

Mass spectrometry and DNA sequencing are complementary techniques for characterizing hemoglobin variants: the example of hemoglobin J-Oxford

Liquid chromatography–electrospray ionization–mass spectrometry (MS) allows the characterization of most hemoglobin variants and can sometimes be a useful tool to narrow down DNA sequencing analysis. As an example, we report a case of hemoglobin variant J-Oxford, characterized by MS and DNA sequencing analysis.

haematologica 2004; 89:608-609

(<http://www.haematologica.org/journal/2004/5/608>)

Hemoglobin (Hb) variants can be characterized by two levels of investigation. The techniques for the first level are usually gel electrophoresis or cation exchange high performance liquid chromatography (HPLC). The second level is based on mass spectrometry (MS) analysis^{1,2} and/or DNA sequencing.¹ The combination of liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS)³ and DNA sequencing analysis are complementary techniques, as in the case Hb J-Oxford or I-Interlaken variant [α 15(A13) Gly_Asp].^{4,5}

A healthy, Sardinian woman, aged 24, and her parents were referred for comprehensive hematologic investigations, using standard automated methods. With the exception of HbA_{1c}, which was low only in the patient, all the hematologic parameters were normal.

To determine the molecular weight of the hemoglobin chain, LC-ESI-MS analyses were performed by ESI-ion trap-MS (LCQ–ThermoQuest–Finnigan, USA). The protein was digested by incubating the hemolysate with trypsin (Sigma Chemical Co., USA) and the abnormal peptides were sequenced by tandem mass spectrometry.

Genomic DNA was extracted with a Nucleon Bacc 2 kit (Amersham Inc., USA). Amplification of α 1 and α 2 Hb genes was performed by polymerase chain reaction (PCR). The forward primer 5'-CGCGCTCGCGGCCCGGCAC-3' was used for the α 1 and α 2 Hb genes and then two different primers were used for the α 1 gene (5'-GGGGGGAGGCCAAGGGCAAGAA-3') and for the α 2 gene (5'-GGGAGGCCCATCGGGCAGGAGGAAC-3'). DNA sequencing was performed by Sanger's method.⁶

HPLC revealed an anomalous peak eluting about halfway between HbF and HbA₀ which amounted to 23.7% of the total Hb. Gel electrophoresis showed an anomalous band migrating faster than HbA₀ and amounting to about 25% of the total Hb. The same profile was observed for the mother but not for the father.

The chromatographic trace of the patient (Figure 1A) did not show any anomalous peaks with respect to the trace from a control (Figure 1B). However, the mass spectrum of the non-homogeneous peak corresponding to normal α -chain showed two molecular ions: one at m/z 15126Da, corresponding to normal α -chain and another at m/z 15184Da (Δ +58+1Da), corresponding to a variant chain (window in Figure 1A). The amount of this ion, about 25% of the total α -chains, confirmed the HPLC result. The total ion chromatogram (TIC) of the tryptic digest of the abnormal chain showed a single variant peptide, not present in the normal α -chain peptides, with a m/z 1051 [M^{++}] (M^+ =2100Da). For sequence analysis, the precursor ion at m/z 1051 was selected for collisional activation. The amino acid sequence of the variant peptide, assessed on the basis of the product ions present in the mass spectrum

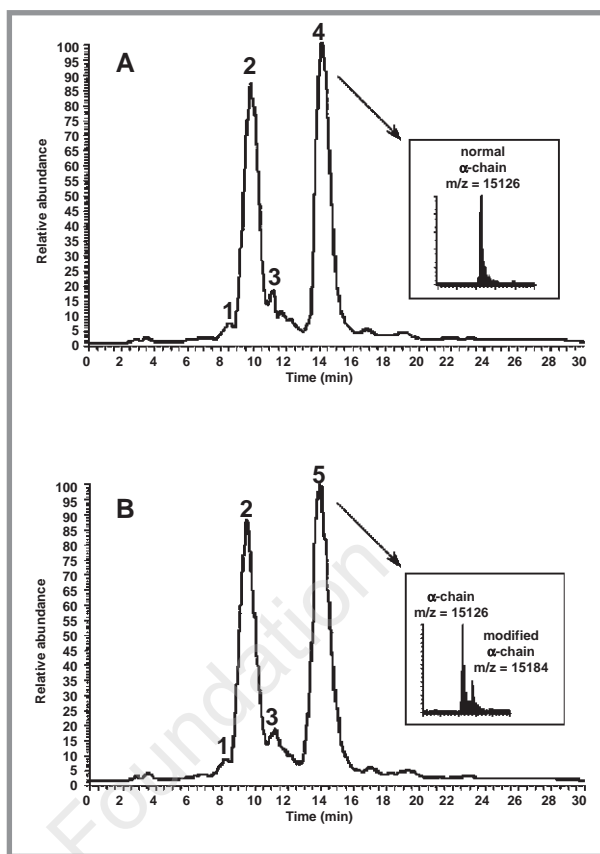


Figure 1. LC-MS analysis of whole hemoglobin chains. A: Chromatogram of hemoglobin of the patient with the deconvolution of a non-homogeneous peak showing the normal (m/z 15126) and variant (m/z 15184) α -chains (in the window). In particular: peak 1 = γ -glycated chain, peak 2 = β -chain, peak 3 = δ -chain, peak 4 = α -chain and peak 5 = non-homogeneous peak corresponding to normal and modified α -chains. B: chromatogram of normal hemoglobin with the deconvolution of the peak (in the window) corresponding to normal α -chain.

and with the aid of the Sequest[®], program, only partially clarified the primary structure. The variant α -chain contained a mutation in the region between amino acids 12-31 possibly hampering enzymatic hydrolysis at lysine 16.⁴ The mutation at the amino acid lysine 16 or glycine 15 was pinpointed by MS and led us to formulate two different hypotheses. The absence of cleavage at lysine 16 with (Δ +58Da) could be due either to substitution of lysine 16 (MW=146Da) with tryptophan (MW=204Da), which implies a rare event as a double nucleotide mutation at the DNA level (AAG \rightarrow TGG), or to the substitution of glycine 15 (MW=75Da) with an amino acid impairing the lysine cleavage, such as aspartic acid (MW=133Da), which implies a more probable single nucleotide mutation (GGT \rightarrow GAT).

To define the amino acid sequence of the abnormal peptide, we designed a PCR strategy to selectively amplify the α 1 and α 2 globin chains of the patient and of her parents, based on the result of MS analysis. DNA sequencing analysis demonstrated that the codon GGT encoding glycine (G) 15 was mutated to codon GAT encoding for aspartic acid (D), which was one of the two possible structures suggested by the MS (Figure 2). The aspartic acid flanking lysine 16 was responsible for the missing tryptic cleavage.⁴ This substitution is characteristic of the variant J-Oxford or I-Interlake

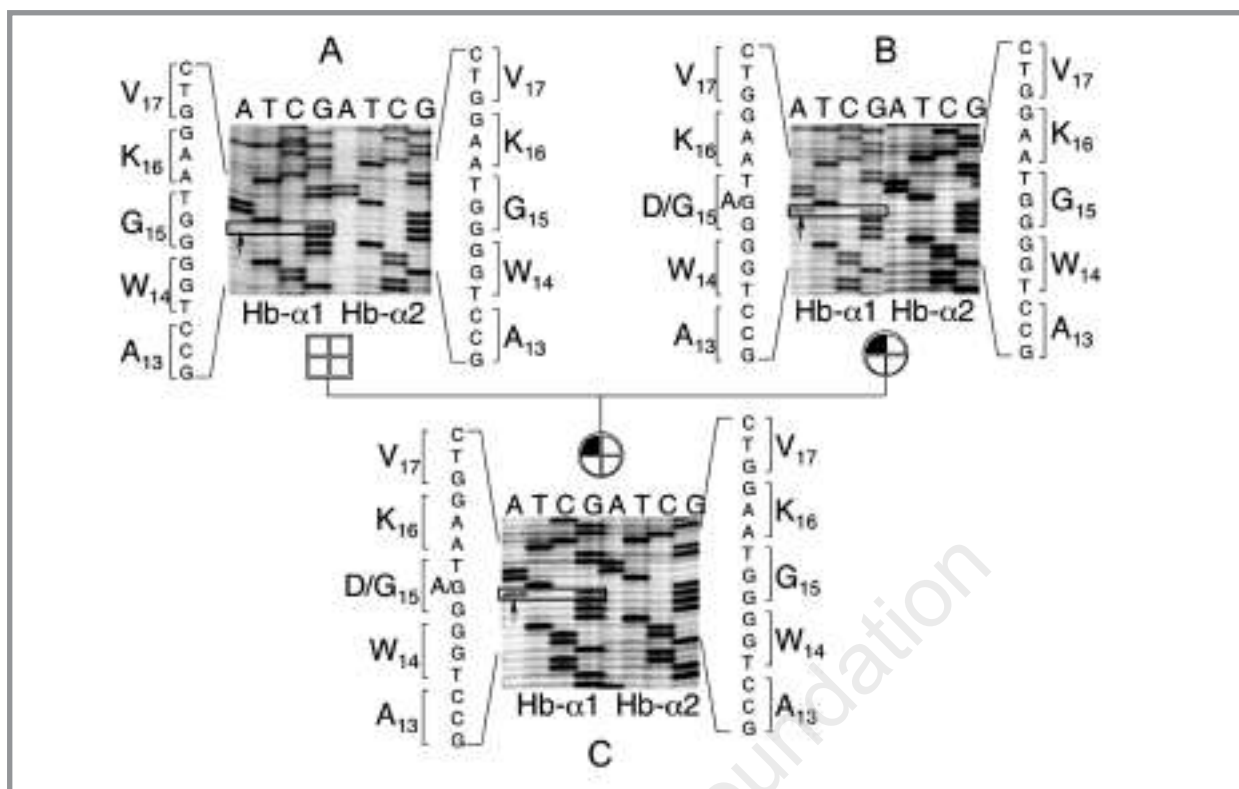


Figure 2. Sequence analysis of amplified DNA by Sanger's method. **A:** Normal sequence (father), **B:** Mother and **C:** Patient. **B** and **C** show the mutated sequence of the $\alpha 1$ gene. The squares emphasize the site of the mutation. The sites of mutation are boxed. Arrows indicate where both adenine and guanine are present in the patient and her mother instead of only guanine as in the father (**A**).

[$\alpha 15(A13)$ Gly \rightarrow Asp]. In addition, the DNA analysis revealed that only $\alpha 1$ gene was mutated (Figure 2).

In conclusion, the MS methods currently available allow the characterization of proteins and peptides.^{3,8,9} In some cases, they can pinpoint the mutated region of the protein and allow the right primers to be designed to focus DNA sequencing analysis on a specific region of the gene. In the example reported here, the position of the substitution prevented trypsin cleavage at Arg/Lys residues, thus hampering the definitive characterization of the variant chain by MS.⁴ We have shown the advantages of coupling mass spectrometry to DNA sequencing analysis in the identification of particular hemoglobin variants.

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Acknowledgments: this paper is dedicated to the memory of Prof. Giovanni Galli, the master who inspired this investigation and gave us constant encouragement during our careers. We are grateful to Miss Elda Desiderio-Pinto for her precious administrative support.

Key words: hemoglobin variants, mass spectrometry, DNA sequencing.

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