age category, gender, and HbF concentration ≥10% on the hematological parameters were evaluated. The level of significance was set at $P < 0.05$ in all analyses.

Results

The patients had a median (± SD) hemoglobin concentration of 8.369 ± 1.632 g/dl, median hematocrit of 25.044 ± 5.03%, median cell volume of 88.488 ± 10.033 fl, median cell hemoglobin of 30.095 ± 4.195 pg, and median cell hemoglobin concentration of 33.905 ± 1.679 g/dl. These parameters did not vary significantly between age and gender categories. However, patients with HbF ≥10% were found to have significantly higher Hb concentrations compared to patients of the group with Hb <10% (median: 7.8 *vs* 9.0; P = 0.002) and hematocrit values (median: 24.00 *vs* 28.00; P = 0.003).

The hematological data and the ßS-haplotypes/genotypes obtained for the 80 sickle cell disease patients analyzed are listed in Table 1.

The hematological data, including the different proportions of HbF found, are reported in Table 2. Median age was significantly higher among the CAR/CAR and CAR/BEN genotypes. The median HbF levels among the CAR/CAR, CAR/BEN, BEN/BEN and CAR/atypical (Atp) genotypes are shown in Figure 2. The patient group presenting HbF ≥10% consisted of 25 (31.2%) individuals; the genotype was CAR/BEN in 12, CAR/CAR in four, BEN/BEN in seven, SEN/Atp in one, and Atp/Atp in one. There was no CAR/Atp or BEN/SEN genotype in this group. In the group with HbF higher than 10%, eight subjects presented HbF ≥15%, with the genotype being CAR/CAR in one of

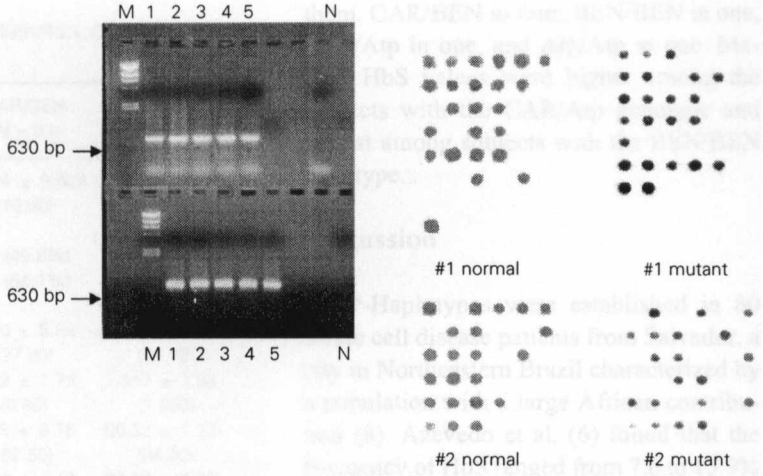

Figure 1. Gamma-globin gene amplification and dot-blot analyses of CAR ßS-haplotype. Sickle cell disease patients, heterozygous for the CAR haplotype, have a positive signal with normal and mutant probes. Homozygous patients have a positive signal only with a mutant probe and negative patients for this haplotype have a signal only with a normal probe. Lanes 1-5 show a 630-bp PCR fragment from the $^{G}\gamma$-globin. M = λ *Hin*dIII marker; N = negative control.

Table 1. Hematological data of the 80 sickle cell disease patients (40 males and 40 females) from Salvador, Bahia, Brazil, and ßS-haplotype frequencies and genotype frequencies found among the 80 sickle cell disease patients.

	Mean ± SD (median)
Age (years)	13.179 ± 9.715 (10.55)
Hematological data	
Ht (%)	25.044 ± 5.030 (24.00)
Hb (g/dl)	8.369 ± 1.632 (8.300)
MCV (fl)	88.488 ± 10.033 (91.00)
MCH (pg)	30.095 ± 4.195 (30.00)
MCHC (g/dl)	33.905 ± 1.679 (34.00)
HbF (%)	8.253 ± 4.636 (8.20)
HbS (%)	89.876 ± 4.476 (90.00)
Haplotypes/genotypes	
CAR/CAR	17 (21.25%)
BEN/BEN	17 (21.25%)
CAR/BEN	37 (46.25%)
CAR/Atp	6 (7.5%)
BEN/Atp	1 (1.25%)
BEN/SEN	1 (1.25%)
Atp/Atp	1 (1.25%)

Hb = hemoglobin; HbF = fetal hemoglobin; HbS = sickle cell hemoglobin; Ht = hematocrit; MCH = median cell hemoglobin; MCHC = median cell hemoglobin concentration; MCV = median cell volume; Atp = atypical; BEN = Benin; CAR = Central African Republic; SEN = Senegal.

Table 2. Characterization of patients with the CAR/CAR, BEN/BEN, CAR/BEN and CAR/Atp genotypes.

	CAR/CAR (N = 17)	BEN/BEN (N = 17)	CAR/BEN (N = 37)	CAR/Atp (N = 6)
Age	16.72 ± 12.89 (11.05)	8.213 ± 4.651 (6.70)	14.34 ± 9.904 (10.60)	7.267 ± 1.193** (7.80)
Gender (N, %)				
Male	10 (58.8%)	8 (47.0%)	17 (45.9%)	3 (50.0%)
Female	7 (41.2%)	9 (53.0%)	20 (54.1%)	3 (50.0%)
Hematological data				
Ht (%)	23.67 ± 2.934 (23.50)	24.44 ± 5.49 (24.50)	26.00 ± 5.82 (27.00)	23.667 ± 3.79 (22.00)
Hb (g/dl)	7.88 ± 0.824 (7.950)	8.15 ± 1.89 (8.350)	8.79 ± 1.79 (8.80)	7.867 ± 1.03 (7.600)
MCV (fl)	86.29 ± 8.18 (88.00)	90.54 ± 12.39 (91.00)	86.78 ± 9.76 (87.50)	90.33 ± 7.23 (94.00)
MCH (pg)	29.00 ± 3.21 (29.50)	30.91 ± 4.99 (32.00)	29.72 ± 4.43 (30.00)	30.33 ± 3.79 (32.00)
MCHC (g/dl)	33.25 ± 1.49 (33.50)	34.18 ± 1.08 (34.00)	34.11 ± 2.11 (34.00)	33.33 ± 1.53 (33.00)
HbF (%)	7.544 ± 4.342 (7.350)	9.882 ± 3.558 (9.600)	8.146 ± 4.631 (7.800)	4.180 ± 2.250* (4.000)
HbS (%)	90.434 ± 4.01 (90.530)	88.010 ± 3.66 (88.170)	90.005 ± 4.51 (90.44)	94.432 ± 2.10* (94.490)

Data are reported as means ± SD (median). Atp = atypical; BEN = Benin; CAR = Central African Republic. For other abbreviations, see legend to Table 1.
*P < 0.05 for HbF and HbS concentrations between the different genotypes (Kruskal Wallis test). **P < 0.05 for age between the different genotypes (ANOVA + chi-square test).

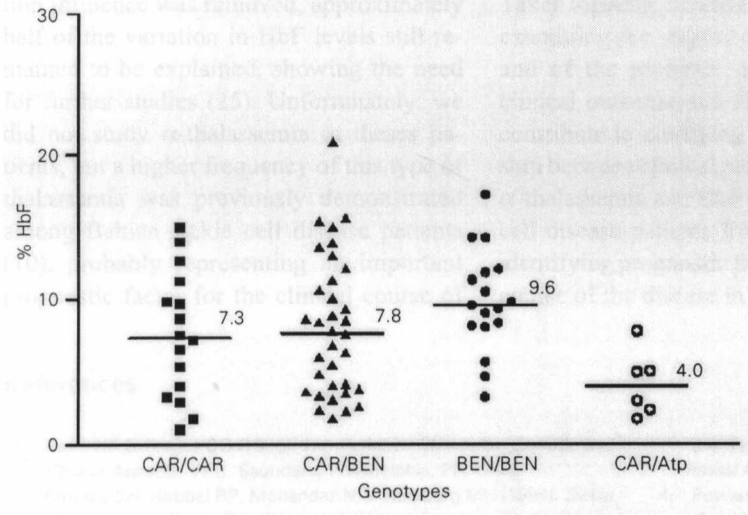

Figure 2. Distribution of median fetal hemoglobin (HbF) levels among the CAR/CAR (N = 17), CAR/BEN (N = 37), BEN/BEN (N = 17) and CAR/Atp (N = 6) genotypes in sickle cell disease patients from Salvador, Bahia, Brazil. For abbreviations, see legend to Table 2.

them, CAR/BEN in four, BEN/BEN in one, SEN/Atp in one, and Atp/Atp in one. Median HbS values were higher among the subjects with the CAR/Atp genotype and lowest among subjects with the BEN/BEN genotype.

Discussion

β^S-Haplotypes were established in 80 sickle cell disease patients from Salvador, a city in Northeastern Brazil characterized by a population with a large African contribution (8). Azevedo et al. (6) found that the frequency of HbS ranged from 7.6 to 15.9% in different population groups of Salvador. In the present study, the CAR/BEN genotype was predominant. Unexpectedly, the BEN and CAR homozygous genotypes were found to occur at similar frequencies, mainly considering the high presence of the CAR haplotype. Verger (11) emphasized that from 1678 to 1814, only 39 of 1770 ships that exported tobacco from Bahia went to the Congo and Angola, where they captured slaves representing a possible contribution of Africans from Atlantic Central Africa. All the other ships went to Coast of Mine ports. The slave traffic from Atlantic Central Africa was supposedly intensified between 1815 and 1824 (11), a fact that can explain our results. No Saudi Arabian or Cameroon haplotypes were identified in the study sample, and only one SEN haplotype was encountered.

In the United States and Jamaica, the BEN haplotype is predominant, a result of the preference for the traffic of Midwestern Africans to these regions during the British Atlantic slave trade (4,17,18). In contrast, haplotype studies on the Cuban and Puerto Rican populations have found a predominance of genes from the Bantu haplotype, suggesting a different African origin of these populations (5,19-21).

The distribution of β^S-haplotypes in the Brazilian State of São Paulo (Southeastern

Brazil) and Pará (Northern Brazil) showed high frequencies of the CAR haplotype, i.e., 62.2 and 65.9%, respectively (9,22-24). Analyses of $ß^S$-haplotypes from the Amazon region have indicated a 60% frequency of the CAR haplotype, a 30% frequency of the SEN haplotype, and a 10% frequency of the BEN haplotype (24).

Populations with a high frequency of BEN/CAR heterozygotes, as reported for Bahia, provide an excellent cohort for the study of the effect of $ß^S$-haplotypes on the clinical course of sickle cell anemia. An important finding of the present study was the high concentration of HbF among individuals with the CAR/CAR genotype, which normally present a median HbF value below 5.0% (4). It is well known that HbF levels in sickle cell anemia could be influenced by age, gender, α-globin gene number, ß-globin haplotype, and the X-linked F-cell production locus that regulates the production of HbF-containing erythrocytes (F cells) (25).

In a previous study, the F-cell production locus accounted for 40% of the overall variation of HbF levels and the ß-globin haplotype was associated with 14% of the remaining HbF variation; when the F-cell production influence was removed, approximately half of the variation in HbF levels still remained to be explained, showing the need for further studies (25). Unfortunately, we did not study α-thalassemia in theses patients, but a higher frequency of this type of thalassemia was previously demonstrated among Bahian sickle cell disease patients (10), probably representing an important prognostic factor for the clinical course of the disease.

The presence of high HbF levels in the CAR/CAR genotype could be explained by sequence variation in regulatory regions of the 5' HS2 and 5' flanking region of the γ-gene expression, as previously discussed by Lanclos et al. (26).

In addition, we also identified an individual with the SEN haplotype, a fact that may suggest that Bahia State also had a gene flow from Atlantic West Africa, as was the case for other Brazilian states (24). Internal migration is unlikely since the patient's ancestors were from Salvador. The low frequency of the SEN haplotype could be explained by the absence of SEN carriers looking for medical care or a recent origin of the $ß^S$ SEN mutation in this population (27). The atypical haplotypes showed different distributions and could be found associated with the CAR and BEN genotypes, indicating the occurrence of diverse genetic mechanisms that could be responsible for the variation of HbF concentrations among the atypical haplotype carriers (28-30).

The present results are relevant to the study of slave traffic routes in Brazil and of the African origins of the Bahian population. Taken together, the data indicate that studies examining the impact of the $ß^S$-haplotypes and of the presence of α-thalassemia on clinical outcome and HbF expression may contribute to clarifying a possible relationship between clinical picture, $ß^S$-haplotypes, α-thalassemia and HbF production in sickle cell disease patients from Salvador, Bahia, identifying prognostic factors for the clinical course of the disease in this population.

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10.3 Termo de Consentimento

TERMO DE CONSENTIMENTO

Eu, _____, com 18 anos de idade ou mais (nome do pai ou responsável), detentor de integral competência, dou consentimento para participar como voluntário do estudo denominado "**Anemia falciforme em Salvador-Bahia: caracterização fenotípica, molecular e de seqüências gênicas potencialmente importantes na expressão dos genes gama da hemoglobina fetal**", sob a coordenação de Dra. Marilda de Souza Gonçalves. As implicações de sua participação voluntária, incluindo a natureza, duração e objetivo do estudo, os métodos e meios através dos quais deve ser conduzido e as inconveniências e riscos que podem ser naturalmente esperados foram explicados por _____(nome do investigador no(a)_____(endereço e telefone).

Entendo também que eu tenho permissão para a qualquer momento revogar o meu consentimento e retirar o paciente do estudo sem sofrer nenhuma punição ou perda de direitos. Entretanto, o paciente poderá ser solicitado a realizar exames , caso o médico que o assiste, julgue-os necessários para sua saúde e bem estar. Minha recusa em permitir que meu filho ou tutelado participe do estudo não resultará em punições ou perdas de benefícios a que ele/ela tenha direito.

Nome do responsável (letra de forma)_____

Assinatura do responsável_____Data___/___/___

Endereço_____

Número de identidade_____

Número no estudo_____

Explicação do termo de consentimento

Título do estudo

"Anemia falciforme em Salvador-Bahia: caracterização fenotípica, molecular e de seqüências gênicas potencialmente importantes na expressão dos genes gama da hemoglobina fetal".

Investigador principal

Dra. Marilda de Souza Gonçalves – CPqGM – FIOCRUZ / FAR - UFBA

Informações sobre a sua participação

A anemia falciforme é uma doença grave e os indivíduos transmissores da herança representam 7% da nossa população. Por isso, é importante realizar estudos que possam ajudar a compreender melhor esta doença e também colaborar para o surgimento de um novo tipo de tratamento.

Por esse motivo, você está sendo convidado a participar de uma pesquisa médica, que envolverá diagnóstico, assistência e informações sobre esta anemia hereditária. É de grande importância que você entenda os princípios gerais que se seguem e que serão aplicados a todos os participantes do nosso estudo: a) sua participação é totalmente voluntária; b) você poderá interromper sua participação antes ou em qualquer momento do estudo. Sua recusa em participar não envolverá punições ou perda de seus direitos constituídos; c) depois de lidas as explicações, você pode fazer qualquer pergunta necessária ao seu entendimento

Objetivo do estudo

O objetivo deste estudo é fornecer o diagnóstico desta anemia, com as suas características, fornecendo acompanhamento médico aos portadores e proporcionando uma melhor qualidade de vida aos mesmos. Caso você concorde em participar, deve permitir a coleta de 5 mL de sangue, que será realizado pela equipe responsável no CPqGM - FIOCRUZ

O tempo previsto para a realização do nosso estudo será de aproximadamente 04 (quatro) anos. Entretanto, todos os portadores da anemia serão assistidos pela equipe médica do HEMOBA para acompanhamento clínico e aconselhamento genético.

O sangue será coletado através da utilização de materiais novos, estéreis e descartáveis, por pessoal habilitado e especializado.

Benefícios

A participação neste projeto proporcionará benefícios aos indivíduos portadores desta anemia, uma vez que possibilitará a realização do acompanhamento clínico, laboratorial e aconselhamento genético.

Os registros da sua participação ou da criança no estudo serão mantidos confidencialmente, sendo do conhecimento dos participantes do projeto e do médico que o acompanha.

Novos achados significativos

Qualquer informação importante que surgir durante a sua participação no estudo e que possa contribuir para o melhor desenvolvimento clínico da doença em estudo será levada imediatamente ao seu conhecimento e do seu médico.

Pessoas e locais a serem contactadas para a obtenção de respostas, e formulação de perguntas e maiores informações

Por favor entre em contato com uma das pessoas abaixo caso você necessite de maiores esclarecimentos.

Dra. Marilda de Souza Gonçalves – Coordenadora do projeto – Laboratório de Biologia Molecular do CPqGM – FIOCRUZ Tel: 356-8783 R- 265

Elisângela Vitória Adorno - Farm. Bioquímica que desenvolverá a tese de doutorado com a realização do presente projeto no Laboratório LPBM – CPqGM – FIOCRUZ Tel : 356-8783 R- 265

Caso você não tenha entendido alguma parte deste documento/explicação, pergunte ao investigador antes de assinar

Atesto o recebimento da cópia deste acordo, que é constituído pelos termos de explicação e de consentimento.

Assinatura do paciente_____ Data____/____/____

Nome do paciente (letra de forma)_____

Assinatura da testemunha 1_____ Data____/____/____

Nome da testemunha 1 (letra de forma)_____

Assinatura da testemunha 2_____ Data____/____/____

Nome da testemunha 2 (letra de forma)_____

10.4 Questionário

QUESTIONÁRIO EMPREGADO NOS PACIENTES OU RESPONSÁVEIS

Nome:_____

Endereço:_____

Telefone:_____Nascimento:___/___/___ Profissão:_____

01. Grupo Racial:

( ) Branco ( ) Mulato Claro ( ) Mulato Médio ( ) Mulato Escuro ( ) Negro

02. Conhece casos de Anemia Falciforme na família?_____

03. Já fez transfusão sangüínea?_____

Em caso de SIM, quantas vezes?_____

Qual o período da última transfusão?_____

04. Já esteve internado por causa da anemia?_____

Em caso de SIM, quantas vezes?_____ Qual o período?_____

05. Já esteve internado por probelma de infecção? _____

Em caso de SIM, quantas vezes?_____ Qual o período?_____

Qual o tipo de infecção?_____

06. Já teve Acidente Vascular Cerebral (AVC)?_____

Em caso de sim, qual o período? _____