

tions recommended by the manufacturer. Family studies allowed the unequivocal association of Hb Porto Alegre with Mediterranean haplotype I⁸, and with framework 1⁸ and also revealed its segregation, in *cis*, with the codon 27 novel intragenic polymorphism.

Because of historical data, we hypothesized whether Hb Porto Alegre described in Brazil could have a Portuguese origin, and in order to answer this question, the same molecular approach was performed in the Brazilian family described by Gonçalves *et al.*³ As we expected, the Brazilian case presented the same, above described, genetic background.

As suggested by Malcorra-Azpiazu and colleagues in 1993,⁵ the several cases of Hb Porto Alegre described in the Canary Islands could be indicative that the mutational event giving rise to this variant had occurred there. Subsequently, the mutation could have spread to Spanish colonies in South America. This hypothesis is, in fact, compatible with historical data. The indigenous inhabitants of the Canary Islands – the Guanches – were occasionally visited by a variety of groups: Romans (40 B.C.), then the Arabs (999), and then in the 13th and 14th centuries many European conquerors, including the Portuguese. In the 15th century the Islands was contended between the Portuguese and Spaniards until 1479, when the *Treaty of Alcáçovas* declared Spanish sovereignty over the islands. These islands became an important base for voyages to the Americas.⁹ However, knowing that Hb Porto Alegre has been found in the Portuguese population, another hypothesis could be forwarded. It is possible that Portuguese carriers of Hb Porto Alegre left progeny on Canary Islands and that the mutation subsequently expanded to the South America in the 15th and following centuries. Furthermore, it is known that after the discovery of Brazil by the Portuguese in 1500, a massive transmigration of the Portuguese population to Brazil occurred until the mid 20th century.¹⁰ In conclusion, based on our molecular findings, and considering the above historical data, we propose that Hb Porto Alegre had one original mutational event in the Portuguese population and that it was spread to South America, namely to Brazil.

This hypothesis could find support from studies of the genetic background of the Spanish and Cuban Hb Porto Alegre cases and efforts were made to contact the laboratories where these cases had been characterized. We have obtained information that in Cuba another case of the variant was detected, curiously in a family of Portuguese origin with the surname Ferreira (*G. Martinez, personnel communication*). Unfortunately, none of the above mentioned cases of Hb Porto Alegre was accessible for molecular studies.

Paula Faustino,* Armandina Miranda,[†] Isabel Picanço,*
Elza M Kimura,[‡] Fernando Ferreira Costa,[§]
Maria de Fátima Sonati[¶]

*Laboratório de Biologia Molecular, Centro de Genética Humana, Instituto Nacional de Saúde Dr Ricardo Jorge (INSA), Lisboa, Portugal; †Laboratório de Hematologia, Centro de Biopatologia, INSA, Lisboa, Portugal; ‡Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil; §Centro de Hematologia e Hemoterapia, UNICAMP, Campinas, Brazil

Acknowledgments: the authors thank Sónia Pedro for automatic sequencing. We also thank G. Martinez (Cuba), A. Kutlar (USA), A. Villegas and A. González (Spain) for information given about their Hb Porto Alegre studies.

Key words: hemoglobin variant, hemoglobinopathies, HBB gene.

Correspondence: Paula Faustino, PhD, Centro de Genética Humana, Instituto Nacional de Saúde Dr Ricardo Jorge, Av. Padre Cruz 1649-016 Lisboa, Portugal. Phone: international +351.217519234. Fax: international +351.217526410. E-mail: paula.faustino@insa.min-saude.pt

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Red Cell Disorders

Simultaneous detection of α -thalassaemia and β -thalassaemia by oligonucleotide microarray

In this study, we describe a reliable microarray-based assay for the simultaneous detection of α/β -globin genotypes. The efficiency and specificity of this method were evaluated by blinded analysis of 1,880 samples. The assay provides unambiguous detection of complex combinations of heterozygous, compound heterozygous and homozygous α/β -thalassaemia genotypes.

haematologica 2004; 89:1010-1012

(<http://www.haematologica.org/2004/8/1010>)

Thalassaemia is most common genetic disorder in Asia. The approach to dealing with the problem of thalassaemia is to prevent and control the birth of new cases. This requires an accurate identification of couples at high risk of thalassaemia. A previous study has shown that microarrays can be used to detect thalassaemia.¹ In this study, we have developed a microarray-based assay for rapid detection of the common severe thalassaemia defects found in Asia. This screening test can accurately give a specific diagnosis of the thalassaemia genotype. To identify mutations of α/β -thalassaemia, a total 38 of oligonucleotide probes are immobilized on slides and a single hybridization is performed with fluorescence-labeled multiplex polymerase chain reaction (PCR) products. Hybridization is detected by fluorescence scanning, and the α/β -globin genotypes are assigned by quantitative analysis of the hybridization results.

A pair of probes was designed for each point mutation, one complementary to the normal sequence and the other to the variant. The probes are 15~20mer oligonucleotides with the mismatch base placed in the center of the

Table 1. Sequences of oligonucleotide probes for thalassaemia.

Probes	Mutation	Sequences
A1	(-28)(-29)-W	GGG CAT AAA AGT CAG
A2	(-28)-M	GGG CAT <u>A</u> GA AGT CAG
A3	(-29)-M	TGG GCA T <u>G</u> A AAG TCA
B1	β (14-15)-W	CTG CCC TGT GGG GCA AG
B2	β (14-15)-M	CTG CCC T <u>G</u> G TGG GGC AA
C1	β (17)-W	GTG GGG CAA GGT GAA
C2	β (17)-M	GTG GGG C <u>T</u> A GGT GAA
D1	HbE26-W	TGG TGG TGA GGC CCT
D2	HbE26-M	TGG TGG T <u>A</u> A GGC CCT
E1	β (27-28)-W	GTG AGG CCC TGG GC
E2	β (27-28)-M	TGA GGC C <u>C</u> C TGG GC
F1	IVS(I-1)-W	TGG GCA GGT TGG TAT
F2	IVS(I-1)-M	TGG GCA G <u>T</u> T TGG TAT
G1	β (71-72)-W	GTG CCT TTA GTG ATG GC
G2	β (71-72)-M1	GTG CCT TTA AGT GAT GG
G3	β (71-72)-M2	GTG CCT T <u>T</u> I AGT GAT GG
H1	IVS(I-5)-W	CAG GTT GGT ATC AAG
H2	IVS(I-5)-M	CAG GTT G <u>C</u> T ATC AAG
I1	β (37)-W	TAC CCT TGG ACC CA
I2	β (37)-M	TAC CCT T <u>A</u> G ACC CA
J1	β (41)-W	CCA GAG GTT CTT TGA G
J2	β (41)-M	CAG AGG T <u>T</u> T CTT TGA G
K1	β (41-42)-W	CAG AGG T <u>T</u> C TTT GAG T
K2	β (41-42)-M	CAG AGG TTG AGT CCT T
L1	β (43)-W	GGT TCT TTG AGT CCT TT
L2	β (43)-M	GGT TCT TTT AGT CCT TT
M1	IVS(II-654)-W	TGC TAT TGC CTT AAC
M2	IVS(II-654)-M	TGC TAT TAC CTT AAC
N1	IVS(II-5)-W	AGG GTG AGT CTA TGG
N2	IVS(II-5)-M	AGG GTG ACT CTA TGG
O1	CS-W	CCA GCT TAA CGG TAT
O2	CS-M	CCA GCT TGA CGG TAT
P1	QS-W	CTT GTC CAG GGA GGC
P2	QS-M	CTT GTC CGG GGA GGC
Q1	RT1	CAG AGA GAA CCC AGG
Q2	RT2	GGG AAA AAA CTC AGG
Q3	SEA	CAT CAC TCA GCT TCT C
Q4	LT	GTT TCG CTG TTG TTT TCC

Each oligonucleotide probe has a 8 dT linker and amino-group at its 5'-end. The mutation sites in each probe are in underlined. The probes detect 16 β -thalassaemia mutations [β (41-42)(-TCTT), IVSII-654(C->T), β 17(A->T), -28(A->G), β (71-72)(+A), β (71-72)(+T), HbE26(G->A), -29(A->G), β (27-28)(+C), IVS1-1(G->T), IVS1-5(G->C), β (14-15)(+G), IVSII-5(G->C), β 41(+T), β 37(G->A), and β 43(G->T)] and 5 α -thalassaemia mutations [three deletions of - $\alpha^{3,7}$, - $\alpha^{4,2}$, and - α^{SEA} ; two nondeletions of $\alpha^{2\text{Hong_Sea}}$ codon a125(T->C) and $\alpha^{\text{constant Spring}}$ codon α 142(T->C)].

sequence. For deletion of α -thalassaemia, the probes were designed to detect whether a gap-PCR product exists or not. All probes are shown in Table 1. These probe oligonucleotides were immobilized on the surface of a silylated-slide(CSS-100, CEL, USA) in triplicate.

Multiplex PCR reaction was performed for genotyping α/β -thalassaemia using the LA-Taq PCR kit (TaKaRa, Japan). Gap-PCR, in multiplex PCR, were used to simultaneously amplify the α -globin genes with four primer pairs: ctttccctaccagagccagggt and gcccatgctggcaggtttctgagg for - $\alpha^{3,7}$, tagagcattggtggtcatgc and tctgccacccttctctgag for - $\alpha^{4,2}$,

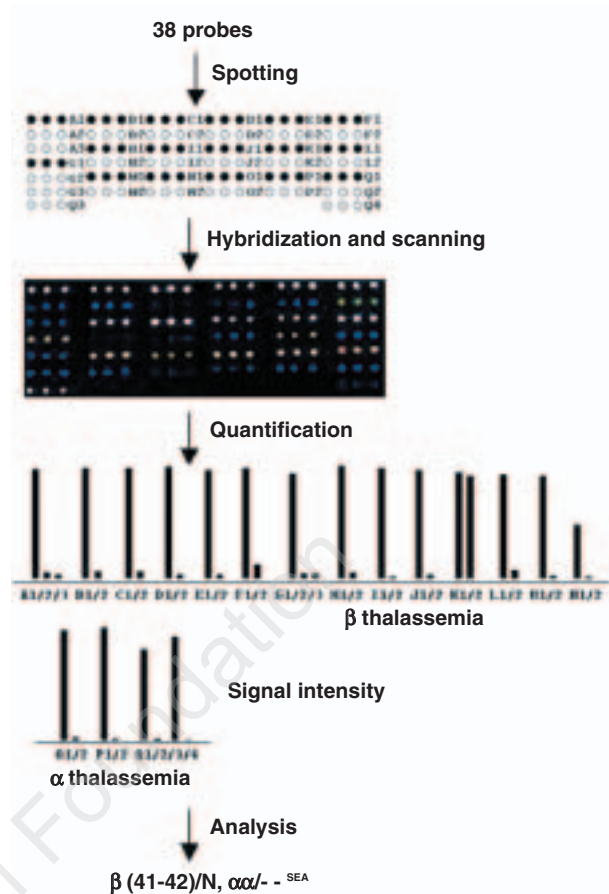


Figure 1. The procedure for analysis of hybridization data is illustrated for the sample (β (41-42)/N heterozygote, $\alpha\alpha/-\text{SEA}$). All oligonucleotide probes were spotted and named (refer to Table 1 for probes). After hybridization, signal intensities were quantified.

cttcgctgactccaataaaa and ttgagacgatgctgtgctttg for - α^{SEA} , ctttccctaccagagccagggt and ccattgttggcattccgggaca for the $\alpha 2$ gene ($\alpha\alpha$). Meanwhile, primers (agggttggccaattactacc and ccagccttatccaaccata) for β thalassaemia were designed to amplify the fragment containing all mutations. Since each of the - $\alpha^{3,7}$, - $\alpha^{4,2}$, - α^{SEA} deletions either partially or completely removes the $\alpha 2$ gene, its positive amplification was used to indicate heterozygosity when a deletion allele was also present.² The multiplex PCR product was used for linear amplification with fluorescently labeled primers: 5'-Cy5-gcgatctgggctctgtgttct for a 253-base-long fragment of - α^{SEA} , 5'-Cy5-ctctcaggg cagaggatcac for a 384-base-long fragment of - $\alpha^{3,7}$ mutant and a 378-base-long fragment of normal $\alpha 2$ gene, 5'-Cy5-gcagaggttgacgtgagccta for a 155-base-long fragment of - $\alpha^{4,2}$, 5'-Cy5-tctcccctt cctatgacatga for a 555-base-long fragment of exon 1 and exon 2 of the *HBB* gene, 5'-Cy5-atgcctctt tgaccattct for a 183-base-long fragment of exon 3 of the *HBB* gene. Short single-stranded products generated from linear PCR demonstrated much higher hybridization efficiency when applied to the oligonucleotide array than did long double-stranded PCR products.³

Following hybridization and washing, the microarray slide was scanned on a laser scanner (ScanArray5000, General Scanning, USA). The fluorescent signal from each spot was

calculated using the Genepix Pro3.0 software (Axon, USA). In quantitative analysis, the wild-type-to-mutant signal ratios (W/M) is the spot of wild type probe (denoted as W) to mutant probe (denoted as M), can be employed to detect the genotype of point mutations. We employed an intensity ratio of 5:1 as the threshold for categorizing the positive and negative signals. Used to detect point mutations, the W/M produced unequivocal assignment of samples genotype, with W/M > 5 for normal subjects, ~1 for heterozygotes, and < 0.2 for homozygous mutations. The Q-probe group was used to detect three deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $-\alpha^{SEA}$). Q1 probe was complementary to a 378-base-long fragment of normal $\alpha 2$ gene produced by 5'-Cy5-ctctcagggcagag-gatcac. Q2 probe was complementary to a 384-base-long fragment of the mutation ($-\alpha^{3.7}$) produced by 5'-Cy5-ctctcagggcagag-gatcac. Q3 probe was complementary to a 253-base-long fragment produced by linear amplification of PCR product ($-\alpha^{SEA}$) by 5'-Cy5-gcgatctggcctctgtgtct. Q4 probe was complementary to a 155-base-long fragment produced by linear amplification of PCR product ($-\alpha^{4.2}$) by 5'-Cy5-gcagaggttgagctgagcta. When the signal of a deletion ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $-\alpha^{SEA}$) allele was present, the Q1 probe was used to provide a reliable identification of heterozygous, compound heterozygous and homozygous α thalassemia genotypes. Again, we employed an intensity ratio of 5:1 as the threshold to categorize the positive and negative signals in the Q probe group. The hybridization result with a sample ($\beta(41-42)$ heterozygote, $\alpha\alpha/-\alpha^{SEA}$) is shown in Figure 1. It is apparent from these results that the discrimination of single-base mismatches is excellent. In addition, the results demonstrate the microarray's ability to detect and discriminate both homozygous and heterozygous mutations in human genomic DNA.

To demonstrate the accuracy of the method in genotyping thalassemia, we investigated 1,880 unrelated individuals whose thalassemia alleles had been typed previously. There was 100% agreement in all 1,880 samples. Based on these results we conclude that the method described here is a valuable technique, suitable for population screening.

Ye Bang-Ce, Liu Hongqiong,
Zhang Zhuanfong, Lei Zhengsong, Gao Jianling

*State Key Laboratory of Bioreactor Engineering, East China University of Science & Technology, Shanghai, 200237, China;
°Jiangnan Biotechnology Inc. Huzhou, Zhejiang, 313000, China

Funding: This work was supported in part by 863 Program of China (2002AA222043).

Acknowledgments: we are grateful to Dr. Li Dehao (the Family Planning Bureau of Hainan) and Mr. Lin Weixiong (the First Affiliated Hospital of Guangxi Medical University), who kindly provided us samples for this study.

Key words: thalassemia, microarray, genotyping, population screening

Correspondence: Ye Bang-Ce, State Key Laboratory of Bioreactor Engineering, East China University of Science & Technology, Shanghai, 200237, China. Phone: international +86.21.64252250.

Fax: international + 86.21.64253904. E-mail: bcy@ecust.edu.cn

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Lack of mutations in the human telomerase RNA component (*hTERC*) gene in Fanconi's anemia

As some patients with Fanconi's anemia (FA) present excessive telomere shortening correlating with poor outcome, we investigated whether human telomerase RNA component (*hTERC*) mutations also play a role in telomere shortening in 115 FA patients. Only one patient was heterozygous for the G58A polymorphism. No other mutation or deletion was found. We conclude that *hTERC* gene mutations do not contribute to telomere shortening in FA.

haematologica 2004; 89:1012-1013

(<http://www.haematologica.org/2004/8/1012>)

Fanconi's anemia (FA) is an autosomal recessive disorder clinically characterized by progressive pancytopenia due to bone marrow (BM) failure frequently evolving to acute leukemia, a variety of physical abnormalities, and increased predisposition to cancer.¹ The onset of BM failure may be variable, presenting at older ages in some patients due, at least in part, to a myriad of genetic abnormalities. Eleven complementation groups (A to J) of FA cells have been described.² Increased telomere shortening also correlates with BM failure in FA.³ Telomeres are responsible for the integrity and stability of chromosome ends, and telomere length is maintained by telomerase-mediated addition of telomeric repeats.⁴ Excessive telomere shortening is observed in other types of BM failure syndromes, such as dyskeratosis congenita (DC) and acquired aplastic anemia. Mutations in genes encoding telomerase complex components cause the X-linked type of DC (*DKC1* gene) and the autosomal dominant type of DC (*hTERC* gene).^{1,5} Mutations in the *hTERC* gene are also found in a small subgroup of patients with acquired aplastic anemia.⁶⁻¹⁰

In this study, we investigated whether *hTERC* mutations may also contribute to telomere shortening in FA. Peripheral blood samples were collected from 115 patients with FA before bone marrow transplantation and screened for *hTERC* mutations by direct sequencing, as previously described.^{6,7} Seventy-one were Brazilian patients treated at the Federal University of Paraná, Curitiba, PR, Brazil (37 males and 34 females; 42 were White, 25 Mulatto, seven Black and one Asian); the diagnosis was confirmed by diepoxybutane-induced chromosome breakage analysis. Complementation group analysis was available for 39 Brazilian patients (30 belonged to the FANC A group, four to the FANC C group, and five to the FANC G group). The other 44 cases studied were European patients treated at Hôpital Saint-Louis, Paris, France, and had a Caucasian ethnic background. Fifty-seven percent of these patients were male and 43% were females. Their diagnoses were confirmed by cytogenetics and nitrogen mustard-induced chromosome breakage analysis. Complementation group analysis was available for 17 patients (16 were FANC A and one was FANC C). Telomere length was determined in peripheral blood leukocytes by Southern blot in all European patients, as previously reported.³ Nineteen patients had a telomere shortening below 200 bp/year, whereas in 25 patients telomere shortening was greater than 200 bp/year. Blood samples for genetic analysis were collected after informed consent had been obtained for this study which was approved by the respective local Ethics Committees.

One patient (a 12-year old, African-Brazilian male with FANC A complementation group, homozygous for the 3788-