Identification of a Rare Hemoglobin Variant HbJ-Rajappen [α 90 (FG2) Lys $\rightarrow$ Thr] Using Mass Spectrometry

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Abstract Hemoglobin J-Rajappen (α 90) Lys $\rightarrow$ Thr is an α chain variant found in heterozygous state and presents normal hematological blood picture. Due to the ambiguity in results obtained while analyzing by HPLC and alkaline gel electrophoresis, we report this rare case of HbJ-Rajappen using non denaturing gel electrophoresis and matrix assisted laser desorption ionization mass spectrometry. Though HbJ-Rajappen has earlier been reported using different techniques, this is the first report being validated using mass spectrometry technique.

Keywords Hemoglobin J-Rajappen · Mass spectrometry · High performance liquid chromatography · Electrophoresis

Introduction

Molecular characterization of hemoglobin variants is generally done at two levels, the first involving gel electrophoresis or cation exchange high performance liquid chromatography (HPLC) and second level of analysis by mass spectrometry and/or DNA sequencing [1, 2].

Hemoglobin-J variant was discovered in year 1956, and as on date there are more than 40 different hemoglobin J-variants identified and these are available on the website

(<http://globin.bx.psu.edu/hbvar/menu.html>). As on date there are more than 40 hemoglobin-variants identified and these are available on the web site (<http://globin.bx.psu.edu/hbvar/menu.html>). The first case of HbJ-Rajappen was reported by Hyde et al. [3] and later on a single case by Henthorn et al. [4] in their results of a 10 year program in an English Health region.

The present study describes the HPLC, electrophoretic and mass spectrometric findings of HbJ hemoglobinopathy from a 65 year old man who hailed from the state of Kerala in South India.

Materials and Methods

Blood sample collected in Becton Dickinson vacutainer EDTA tube was analysed for a routine blood count using Sysmex XT 1800i Hematology analyser.

As the patient was diabetic, the same sample was analysed for glycated hemoglobin (HbA1c) on a BioRad D10 HPLC instrument as per standard procedures. The sample was later centrifuged at 3,000 rpm for 10 min. at 25 °C and the obtained packed cells were lysed with hemoglobin lysing reagent (SAS-Max Alkaline Hb-10 kit, Helena Bioscience, Europe) and the alkaline gel electrophoresis was carried out. Gel was stained using acid blue stain and subsequently destained at room temperature. The hemoglobin bands were later excised from the gel, destained and desalted by vigorous shaking in a solution containing acetonitrile and 50mM NH_4HCO_3 buffer in 1:1 (v/v) ratio. Gel pieces were dehydrated with acetonitrile for 5 min. In-gel digestion was performed using Trypsin (TPCK treated, SIGMA, USA) in 50 mM NH_4HCO_3 , pH 8.0, at 37 °C for 12 h. Proteolytic peptides were eluted from gel with elution buffer (acetonitrile:water = 60:40 (v/v), 0.1 % TFA).

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MS analysis were performed on a MALDI mass spectrometer (Waters Synapt HDMS). The mass spectrometer was calibrated using an external calibrant, PEG mix (Waters, UK) in the 900–3,500 m/z range. The digested peptides were mixed with the matrix solution, α -cyano-4-hydroxycinnamic acid in 1:1 (v/v) ratio and spotted on a MALDI plate. Mass spectra for proteolytic peptides were recorded in the positive ion mode using a 200 Hz laser. Mass spectral findings were analysed using MassLynx software.

Results

The examination of blood in a diabetic workup from a patient from Kerala, South India, presented an anomalous blood picture. Based on the hematological data, the patient had Hb levels of 12.5 g % and MCV of 68 fl. Peripheral smears showed a mildly hypochromic and microcytic blood picture. BioRad D10 HPLC (Fig. 1a) showed a P3 peak with a retention time of 1.3 min, and a percentage of 23.4 %. Being diabetic his HbA1c level was only 4.4 %, indicating it to be falsely reduced as evidenced by the level of elevated blood glucose of 232 mg %.

Figure 1b also depicts yet another patient with an elevated P3 peak, but with a retention time of 1.5 min on the BioRad D10 HPLC system. As this elevated P3 peak suggests presence of a hemoglobin variant HbJ, which is a fast moving hemoglobin, electrophoresis of the sample was carried out.

Figure 2 depicts the presence of HbJ, which has moved ahead of the adult hemoglobin fraction HbA. In agarose gel electrophoresis Hb variants are characterized with respect to their differential electrophoretic mobility in gel [5].

Fig. 1 **a** BioRad HPLC depicting a P3 peak with a retention time of 1.3 min. **b** BioRad HPLC depicting a P3 peak with a retention time of 1.5 min

Fig. 2 **a** A control specimen depicting presence of HbA, HbF, HbS and HbA2. **b** Patient sample depicting the fast moving hemoglobin HbJ and the adult fraction HbA

But in this case co-elution of hemoglobin variant in automated HPLC/comigration of hemoglobin variant in alkaline gel electrophoresis resulted in ambiguity in hemoglobin characterization. Absolute mass measurement of globin chains and peptide mass profiling of proteolytically digested hemoglobin can only remove this above mentioned ambiguity. So a NanoLC-MS bottom up proteomic approach adapted by Mathew et al. [6] for identification and characterization of this hemoglobin variant from the analysis of proteolytic peptide was carried out.

The variant band was isolated from gel and digested with trypsin and analysed in MALDI-MS. The peptide mass fingerprint was compared with that of normal hemoglobin. The appearance of a new peak in the variant digest indicated a probable characteristic peptide for the variant. Using tandem mass spectrometry the characteristic peptide was fragmented and sequenced to identify and locate the mutation. Figure 3 depicts the MALDI-MS spectra of the tryptic peptide finger print. The peptide fragment at 2996.7 Da and

Fig. 3 ESI spectra indicating the presence of m/z 3238.7 (HbJ-Rajappen) to that of Hb. Normal of m/z 2996.7, a difference of 42 Da which is due to K $\rightarrow$ T substitution

3238.7 Da differing by 42 Da indicates the specific fragment harboring the putative K to T mutant.

library, and it is only based on the MS findings are we able to identify the site of mutation.

Discussion

The HbJ family consists of a large group of α and β variants that migrate faster than HbA on alkaline electrophoresis because of an amino acid substitution, resulting most often in gain of a negative charge and less often in the loss of a positive charge on the Hb molecule. However the retention time by HPLC alone was superior to electrophoresis in the identification of this variant HbJ-Rajappen, a member of HbJ family.

The purpose of this report is to also describe this patient with abnormal Hb and to draw attention to the presence of this abnormal hemoglobin when a diabetic patient shows a discrepancy between levels of HbA1c and plasma glucose.

Also to emphasize on this technique wherein using alkaline gel electrophoresis and MALDI-MS in combination, hemoglobin variant can be characterized. The retention time of this variant has not been defined in the variant

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