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Utilization of different methodologies for the characterization of Hb Hasharon heterozygotes

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ABSTRACT. Hb Hasharon has an electrophoretic mobility similar to that of Hb S in cellulose acetate and a mobility between Hb S and C at acid pH. In high-performance liquid chromatography, Hb Hasharon shows a distinct chromatographic profile and retention time. The origin of this variant is a mutation in codon 47 (GAC → CAC) of the α2-globin gene, resulting in the replacement of asparagine by histidine during the translation process. Ten blood samples from individuals suspected of being Hb Hasharon carriers were analyzed. In addition to classic laboratory tests and high-performance liquid chromatography, molecular analysis by polymerase chain reaction with restriction fragment length polymorphism designed in the laboratory was performed to confirm this mutation. The study of these cases showed that a combination of classical and molecular methodologies is necessary in the diagnosis of hemoglobinopathies for a correct hemoglobin mutant identification. The accurate identifica-

tion of hemoglobin variants is essential for genetic counseling and choice of therapy.

Key words: Hb Hasharon, Laboratory diagnosis, Hemoglobin variants

INTRODUCTION

Over 900 hemoglobin variants have already been described, and most of them are caused by substitutions which do not change the electrical charge of the protein. In alkaline pH electrophoresis, several variants show co-migration, such as Hb S and Hb D (Grignoli et al., 1999; Wajcman et al., 2001). Hb Hasharon migrates to the same position as Hb S in cellulose acetate electrophoresis. However, at acid pH, this variant moves to a position between Hb S and Hb C (THALASSEMIA.com, 2002). In high-performance liquid chromatography (HPLC), the chromatographic profile and retention time in the automated system are specific for this abnormal hemoglobin.

This mutation originates from the GAC → CAC transversion at codon 47 of the α2-globin gene, resulting in the replacement of asparagine by histidine. Heterozygotes are asymptomatic and are found among Ashkenazi Jews and in Italian families (THALASSEMIA.com, 2002).

MATERIAL AND METHODS

The cases comprised 10 peripheral blood samples from five men and five women who were suspected of being Hb Hasharon carriers. Their ages ranged between 7 months and 27 years. After receiving informed consent, the patients' peripheral blood was collected in sterile tubes with 5% EDTA. An investigation of abnormal hemoglobins was carried out because the electrophoretic profiles of the samples were similar to Hb S. Screening tests consisted of osmotic globular resistance in NaCl solution at 0.36% (Silvestroni and Bianco, 1975), analysis of red blood cell morphology (Bonini-Domingos, 1993) and cellulose acetate electrophoresis using a Tris-EDTA-borate buffer at pH 8.5 (Marengo-Rowe, 1965). Gel electrophoresis in phosphate buffer, pH 6.2 (Vella, 1968), and globin chain electrophoresis on cellulose acetate using Tris-EDTA-borate, urea and mercaptoethanol buffer (Schneider, 1974) were carried out to confirm the results. HPLC was performed in a BIO-RAD VARIANT automated analyzer with the "β-thalassemia short program" which identified hemoglobin variants and determined their levels.

DNA was obtained from white blood cells by the phenolic extraction method for molecular analysis (Pena et al., 1991). Exon 2 of α-globin was amplified by polymerase chain reaction (PCR) for the diagnosis of Hb Hasharon (Saiki et al., 1988). The primer sequence used in the amplification reaction was: primer sense - 5' AGG CCC TGG AGA GGT GAG 3' and primer anti-sense - 5' GGA GCT GTG CAG AGA AGA GG 3'. Ten microliters of the amplified product was submitted to the action of *Taq*αI restriction endonuclease according to the manufacturer's instructions, and the product of the digestion was analyzed by electrophoresis on agarose gel with ethidium bromide staining. The mutation that causes Hb Hasharon is a single base substitution in codon 47 of the α-gene. This alteration, GAC → CAC, removes one of the *Taq*αI

recognition sites normally located in this DNA region. Thus, the amplified fragment of DNA of 481 bp is cleaved by *Taq*αI endonuclease generating three fragments (117, 175 and 189 bp) for the normal allele, while the Hb Hasharon allele generates two fragments (117 and 364 bp).

RESULTS

In the screening tests, all samples showed negative globular osmotic resistance in 0.36% NaCl, which is the selective test for thalassemias. Slight alterations in red blood cell morphology, such as microcytosis and hypochromia, were found in all individuals. In alkaline pH electrophoresis, the samples displayed two fractions, one at the Hb A position and another similar to Hb S. In electrophoresis on acid agar gel, the samples showed one fraction at the Hb A position and another at Hb S position or lower.

All the samples showed one fraction in an α-mutant position in globin chain electrophoresis, with the exception of one sample, which was not tested by this method due to inadequate blood sample size.

HPLC data are shown in Table 1. Besides the normal Hb fractions frequently encountered, such as Hb F, P2, P3, A0, and A_2, the samples showed small fractions eluted in the D, S or C windows and larger fractions corresponding to the Hb Hasharon chromatographic profile eluted in an "Unknown Window" (Unknown 1 and 2). Most samples eluted at the position of "Unknown 1" with a mean of 27.85% and a standard deviation of 1.85 and a retention time of 4.80 min. In one sample, however, the abnormal hemoglobin was eluted in "Unknown 2" with a mean of 28.5% and a retention time of 4.80 min. The chromatographic profile characteristics are shown in Figure 1. These findings as a whole suggested the probable existence of Hb Hasharon.

Molecular confirmation was made by the amplification of the α-globin gene exon 2 by PCR and enzymatic digestion by *Taq*αI restriction endonuclease. DNA samples were analyzed and fragments corresponding to the mutant allele for Hb Hasharon and the normal allele were found. Nine samples displayed heterozygous states with four fragments, where 117 bp was the control of the enzymatic reaction, 175- and 189-bp fragments corresponded to normal allele and one fragment of 364 bp represented the mutant allele. The mutation of the first base of codon 47 abolishes a specific site for *Taq*αI restriction endonuclease, and because of this, there is no enzymatic digestion in this DNA region. One of the samples was not successfully amplified despite the availability of ample genetic material and several attempts of amplification.

DISCUSSION

Hemoglobin electrophoresis on cellulose acetate at alkaline pH is the most commonly used method for the identification of abnormal hemoglobins; however, its sensitivity is limited, especially for variants with similar electrical charges at this pH, such as Hb S, Hb D, Hb G-Philadelphia and Hb Hasharon (Hempe and Craver, 2000). Acid agar gel and globin chain electrophoresis allow the discrimination of some isoforms. Hb D and Hb G migrate in a manner similar to that of Hb A at acid pH and Hb Hasharon shows one fraction near Hb S, at an intermediate position between Hb S and Hb C. In globin chain electrophoresis, Hb S and Hb D are mutants of the β-chain and show similar migrations at alkaline pH and are differentiated from Hb G-Philadelphia and Hb Hasharon variants which are mutants of the α-globin chain.

Table 1. Percentage and retention time of hemoglobin fractions in individuals analyzed by HPLC.

Samples	F*		P2		P3		A0		A2		D		S		Unknown 1		Unknown 2	
	%	RT	%	RT	%	RT	%	RT	%	RT	%	RT	%	RT	%	RT	%	RT
AR-015			0.9	1.34	3.3	1.68	59.3	2.44	1.8	3.64			0.7	4.41	30.6	4.86		
AR-061	3.3	1.10	3.6	1.32	2.7	1.68	61.6	2.55	2.0	3.64					29.9	4.80		
AR-138	0.3	0.95	4.3	1.32	5.8	1.65	58.9	2.65	2.1	3.62	0.7	4.26	1.4	4.64	26.7	4.80		
AR-146	0.6	1.11	3.5	1.32	3.4	1.66	60.6	2.63	1.9	3.62	0.7	4.25			0.7	4.67	28.5	4.80
AR-178	0.9	1.10	4.5	1.31	3.2	1.72	62.8	2.64	2.1	3.61	0.8	4.24			25.6	4.80		
AR-185	0.4	1.12	3.6	1.33	2.3	1.73	61.8	2.65	2.0	3.64	0.8	4.27			28.7	4.80		
AR-186	1.6	1.12	4.0	1.32	2.7	1.75	60.9	2.65	1.6	3.65	0.8	4.27			28.3	4.80		
AR-190	0.6	0.95	4.6	1.32	0.9	1.66	54.8	2.62	2.0	3.66	0.4	4.28	2.6	4.64	25.7	4.80		
AR-222	1.0	1.10	4.1	1.30	4.6	1.65	59.0	2.66	1.9	3.59			1.1	4.64	25.5	4.80		
AR-223	0.2	0.94	4.0	1.30	4.2	1.64	58.8	2.68	1.9	3.61	0.7	4.23	1.1	4.62	29.0	4.80		

*F: hemoglobin fetal window; P2: glycosylated hemoglobin A window; P3: acetylated hemoglobin A window; A0: hemoglobin A window; A2: hemoglobin A_2 window; D: hemoglobin D window; S: hemoglobin S window; Unknown 1 and 2: hemoglobin unknown windows; %: percentage of each hemoglobin; RT: retention time is the time from sample injection until hemoglobin peak.

ANALYTE ID	%	TIME	AREA
F	1.6	1.12	37083
P2	4.0	1.32	88585
P3	2.7	1.75	59682
A0	60.9	2.65	1345176
A2	1.6	3.65	31174
D-WINDOW	0.8	4.27	17304
Unknown 1	28.3	4.80	624962
	TOTAL AREA		2203966
F	1.6%	A2	1.6%

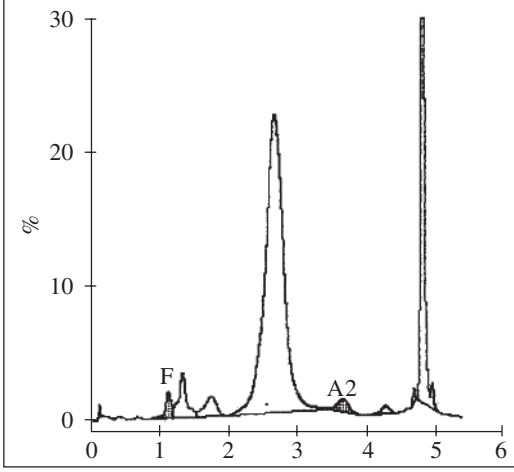

Figure 1. Chromatogram obtained by VARIANT-BIO-RAD, HPLC, for Hb Hasharon heterozygous.

Although these variants migrate differently in electrophoresis at acid pH, small variations can occur due to the conditions of the gel or sample size. Because of this, the utilization of more sensitive methods is important to correctly identify Hb Hasharon.

HPLC has been increasingly used in the last years to separate and measure various normal and abnormal hemoglobin fractions. The VARIANT analyzer and β-thalassemia short program have been designed to separate Hb A_2 and Hb F and determine their area percentages as a reference in the identification of most common abnormal hemoglobins such as Hb S, Hb C, Hb D, and Hb E, taking 6 min for each blood sample. The identification of these abnormal hemoglobins is made by using the retention time windows specified by the manufacturer (Riou et al., 1997). This method is relatively simple and fast and provides chromatographic information important for the characterization of the hemoglobin components (Kutlar et al., 1993). Hb Hasharon is not eluted in a specific window, but in a window designated as "unknown"; its presence is indicated based on previous laboratory results, such as the electrophoretic profile at different pHs, its elevated percentage in this window, elution profile, chromatographic profile, and confirmation by molecular biology as well.

Thus, various classical and molecular techniques are necessary for the accurate identification of abnormal hemoglobin, because each routine methodology used to identify hemoglobin variants has limitations and advantages, making it impossible to reach a definite diagnosis using

only one method. The HPLC procedure is a fast and sensitive method which has become a tool used for the correct characterization of hemoglobin variants. However, it is important to emphasize the necessity of detailed analysis of the percentages of hemoglobin fractions and principally the retention time of each fraction compared to the chromatographic profile. Taken together, all of these informative results lead to a presumptive diagnosis. Molecular analysis confirms electrophoretic and chromatographic findings.

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