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Identification of the Hb Lepore phenotype by HPLC

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ABSTRACT

Background and Objectives. **Hb Lepore is a structurally abnormal hemoglobin in which the abnormal globin chain is a hybrid or fused globin chain (δβ). Three different Lepore hemoglobins have been identified, differing from each other in the point at which the δβ fusion occurs; Hb Lepore Hollandia (δ22/β50), Hb Lepore Baltimore (δ59/β86) and Hb Lepore Boston (δ87/β116). In Spain only Hb Lepore Boston and Hb Lepore Baltimore have been identified. Hb Lepore is easily detected by electrophoretic and chromatographic studies, whereas the type of Hb Lepore is distinguished by chromatography of tryptic peptides of abnormal δβ chain. In this work, we show an easier chromatography technique for identifying the Hb Lepore phenotype.**

Design and Methods. **Thirteen different unrelated families (23 patients) from different parts of Spain were studied. The existence of Hb Lepore was diagnosed by standard methodology and quantified by ionic exchange HPLC. The globin chains were studied by reversed phase HPLC, which showed us the phenotype of Hb Lepore; this phenotype was corroborated by a gold standard test using molecular biology techniques. The statistical analysis was designed to determine the behavior of the quantitative (hematologic) variables using the independent variable (Hb Lepore Baltimore or Hb Lepore Boston) categorized by Student's *t* test for independent groups. The distribution of the variable was established using theoretical models and the variance homogeneity hypothesis was tested. The validity of the hematologic data was estimated by creating a receiver operating characteristic (ROC) curve.**

Results. **In the study of globin chains by reversed phase HPLC, in 14 patients (8 families) three peaks were eluted; they corresponded to α, β and δβ globin chains. In these cases when DNA was studied by PCR followed by digestion with the restriction enzyme Pvu II, the phenotype of Hb Lepore was identified as being the Boston variant, whereas in the rest of patients (9 in total), the peak identified with hybrid chain globin (δβ) was not present and the molecular study showed that these patients were heterozygotes for Hb Lepore Baltimore.**

Interpretation and Conclusions. **The study of globin chains by reversed phase HPLC is sufficient to know the phenotype of Hb Lepore and thus tedious techniques such as chromatography of tryptic digestion product of δβ abnormal chains are not essential, a particularly important consideration in those laboratories that do not have the possibility of carrying out molecular biology studies. Neverteheless, we should continue to use a gold standard molecular biology test in cases of prenatal diagnosis because this technique is the most exact and reproducible that we have.**

Key words: PCR, Hb Lepore Boston, Hb Lepore Baltimore, HPLC, Phenotype

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Hb Lepore is a fusion hemoglobin that contains mutant non-α globin chains which arise from unequal crossing over resulting in globin chains which contain an amino acid sequence identical to the carboxy-terminal end of the human β globin chain and an amino terminal amino acid sequence which is identical to that found in the δ globin chain.[1]

Three variants of Hb Lepore have been described according to the crossover breakpoint and to the amino acid residues at positions 50, 86 and 87 of the non-α chain: Hb Lepore Boston (δ87/β116),[2] Hb Lepore Baltimore (δ50/β86)[3] and Hb Lepore Hollandia (δ22/β50).[4]

In the heterozygous condition, Hb Lepore constitutes 6-15% of the total hemoglobin, HbA_2 levels are normal or discretely reduced and most subjects have increased HbF levels.[5,6]

Hb Lepore may be easily detected by the electrophoretic analysis of hemoglobins. In alkaline conditions, this hemoglobin separates from HbA and shows similar electrophoretic mobility to that of HbS. Identification and quantification is performed using chromatographic procedures such as ion exchange high power liquid chromatography (HPLC)

although molecular biology techniques are required to establish the Hb Lepore type.[7-9] The present study was designed to explore the possible identification of the Hb Lepore type by analysis of the globin chains using reversed phase HPLC and to establish whether any hematologic variable could be of predictive value for identification of the Hb Lepore phenotype.

Design and Methods

The study population comprised 23 subjects from 13 unrelated families previously diagnosed as Hb Lepore heterozygotes from Valencia, Extremadura, Castilla, la Mancha and Madrid, Spain.

Basic hematologic data, HbA_2, HbF and reticulocyte count were collected by standard methods.[10,11]

Hb Lepore was identified by cellulose acetate electrophoresis at alkaline pH (8.0), isoelectric focusing (IEF) in polyacrylamide (pH=5.5-8.5) and agar gel (pH=6.0). Quantification was performed by ion exchange HPLC.

Analysis of the Hb Lepore globin chains was performed by reversed phase HPLC using Vydac large pore C4 columns and a linear gradient from 47% to 100% of phase B in 80 minutes at a flow-rate of 0.8 mL/min (phase A=30% acetonitrile 0.3% TFA in water; phase B= 70% acetonitrile in water) while molecular procedures, requiring the extraction of genomic DNA from peripheral blood leukocytes[12] and PCR amplification with normal and mutated primers,[7] were used to confirm the phenotype. The resultant DNA fragments were subjected to digestion by the Pvu II restriction enzyme which targets (CAG↓CTG) located in the hybrid sequence of the Hb Lepore Boston fragment.[8]

The DNA samples were inspected for (C→T) polymorphism at position –158 of the $^{G}\gamma$ globin gene.[13,14]

The statistical analysis used Student's *t*-test for independent groups. The distribution of the variable was established using theoretical models and the variance homogeneity hypothesis was tested. The validity of the hematologic data was estimated by a ROC curve. Sensitivity (S), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and positive and negative likelihood ratios were evaluated. By determining the sensitivity and specificity at each point, the cut-off point discriminating the Hb Lepore phenotype was established. Statistical analysis was performed using Windows SPSS v 7.0 and SAS v 6.1 software.

Results

Five families (9 subjects) were Hb Lepore Baltimore carriers while the remaining 8 families (14 subjects) were heterozygous for Hb Lepore Boston.

Table 1 shows the hematologic data obtained divided according to the Hb Lepore phenotype. Microcytosis and hypochromia were observed in all blood samples. HbA_2 was within the normal range, although the Hb Lepore Boston carriers showed higher mean values [2.56% (0.52) vs 1.57% (0.42), p=0.000]. In contrast, higher mean HbF [6.69% (2.86) vs 4.06% (2.42) p=0.039] and Hb Lepore [13.08% (1.69) vs 10.81% (1.59) p=0.012] levels were recorded in the Hb Lepore Baltimore phenotype group.

Table 1. Hematologic data corresponding to Hb Lepore Baltimore and Hb Lepore Boston carriers.

	Phenotype	*N*	*Mean*	*SD*	*SE of the mean*	*p*
RBC (10^{12}/L)	Baltimore	9	5.54	0.60	0.20	0.768
	Boston	11	5.64	0.90	0.27	
Hb (g/dL)	Baltimore	9	13.08	1.69	0.56	0.429
	Boston	11	12.46	1.68	0.51	
PCV (L/L)	Baltimore	9	0.40	4.96	1.65	0.567
	Boston	11	0.38	4.98	1.50	
MCV (fL)	Baltimore	9	71.37	3.95	1.32	0.143
	Boston	11	68.40	4.71	1.42	
MCH (pg)	Baltimore	9	23.50	1.49	0.50	0.092
	Boston	11	22.21	1.75	0.53	
RDW (%)	Baltimore	9	15.6	21.53	0.51	0.387
	Boston	11	16.15	0.93	0.28	
Retic. (10^9/L)	Baltimore	9	12.99	7.65	2.55	0.733
	Boston	11	14.49	11.59	3.50	
HbA2 (%)	Baltimore	9	1.57	0.42	0.14	0.000
	Boston	10	2.56	0.52	0.16	
HbF (%)	Baltimore	9	6.69	2.86	0.95	0.039
	Boston	11	4.07	2.42	0.73	
Hb Lepore (%)	Baltimore	8	13.07	1.69	0.60	0.012
	Boston	9	10.81	1.59	0.53	

The maximum sensitivity and specificity cut-off estimated for HbA_2 was ≥2%. This corresponded to a sensitivity of 90% (CI 59.4-99.7), a specificity of 88.9% (IC 55.9-99.7) and a 90% PPV and 88.9% NPV of post-test likelihood ratio 8.1.

Since the HbF level was high in each subject, the polymorphism (C→T) at position –158 of the $^{G}\gamma$ globin gene was evaluated. This served to establish that 9 subjects were heterozygous for the gene responsible and the remainder (12 subjects) showed no polymorphism. When this was related to the particular Hb Lepore phenotype, it was observed that 77.8% of the subjects with Hb Lepore Baltimore showed polymorphism in one gene while in 83.3% of the Hb Lepore Boston subjects, this mutation was absent from both alleles.

Reversed phase HPLC analysis of the globin chains discriminated the type of Hb Lepore. Thus, in the case of Hb Lepore Boston, three peaks corresponding to the α, β and (δβ) Lepore chains were shown. The hybrid globin chain could not be identified in Hb Lepore Baltimore (Figures 1A and 1B).

In each case, the Hb Lepore phenotype was confirmed at the molecular level. In both phenotypes, a

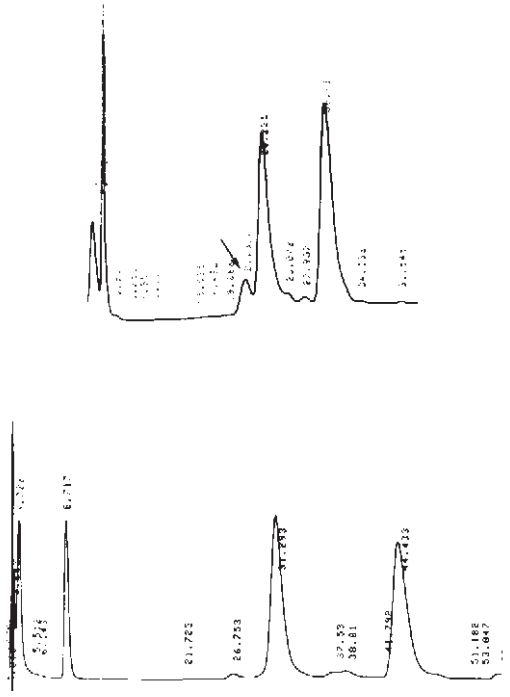

Figure 1. The separation of globin chains by reversed phase HPLC.
A. A chromatogram of Hb Lepore Boston showing a peak in front of the β chain identified as the hybrid δβ chain. B. The chromatogram of Hb Lepore Baltimore shows no anomalous peaks.

915 bp fragment corresponding to the normal β allele and one of 777 bp corresponding to the mutated allele (βδ) were identified. The Pvu II restriction enzyme target (CAG↓CTG) was only identified in the 777 bp fragment corresponding to Hb Lepore Boston. Thus, in this phenotype, enzyme digestion yielded two fragments (one of 603 bp and the other of 174 bp) while in the Hb Lepore Baltimore phenotype only the non-digested 777 bp fragment was detected. The normal 915 bp fragment was present in both hemoglobins.

Discussion

Hb Lepore ($\alpha_2\delta\beta_2$) is a structural hemoglobin variant coded for by a mutated gene formed by the fusion of genes δ and β. The hybrid gene leads to the formation of a reduced quantity of hemoglobin chain due to the unstable nature of the Lepore chain mRNA. Thus, although easily identified in the laboratory, the clinical features of this hemoglobinopathy are similar to those of β thalassemia.[5]

Of the three Hb Lepore types described to date only two have been identified in Spain.[15] The types are differentiated on the grounds of the crossover region which, in turn, conditions the primary structure of the δβ chain. Thus, Hb Lepore Boston has the serine (Ser) and glutamine (Gln) residues at positions 86 and 87 respectively, while the structure of the aberrant chain in Hb Lepore Baltimore involves the replacement of Ser by alanine (Ala) and Gln by threonine (Thr).[16]

The present epidemiologic analysis of hematologic variables shows significant differences in the proportions of HbF, HbA_2 and even Hb Lepore in each type of carrier.

The HbF levels recorded here were discretely higher (5.25±2.88%) than those reported by other authors (Marinucci *et al.* 3.12±2.26%). Despite the fact that the different HbF levels in each Hb Lepore phenotype did not prove to be a predictive factor, it was possible to observe a trend whereby higher HbF levels were observed in Hb Lepore Baltimore than in Hb Lepore Boston (6.69 vs 4.06%, p=0.039). These values were slightly higher than those found by Ribeiro *et al*.[8] in Portugal (4.9 vs 2.75% respectively). This difference is consistent with the possible association of Hb Lepore Baltimore with polymorphism (C→T) of the $^{G}\gamma$ globin gene at position –158 previously related to an increase in HbF[17-19] since, in accordance with the findings of Ribeiro *et al.*, close to 78% of subjects with Hb Lepore Baltimore had this polymorphism in one allele while 83% of the Hb Lepore Boston subjects lack this mutation and have reduced HbF.

In this report, as in most others published to date,[20–22] the percentage of HbA_2 fell within the normal range irrespective of the type of Hb Lepore but, nevertheless, served to distinguish between the Hb Lepore types such that it was 8.1 times more likely to find Hb Lepore Boston in a patient with HbA_2 levels ≥2% than Hb Lepore Baltimore. It was observed that the Hb Lepore phenotype ($\alpha_2\delta\beta_2$) presenting the highest HbA_2 ($\alpha_2\delta_2$) corresponded to the Hb Lepore with the lowest levels. This negative correlation probably reflects the mechanisms of mRNA synthesis which appear to involve competition since δ and δβ chains are affected by the same promotors.

It should be highlighted that, in this hemoglobinopathy, the low (≈15%) proportion of anomalous hemoglobin (Hb Lepore) might be explained by the fact that synthesis of the δβ chain takes place during the early stages of red blood cell development.[17] The proportion of Hb Lepore was higher in the Hb Lepore Baltimore carriers than in subjects with Hb Lepore Boston (13.07% vs 10.81%, p=0.012) although the difference was not sufficient to distinguish between the phenotypes.

Although minor, the structural differences between Lepore Baltimore and Lepore Boston (δβ) chains serve to explain their different elution times when subjected to reversed HPLC. Thus, since the Hb Lepore Baltimore (δβ) chain shares the same amino acid residues at positions 86 (Ala) and 87 (Thr) as the normal β globin chain, the chain elution time is sim-

ilar to that recorded in normal subjects while, as occurs in Hb Knossos [β27(B9)Ala→Ser] in which β^X and β^A globin chains are separated by reversed phase HPLC,[23,24] the replacement of the Ala residue by a Ser residue in the Lepore Boston (δβ) chain permits the separation of this chain and the consequent identification of its phenotype. This occurs because the replacement of a non-polar amino acid (Ala) by a polar uncharged amino acid (Ser) produces a change in the net charge affecting the physico-chemical properties of the globin chain. This leads to a loss in hydrophobicity and the formation of new hydrogen bonds with water due to the new OH group. This may explain the different behavior of each Hb Lepore type.

Despite clinical similarities between Hb Lepore carriers of either type, identification of the phenotype by PCR based methods of analyzing DNA is important for prenatal diagnosis.

Contributions and Acknowledgments

PR and AFG designed the study and wrote the paper. PR performed the molecular studies. JS performed the HPLC studies. EA and SA contributed to the analysis of the data. AdA, MJM and RR sent us several samples for the study. CF performed the statistical analyses. AV is the chief of the Service and contributed to the conception of the study.

The authors are listed in order of the importance of their contribution, except for the last (AV) who is the senior author.

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Disclosures

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