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Protein characterization by LC–MS/MS may be required for the DNA identification of a fusion hemoglobin: The example of Hb P-Nilotic☆

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ABSTRACT

DNA analysis is currently the easiest way to identify a hemoglobin variant in most cases. Nevertheless, in case of complex gene rearrangements, mass spectrometry studies may be required to orientate the DNA diagnosis. The present report shows the use of mass spectrometry techniques prior to DNA analysis for the identification of the rare P-Nilotic fusion hemoglobin. Complete protein analysis is performed by liquid chromatography–tandem mass spectrometry on the abnormal globin chain isolated by reversed-phase liquid chromatography.

1. Introduction

In most hemoglobin (Hb) reference laboratories, identification of a Hb variant is performed first by protein analysis: cation-exchange liquid chromatography (CE-LC), isoelectric focusing (IEF) or capillary electrophoresis (CEP). In a second step, DNA sequencing of the α-, β- and γ-globin genes are used to characterize rare or new Hb variants or to confirm an already known one. However, in the presence of some rare Hb variants, this common strategy can be unsuccessful because DNA sequencing cannot easily solve complex rearrangements without complete protein studies. In such a case, mass spectrometry (MS) studies can be recommended (when available) for Hb identification [1,2]. In the present short communication, we report for the first time the characterization by liquid chromatography–tandem mass spectrometry studies of a rare Hb variant called Hb P-Nilotic which results from non-homologous crossover during meiosis, resulting in a beta-delta fusion gene.

2. Materials and methods

2.1. Protein analysis

EDTA blood samples from three unrelated patients were collected for a complete screening of hemoglobinopathies: CE-LC analyses were carried out on Variant II (Bio-Rad, Hercules, CA, USA) using the 'Short Beta Thal program'; IEF on polyacrylamide gels was performed as previously described [3]; CEP analysis used a Sebia Capillarys apparatus (Sebia, Lisses, France) and reversed-phase liquid chromatography of globin chains (RP-LC) was performed on a column C_4 Uptisphere (4.6 mm × 250 mm, 5 μm particle, average pore size 300 Å, Interchim, Montluçon, France) as previously described [4].

2.2. Mass spectrometry analysis

2.2.1. Sample preparation

Samples were prepared as previously described [4] and injection volume was 20 μL for each RP-LC assay. Globin chain peaks were eluted from the C_4 RP-LC column at a flow rate of 1 mL/min. Chromatographic peaks were collected and dried in a vacuum concentrator before trypsin proteolytic digestion: (i) reduction by 10 μL of 10 mM dithiothreitol in 50 mM NH_4HCO_3 for 15 min at 50 °C, (ii) alkylation by 10 μL of 100 mM iodoacetamide in 50 mM NH_4HCO_3 for 15 min at room temperature in the dark and (iii) tryptic digestion for 45 min at 50 °C with 20 μL of trypsin solution, 0.02 μg/μL

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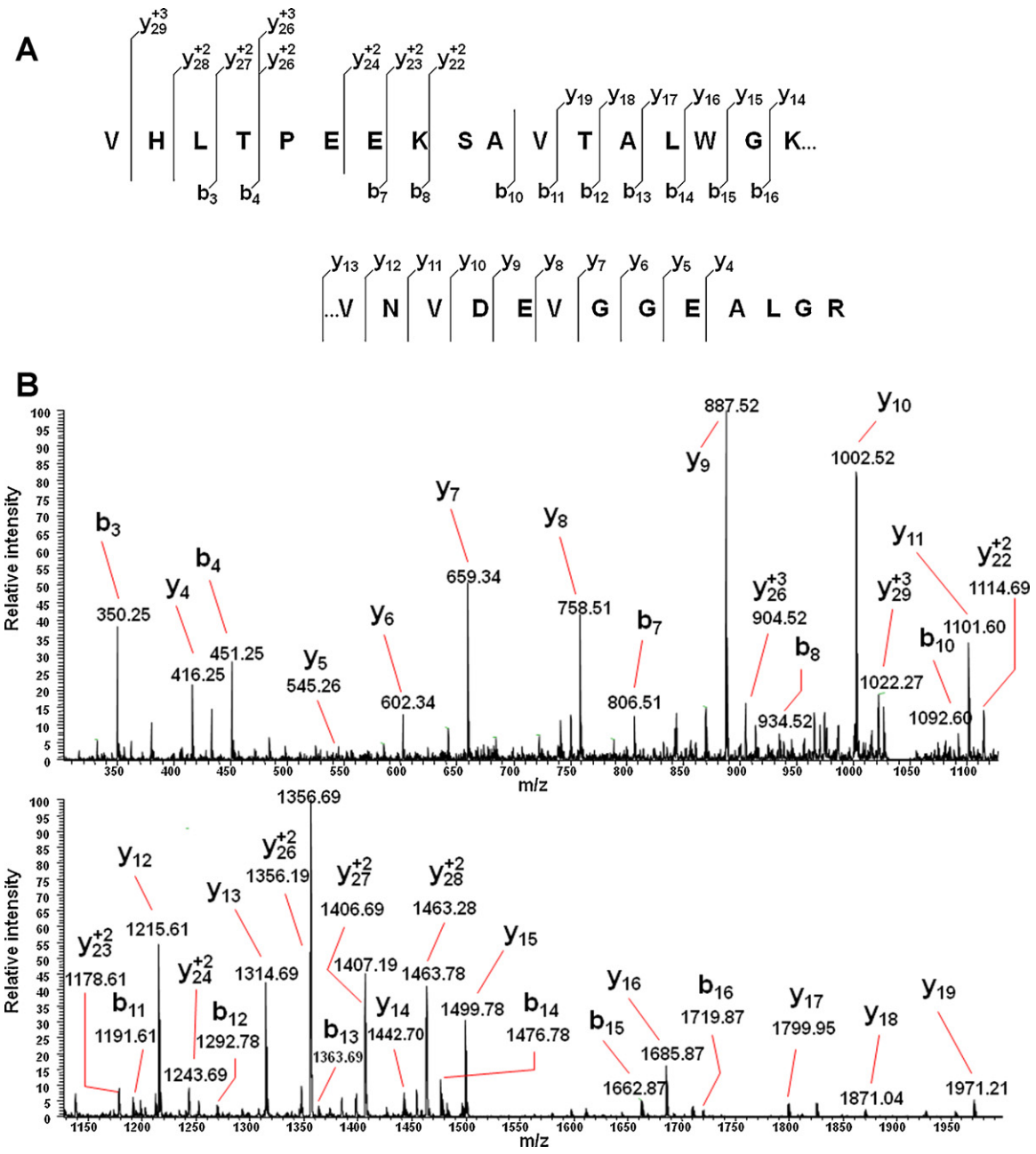

Fig. 1. MS/MS mass spectrum of the triply charged peptide [1–30] at *m*/*z* 1054.4 (experimental monoisotopic mass: 3160.78 Da; theoretical monoisotopic mass: 3160.65 Da). (A) Scheme of the *y* and *b* product ions observed from the amino acid sequence of the tryptic peptide [1–30]. (B) MS/MS mass spectrum of the triply charged peptide [1–30] at *m*/*z* 1054.4 in the *m*/*z* range 300–2000.

in 50 mM NH_4HCO_3 (sequence grade, Promega, Charbonnières, France). The final solution was dried in a vacuum concentrator and redissolved in 30 μL of 0.3% trifluoroacetic acid (Sigma–Aldrich, Saint-Quentin Fallavier, France) for nanoLC–ESI/MS/MS analysis.

2.2.2. Electrospay mass spectrometry (ESI/MS)

Mass measurements of the collected globins were obtained in the electrospray mode on an API 165 instrument (AB SCIEX, Courtaboeuf, France). A 50 μL solution of an aliquot of the collected globin in a methanol/water mixture (50:50) containing 0.1% formic acid (v/v) was analyzed at a flow rate of 5 μL/min. Spectra were acquired at a 5000 V ion-spray voltage and a scan speed of 100 Da/s (scan range, 700–1800 Da).

2.2.3. NanoLC–nanospray-tandem mass spectrometry (LC–ESI/MS/MS)

Experiments were performed on a LTQ Velos (ThermoScientific, Villebon-sur-Yvette, France) instrument in the positive ion mode. The ion source was equipped with a picoTip emitter as nanospray needle (FS360-75-30-CE-5-C10.5, NewObjective, Woburn, MA, USA) operating at 1.5 kV. Typically, two scan events were used: (1) *m*/*z* 400–2000 scan MS with enhanced resolution; (2) data dependent scans MS/MS on the twenty most intense ions from event 1. The spectra were recorded using dynamic exclusion of previously analyzed ions for 0.6 min. The MS/MS normalized collision energy was set to 35 eV.

LC was performed on an Ultimate 3000 nano-LC system (Dionex, Voisins Le Bretonneux, France). Chromatographic separation of peptides was obtained with a C_{18} PepMap micro-precolumn (5 μm;

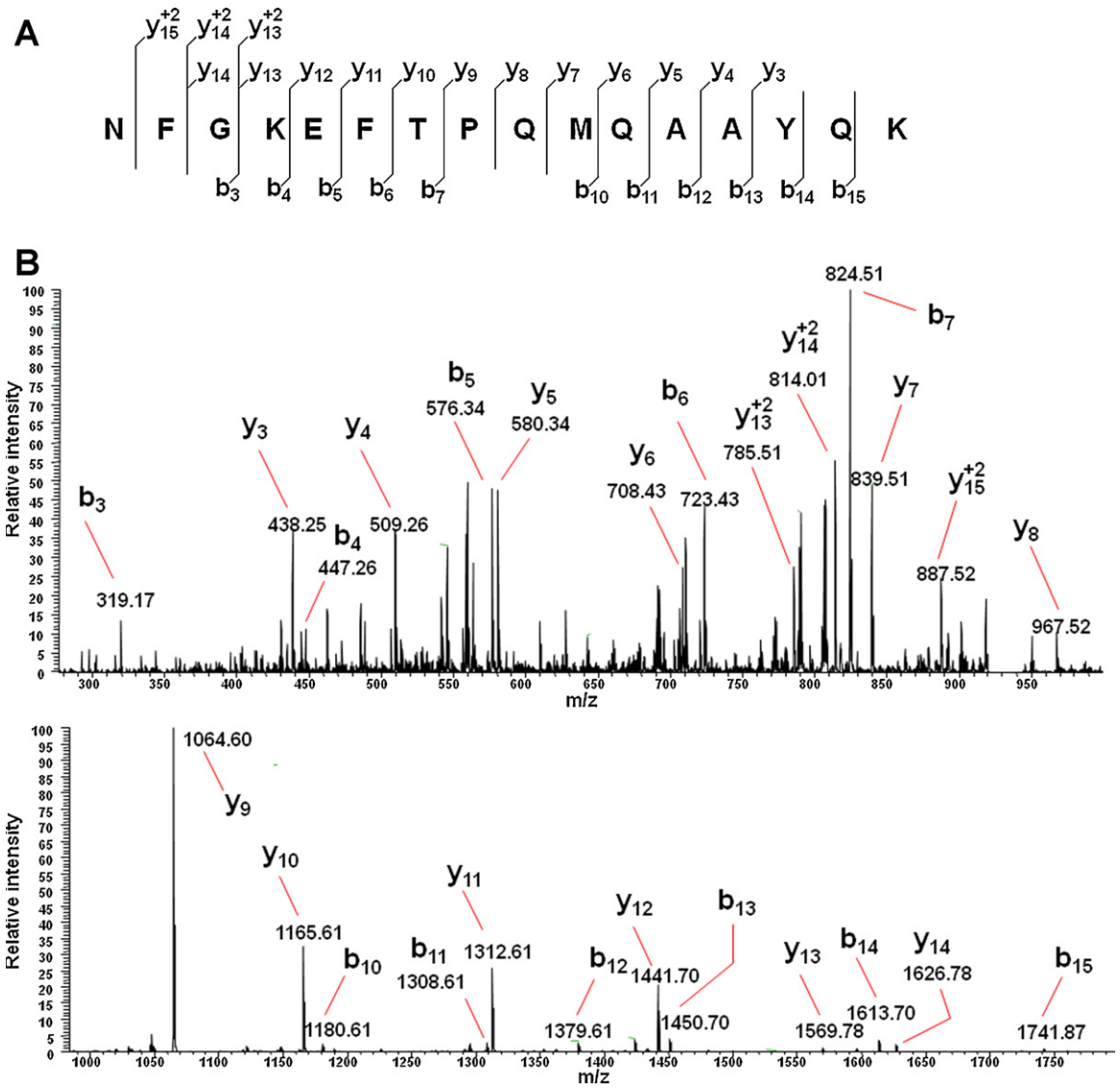

Fig. 2. MS/MS mass spectrum of the doubly charged peptide [117–132] at *m*/*z* 944.5 (experimental monoisotopic mass: 1887.05 Da; theoretical monoisotopic mass: 1886.90 Da). (A) Scheme of the *y* and *b* product ions observed from the amino acid sequence of the tryptic peptide [117–132]. (B) MS/MS mass spectrum of the doubly charged peptide [117–132] at *m*/*z* 944.5 in the *m*/*z* range 290–1800.

0.3 mm × 5 mm) for desalting and a C_{18} PepMap nano-column (3 μm; 100 Å; 75 μm × 150 mm) with a gradient elution at a flow rate of 300 nL/min. Eluent A was a mixture of 95% H_2O, 5% CH_3CN and 0.1% formic acid. Eluent B was a mixture of 20% H_2O, 80% CH_3CN and 0.1% formic acid. The gradient program was from 0% B to 80% B over 60 min and 100% B for 10 min. Protein identifications were performed with the Proteome Discoverer 1.1 software (Thermo-Scientific) using the Mascot search algorithm and the SwissProt (version 2010_06) database.

2.3. Hemoglobin genotypic analysis

The three patients gave their informed written consent for genetic analysis, in agreement with French regulation and the Helsinki declaration. Search for large rearrangements in the β-globin gene cluster was performed by multiplex ligation-dependent probe amplification (MLPA) analysis (MRC-Holland, Amsterdam, The Netherlands) [5] while identification of Hb P-Nilotic was made by a specific polymerase chain reaction (PCR) reported elsewhere [6].

3. Results and discussion

No abnormal peak was detected by CE-LC but IEF and CEP (Z7-Z6 window) revealed a Hb variant at a percentage of 18.7%. RP-LC of globin chains confirmed the presence of an abnormal globin chain eluting between β^A- and α^A-globin chains (see Supplemental data, Fig. 1A). We thus applied our common strategy of identification of the Hb variant by DNA sequencings of the α- and β-globin genes but no missense mutation was found. Consequently, we performed semi-preparative isolation of the abnormal globin chain by RP-LC of globin chains. Deconvoluted ESI/MS gave a 15955.5 Da molecular mass (Supplemental data, Fig. 1B), with an unusual mass increase of +88 Da from the β^A-globin chain (15867.2 Da). Identification and characterization were carried out using LC–ESI/MS/MS of peptides obtained after trypsin digestion of the unknown globin. The proposed sequences were rated as high confidence by Proteome Discoverer 1.1 software and confirmed by manual interpretation of the spectra. Analysis of the MS/MS data from the tryptic peptides allowed to identify two human proteins: β-globin (SwissProt: http://www.uniprot.org/ accession number: P68871) and

Table 1
LC/MS/MS of the hybrid globin tryptic peptides: identification of peptide sequences from the β- and δ-globin.

Ion *m/z* (charge)	Peptide mass[a]	Position	Peptide sequence	β Globin	δ Globin
933.6 (+2)	1865.2	[1–17]	VHLTPEEKSAVTALWGK	+	
657.9 (+2)	1313.8	[18–30]	VNVDEVGGEALGR	+	
1054.6 (+3)	3160.8	[1–30]	VHLTPEEKSAVTALWGKVNVDEVGGEALGR	+	
Not observed		[31–40]	LLVVYPWTQR	+	+
1023.0 (+2)	2044.1	[41–59]	FFESFGDLSSPDAVMGNPK		+
731.2 (+3)	2190.5	[62–82]	AHGKKVLGAFSDGLAHLDNLK	+	+
835.5 (+2)	1669.0	[67–82]	VLGAFSDGLAHLDNLK	+	+
761.4 (+2)	1520.9[b]	[83–95]	GTFSQLSELHCDK		+
563.9 (+2)	1125.8	[96–104]	LHVDPENFR	+	+
664.0 (+2)	1326.0[b]	[105–116]	LLGNVLVCVLAR		+
944.5 (+2)	1887.0	[117–132]	NFGKEFTPQMQAAYQK		+
575.4 (+2)	1148.8	[133–144]	VVAGVANALAHK	+	+
725.5 (+2)	1449.0	[133–146]	VVAGVANALAHKYH	+	+

[a] Experimental monoisotopic mass.
[b] Carbamidomethylated cysteine.

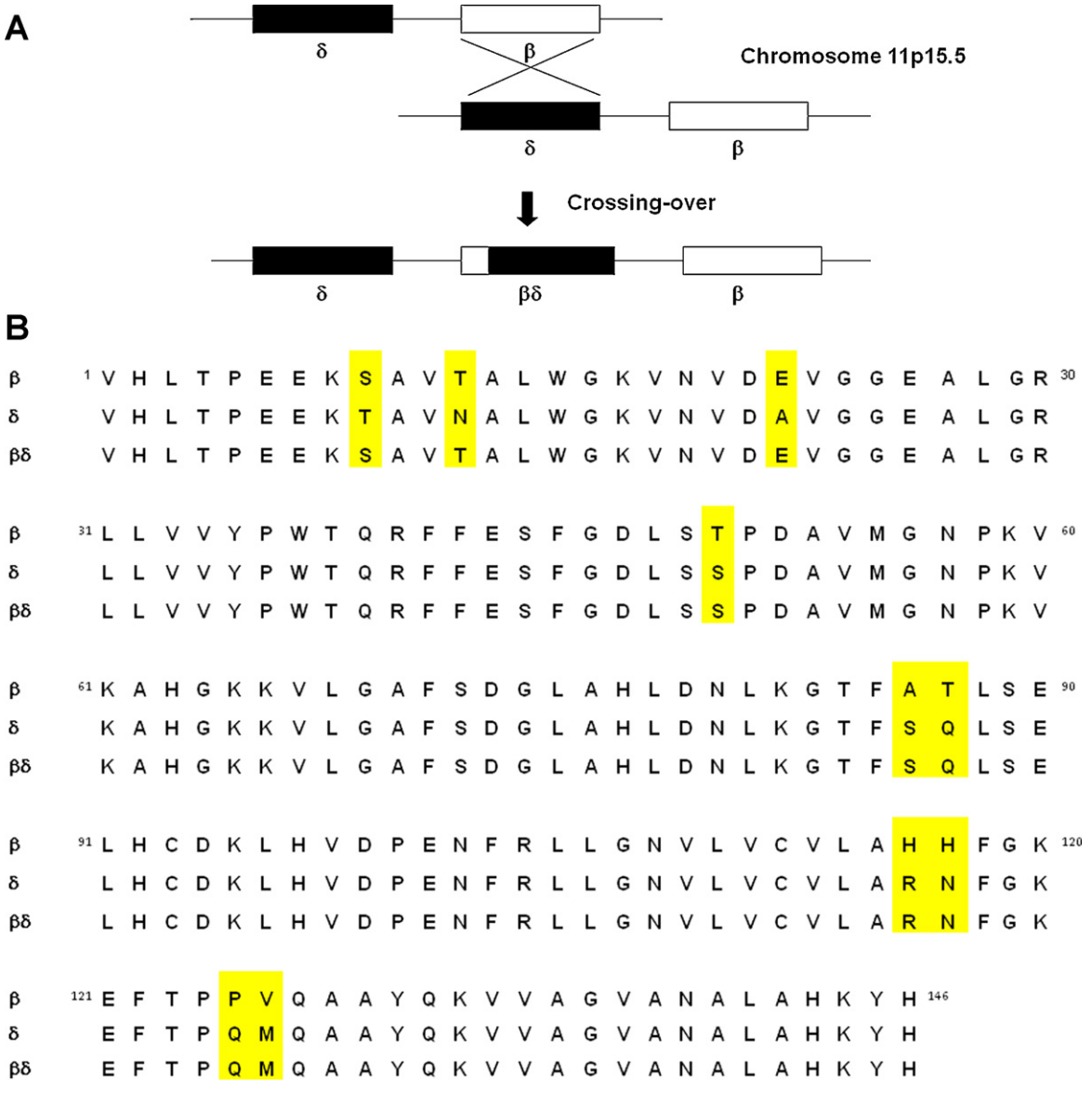

Fig. 3. Schematic DNA mechanism of unequal cross-over resulting in Hb P-Nilotic (anti-Lepore) and amino acid sequences of the β-, δ- and βδ-globin chains. (A) Unequal cross-over resulting in a δ–βδ–β rearrangement on the β-globin gene cluster (chromosome 11p13.3). (B) Amino acid sequences of the β-, δ- and βδ-globin chains. Amino-acid residues (1–30) in βδ-globin chain are issued from β-globin chain. Amino acid residues (31–146) are issued from δ-globin chain. Yellow boxes show the differences in amino acid residues between β-, δ- and βδ-globin chains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

δ-globin (SwissProt accession number: P02042) with both common (six sequences) and specific peptide sequences (three and four sequences for β-globin and δ-globin, respectively). Amino acid sequencing of peptides was done by studying the N-terminal (*b*) and C-terminal (*y*) product ions from MS/MS spectra as exemplified in Figs. 1 and 2 with the MS/MS sequencing results of two characteristic peptides from the β and δ globin parts, respectively. On the β-part of the fusion hemoglobin, three specific amino acid sequences [1–17], [18–30] and [1–30] were determined: The MS/MS spectrum of the [1–30] amino acid sequence was presented as the entire sequence of the β-part of the fusion hemoglobin. The detailed results are shown in Table 1 (LC–MS/MS chromatogram and ion detection are reported in Supplementary data). It was very clear that the hybrid globin was a combination of the two globins β and δ, with the β globin sequence from position 1 to 30 and δ globin for the remaining sequence from 31 to 146. According to Swiss-Prot, such a amino-acid sequence corresponds to Hb P-Nilotic (SwissProt accession numbers: Q14484 and Q14485) which is a βδ fusion Hb (Fig. 3).

Now that we had a clear orientation on the nature of the β-globin gene cluster rearrangement, we could unequivocally confirm it at the DNA level. A literature review gave us specific primers to amplify by PCR the P-Nilotic allele using 5′β-specific primer and 3′δ-specific primer. The double strand DNA sequencing of the resulting amplicon confirmed the presence of Hb P-Nilotic for the three patients. The β-globin MLPA also detected the βδ fusion gene without any other rearrangement. The protein studies on Hb P-Nilotic directed that fusion occurred somewhere between amino acid residue β22 and δ50 (Table 1 and Fig. 3B). However, DNA sequencing showed that fusion occurred between codon β31 and codon δ50 [7]. Hb Lepore (a δβ hybrid) has been the first identification of fusion hemoglobins performed by MS [8–10]. Other fusion hemoglobins resulting from ^{A}γ- or ^{G}γ- and β-genes crossovers have been also identified by MS [11,12]. The identification of one case of anti-Lepore P-Nilotic has been previously described by ESI/MS [13], but no nanoLC–MS/MS study of this variant has been reported to date.

4. Conclusion

The present report emphasizes the importance of protein analysis by mass spectrometry methods for Hb variants resulting from complex gene rearrangements. The use of sophisticated MS methods such as nanoLC–MS/MS may also increase the sensitivity of protein analysis to detect a Hb variant present at a very low level (unstable variant and/or thalassemic variant).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.10.017.

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