Diagnostic Dilemma of HbA1c Detection in Presence of a Hemoglobinopathy: A Case Report

Vijay S. Bhat · Kalyan Kumar Dewan ·
Patnam Rajagopalan Krishnaswamy

Received: 5 April 2010 / Accepted: 7 June 2010 / Published online: 14 September 2010
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Abstract We report a case of a diabetic, heterozygote with near normal hematology, marginally low level of hemoglobin A₂(HbA₂) having an increased level of hemoglobin F(HbF) that was pancellularly distributed among the red cells. BioRad DiaSTAT measurements gave a high glycated hemoglobin A1c(HbA1c) of 31.5% and the BioRad Variant analyzer recorded an HbA1c value which was very low, in discordance with the detected blood glucose levels. Flow cytometry and polymerization chain reaction (PCR) based studies were carried out which revealed the case to be that of the common hereditary persistence of fetal hemoglobin (HPFH)-3, an Asian Indian mutation. Fructosamine estimation and HbA1c by Boronate affinity chromatography were able to resolve the discordant value detected and was able to confirm the diabetes status. The case would have been a diagnostic dilemma, if reported without correlation.

Keywords Glycated hemoglobin A1c(HbA1c) · Flow cytometry · Fluorescein isothiocyanate (FITC) · Gap polymerization chain reaction (Gap-PCR) · Alkaline electrophoresis · Hereditary persistence of fetal hemoglobin (HPFH) · High performance liquid chromatography (HPLC)

Introduction

The measurement of HbA1c in diabetic patients as marker for evaluating long term control of diabetes was first proposed by Koenig et al. in 1976 [1]. Though the reliability of this marker has stood the test of time it is known that hemoglobinopathies could give an aberrant value. The presence of abnormal hemoglobins poses technical challenges in HbA1c determination along with interpretation discrepancy [2]. In addition to this some variant hemoglobin molecules are thought to have a direct influence on the very process of HbA glycation [3].

At birth fetal hemoglobin (HbF- $\alpha_2\gamma_2$) comprises 70% of the total hemoglobin. By the age of 6 months this value falls to <5.0%. The switching of the synthesis of fetal hemoglobin (HbF) characterized by $\alpha_2\gamma_2$, to the adult $\alpha_2\beta_2$ molecule is a precisely timed event and represents an impressive example of ontologically related differential gene expression in man [4]. High levels of HbF production in adult life, is due to deletions or cross over involving β and γ chain production, and this heterogeneous group of disorder is referred to as Hereditary Persistence of Fetal Hemoglobin. We are presenting this case as the diagnostic challenge posed by discordant glucose and HbA1c values could have a treatment implication with anti diabetic drugs.

Materials and Methods

Blood sample collected in Becton & Dickenson EDTA vacutainer tube was used for a complete blood count on an automated cell counter (Sysmex XT 1800i Hematology analyzer), and an aliquot from the same tube was used for analysis on the BioRad DiaSTAT (Bio-Rad, Hercules, CA,

V. S. Bhat (✉)
Department of Biochemistry, Manipal Hospital Diagnostic
Services, Manipal Hospital, Old Airport Road,
Bangalore 560017, India
e-mail: vijay.bhat@manipalhealth.com

K. K. Dewan · P. R. Krishnaswamy
Cauvery Medical Center, 43/2 Sahakarnagar, Bellary Road,
NH-7, Bangalore 560092, India

USA) Low performance liquid chromatography (LPLC) system for estimation of HbA1c. On the patient's subsequent visit after 3 years her HbA1c was tested on the BioRad Variant analyzer. An underestimation of HbA1c was noted in the second visit which prompted an investigation on the cause of this suspiciously low value.

Hemolysates for electrophoresis was prepared following the Helena SAS-Mx Alkaline Hb-10 protocol, and electrophoresis carried out on HELENA SAS-II analyzer at an alkaline pH.

Prior consent and clearance from the Institutional Ethics Committee was obtained.

A flow cytometric analysis based on the original procedure described by Davis et al. [5] was enumerated on a ABBOTT Cell-Dyn Sapphire (Abbott GmbH & Co.KG, Wiesbaden, Germany) 1-colour flow cytometer, as a replacement for the Kleihauer-Betke test [6]. In brief, RBCs were fixed with glutaraldehyde and permeabilized by Triton X-100 (Sigma, St.Louis, MO), and a mouse monoclonal antibody against hemoglobin F was conjugated to Fluorescein isothiocyanate (FITC) from Caltag Laboratories, Burlingame, CA. As control, fetal red cells from Caltag® Fetalrol were used. In parallel to this, a Kleihauer–Betke's acid elution technique, based on the differential elution of fetal and adult hemoglobin was also performed.

A Fructosamine test was also conducted on the serum sample, which was estimated by a colorimetric assay using the Fortress diagnostics kit from UK. This quantitative assay is based on the ability of ketoamines to reduce nitrotetrazolium blue (NB) to formazan in an alkaline solution. Based on the study of Mendlovic et al. [7], HbA1c levels were calculated from the Fructosamine value by using the formula

$$\text{HbA1c} = 0.017 * \text{Fructosamine} + 1.61$$

This formula is based on official advice for Quality and Outcomes Framework guidance in the UK and by the United States National Quality Measures Clearinghouse.

HbA1c estimation was also carried out on the ultra2™ HbA1c analyzer (Primus Corp., USA), which is based on boronate affinity chromatography method.

For molecular characterization of the HbF phenotype, a Gap-polymerase chain reaction based strategy described by Craig et al. [8] was adopted, with no modification in procedure. Genomic DNA was extracted from the blood sample using the DNEasy kit (Qiagen). For the Gap-PCR method three oligonucleotide primers (SIGMA) were used in a single amplification reaction which generates a deletion specific product in the presence of the target deletion and a normal control band of a different size in the presence of the normal allele.

Details of the PCR primers (with sequence 5' → 3') being,

A: GACATGGACTATTGTTCAATGA,
B1: CTTTGCTGTTTCAGGCTTAATTT, and
B2 : GACACAGAGCAGTGATTGGTGCA.

PCR amplification was performed using a DNA thermal cycler (PTC-100 from M.J. Research).

Results

The patient had visited the hospital to attend a routine health check that included laboratory tests for diabetes. Cation exchange liquid chromatography analysis of hemoglobin using a BioRad DiaSTAT system to estimate glycated hemoglobin, showed a large peak at the position of HbA1c, comprising 31.5% of the total hemoglobin (Fig. 1a). The other observations on cell counter were: total Hb: 10.4%, MCV: 90.9 fl, RDW: 23.0%.

Blood glucose at this visit was 218 mg dl⁻¹. Since the HbA1c level was exceptionally high and the patient's clinical as well as glycemic state did not correlate with the finding, she was prescribed medicine for diabetes based on her blood glucose values, alone. The patient returned after 3 years for a diabetic check up, during which her HbA1c levels were estimated using a BioRad Variant analyzer. A value of 2.8% was obtained along with an additional peak of HbF of 15.9% as seen in (Fig. 1b). Her fasting blood glucose was 223 mg dl⁻¹.

Boronate affinity chromatography on the Primus analyzer provided an HbA1c value of 7.2% which correlated well with her glycemic control.

Alkaline hemoglobin electrophoresis (Fig. 2, Lane 3), meanwhile revealed the presence of a significant HbF band which confirmed that the patient was a carrier of HbF.

Fructosamine was also estimated in the sample and was found to be 330 umol (normal range being 205–260 umol) which is a measurement of the average blood glucose concentration during the past 2–3 weeks.

By implementing the acid elution method, we could see that the F cells were densely stained with erythrosine and the cells containing HbA appear as ghost cells (Fig. 3a). Flow cytometric analysis (Fig. 3b) showed a pan cellular distribution of HbF corresponding to a single peak with intermediate fluorescence.

Based on this finding we proceeded with the analysis of the specific mutation accounting for the HbF mutation. This Gap-PCR technique was based on the methodology of Craig et al., a 1 kb DNA marker θ *Hae*III was used, and the PCR amplicon size for the normal allele was 841 bp (primers being A–B1) and that for the mutation 604 bp (primers being A–B2). Figure 4 depicts the Gap PCR pattern obtained in case of normal allele and the heterozygote for HPFH mutation, along with DNA ladder.

Fig. 1 **a** HbA1c value obtained on Bio-Rad DiaSTAT LPLC analyzer. **b** A1c result of same patient sample in HbA1c mode of Bio-Rad Variant analyzer

Alkaline Gel Hemoglobin Electrophoresis

Fig. 2 Alkaline gel electrophoresis showing *Lane 1*: normal sample with HbA and HbA2, *Lane 2*: sample with only HbF. *Lane 3*: patient showing HbA, HbF and reduced HbA2. *Lane 4*: Control with HbA, HbF, HbS and HbA2

Discussion

Glycohemoglobin assays are important for evaluating long term glycemic control in patients with diabetes. Ion exchange HPLC is commonly used for measuring hemoglobin A1c, however hemoglobin variants (Hb Var) or increased fetal hemoglobin concentration affect the quantification of HbA1c by HPLC. There are at least two ways in which abnormal Hb may affect HbA1c values. One is the presence of an abnormal peak on chromatography, making the estimation of the fraction of HbA1c unreliable. Secondly, some abnormal forms of Hb (e.g. Thalassemia and sickle cell trait) make red blood cells more susceptible to hemolysis. Increased hemolysis corresponds with decreased red cell lifespan consequently decreasing the time available for glycosylation of Hb chains. The two effects may coexist. Given the fact that an HbA1c

Fig. 3 **a** Smear depicting the intracellular distribution of HbF as detected by Kleihauer–Betke’s acid elution method. **b** Flow cytometric analysis showing pan cellular distribution of HbF in the case of patient

Fig. 4 Molecular characterization of HPFH mutation using Gap-PCR. *Lane 1:* normal human sample control DNA, *Lane 2:* patient, heterozygote for HPFH-3, *Lane 3:* DNA marker (1 kb marker) θ *Hae*III

deviation of 1% represents a change of 25–35 mgs% in the average blood glucose concentration, a false increase or decreased HbA1c result may have a substantial influence on the instruction given to attain strict glycemic control.

In case of this patient HbA1c of 31.5% by LPLC was implausibly high. This raised suspicion of a possible hemoglobin variant. At this point we disregarded the HbA1c value and it was only the blood glucose value which was used for the treatment. It was only on subsequent visit, that Boronate affinity excluded an HbA1c of 31.5% and confirmed that the subject was heterozygous for a hemoglobinopathy.

A considerable number of deletions of variable size and position that involve the β -globin gene complex on chromosome 11 are associated with the clinical entities of HPFH and $\delta\beta$ thalassemia.

HPFH includes a wide range of conditions, but typically it is characterized in heterozygotes by levels of HbF of up to 30% with normal red blood cell indices, while heterozygotes for $\delta\beta$ thalassemia tend to have elevated levels of HbF that are lower (5–20%) and accompanied by hypochromic, microcytic red blood cell indices.

The cellular distribution of hemoglobin F is important for evaluating persistently elevated hemoglobin F levels, such as found in HPFH or $\delta\beta$ thalassemia, and this distribution is traditionally evaluated by using Kleihauer–Betke (K–B) acid elution test. We evaluated a flow cytometric method using an anti-hemoglobin F antibody as a replacement for the K–B test and have found that the

HPFH trait gave a single peak with intermediate fluorescence which is comparable to the homocellular pattern obtained by the K–B test [9].

Molecular characterization of this HPFH mutation was done by using Gap-PCR. PCR primers were designed to flank a known deletion generating a unique amplicon that is smaller in mutant sequence compared with the wild type. When the deletion is vast (more than 2 kb), it is technically difficult to generate a product in the wild type. As a control, a primer was included that annealed within the deleted sequence to generate an additional product with one of the other primers flanking the deletion.

Weykamp et al. [10] compared the A1c results of several hemoglobin variants in 102 laboratories using 16 methods of A1c assessment. They found significant differences in results between laboratory and the methods used. For diabetics who are harboring Hb variant(s), proper care needs to be taken by the laboratory while opting for a method to test for A1c which has the least interference. Boronate affinity chromatography can be a method of choice, because though Mass Spectrometry being a referral method, the cost of the equipment is beyond the reach of most laboratories. This case report highlights the issue of potential interference from elevated HbF levels that affect HbA1c values. The underestimation of HbA1c could result in suboptimal therapy and thereby increased risk of diabetic complications by making it seem that adequate glycemic control is being maintained when it is not. The current work also highlights the usage of Flow cytometry as an alternative in identifying the cellular distribution of fetal cells.

In this case study we have also made use of the Fructosamine assay and correlated the values with that of HbA1c obtained by Boronate affinity chromatography.

References

1. Koenig RJ, Peterson CM, Jones RL, Saudek C, Lehrman M, Cerami A. Correlation of glucose regulation and hemoglobin A1c in diabetes mellitus. *N Engl J Med*. 1976;295(8):417–20.
2. Schnedl WJ, Krause R, Halwach-Baumann G, Trinker M, Lipp RW, Kreis GJ. Evaluation of HbA1c determination methods in patients with hemoglobinopathies. *Diabetes Care*. 2000;23: 339–44.
3. Sacks DB. Haemoglobin variants and hemoglobin A1c analysis: problem solved? *Clin Chem*. 2003;49:1245–7.
4. Sampietro M, Thein SL, Contreras M. Molecular studies of β -thalassemia heterozygotes with raised HbF levels. *Hemoglobin*. 2000;24(3):203–20.
5. Davis BH, Olsen S, Bigelow NC, Chen JC. Detection of fetal red cells in fetomaternal hemorrhage using a fetal hemoglobin monoclonal antibody by flow cytometry. *Transfusion*. 1998;38: 749–56.
6. Kleihauer E. Determination of fetal hemoglobin: elution technique. *Crit Rev Clin Lab Sci*. 1974;5(1):50–52.

7. Mendlovic DB, Whitehouse FW, Foreback CC. The why and wherefore of fructosamine. *Henry Ford Hosp Med J*. 1992;40: 149–51.
8. Craig JE, Barnetson RA, Prior J, Raven JL, Thein SL. Rapid detection of deletions causing $\delta\beta$ thalassemia and hereditary persistence of fetal hemoglobin by enzymatic amplification. *Blood*. 1994;83(6):1673–82.
9. Hoyer JD, Penz CS, Fairbanks VF, Hanson CA, Katzmman JA. Flow cytometric measurement of hemoglobin F in RBCs. *Am J Clin Pathol*. 2002;117:857–63.
10. Weykamp CW, Penders TJ, Muskier FA, van der Slik W. Influence of hemoglobin variants and derivatives on glycohemoglobin determinations, as investigated by 102 laboratories using 16 methods. *Clin Chem*. 1993;39:1717–23.