Case Report

Hülya Ünal*, Aysenur Atay, Muammer Yücel, Figen Narin, Serdar Ceylaner and Duran Canatan

First observation of hemoglobin G-Norfolk in the Turkish population

https://doi.org/10.1515/tjb-2019-0476
Received November 13, 2019; accepted February 25, 2020; published online February 12, 2021

Abstract

Objectives: Hemoglobinopathies are inherited transition blood diseases associated with globin chains of the hemoglobin. However many mutations have been defined, there may be many of them not defined yet. We here report the first case of those mutations, named Hb G-Norfolk in Turkey.

Case presentation: A 15 years-old male patient with erythrocytosis was referred to our laboratory for the evaluation of hemoglobinopathy. In chromatographic analysis, an unidentified peak was observed. A similar identification for variant Hb could not be obtained from High-Performance Liquid Chromatography (HPLC) analyzer’s data library. No definitive diagnosis could also be made by different analyzer. Family screening and molecular genetic DNA sequence analysis were carried out.

Conclusions: Although there were not found any beta gene mutation of neither the patient nor his family, analyses of alpha genes A1 and A2 were performed and abnormal hemoglobines were detected for all of them. This change in the HbA2 gene was at codon85 GAC>AAC (Asp>Asn) in the heterozygous state, known as Hb G-Norfolk [HbA2:c256G>A p.Asp85Asn] based on HbVar database.

Abnormal Hb bands detected by HPLC with clinical findings such as erythrocytosis or cyanosis should be investigated by sequence analysis to corroborate alpha and/or beta-globin gene mutations for avoiding misdiagnosis and misinterpretation.

Keywords: abnormal hemoglobins; alpha chain variants; Hb G-Norfolk; HPLC; oxygen affinity.

Introduction

Hemoglobinopathies are inherited transition blood diseases associated with structural changes or synthesis disorders of the globin chains of the hemoglobin (Hb) molecule. Nowadays, hemoglobinopathies can be seen in every region of the world due to increasing migrations. It is estimated that more than 300,000 children are born each year with a severe inherited disorder of Hb and approximately 80% of these births occur in low- or middle-income countries [1]. The number of abnormal Hb according to HbVar database is 1,347 [2]. In Turkey, which is located in the Mediterranean area, thalassemia and abnormal hemoglobins are common. Hb S, D, E, and Hb O-Arab are the most common abnormal hemoglobins in Turkey [3].

Thus far, more than 400 alpha-globin chain mutations have been identified according to Hbvar database. Most of the alpha chain mutations don’t have clinical results, and that is why the definition of the mutations are difficult. Additionally, clinical outcomes such as erythrocytosis or cyanosis may occur in some alpha mutations which alter the oxygen affinity of Hb [4]. Functional studies demonstrated a high oxygen affinity which is difficult to interpret on a stereochemical basis. The definitive diagnosis of hemoglobinopathy has been done with molecular methods [5]. To date, Hb G-Norfolk [HbA2:c256G>A p.Asp85Asn] based on HbVar database have not been reported in Turkey. In this study, we present a family with Hb G-Norfolk from Turkey.

Patient and Methods

A 15-year-old male patient with erythrocytosis that was detected during the routine examination, was referred to...
our laboratory for the evaluation of hemoglobinopathy. High-Performance Liquid Chromatography (HPLC) analysis was performed by analyzer with beta thal short program (Bio-Rad variant II, Bio-Rad Laboratories, Munich, Germany) for the determination of variant Hb. In the chromatographic analysis, an unidentified peak was observed with an area of 18.1% and Retention time (RT) of 3.93 (Figure 1A). When the HPLC analyzer’s data library

Table 1

| Peak Name | Calibrated Area | Area % | Retention Time (min) | Peak Area |
|-----------|-----------------|--------|----------------------|-----------|
| Unknown   | ---             | 0.0    | 0.96                 | 1113      |
| F         | ---             | 0.2    | 1.06                 | 4998      |
| Unknown   | ---             | 0.7    | 1.22                 | 20964     |
| F2        | ---             | 3.4    | 1.31                 | 103434    |
| F3        | ---             | 3.9    | 1.66                 | 117306    |
| Ag        | ---             | 71.9   | 2.40                 | 2163981   |
| A2        | 1.3*            | ---    | 3.62                 | 36107     |
| G-window  | ---             | 18.1   | 3.03                 | 143176    |
| G-window  | ---             | 0.6    | 4.44                 | 17212     |

Total Area: 3,010,181

F Concentration = 0.2 %
A2 Concentration = 1.3% *

*Values outside of expected ranges

Analysis comments:
was examined, a suitable identification for variant Hb could not be obtained. Additionally, any identification of variant could not be obtained by another one HPLC analyzer (Primus Ultra2TM, Trinity Biotech, Kansas City, MO) (Figure 1B). To obtain definitive diagnosis, family screening and molecular genetic analysis phases were planned.

**Results**

Evaluation of family members shows that they (mother and sister) also have an unknown peak with the similar area and RT (18.3% and 3.96, 17.8% and 3.96 respectively) (Figures 2A and B). The evaluation could not be applied for the father since the parents were divorced and we could not attain

![Figure 2: (A) HPLC graphic of the mother's hemoglobin fractions on Bio-Rad variant II Turbo using beta-thal short program. (B) HPLC graphic of the sister's hemoglobin fractions on Bio-Rad variant II Turbo using beta-thal short program.](image)

**Table 1:** Summary of hematological and biochemical parameters for the patient and his family.

| Parameters                  | Patient | Sister | Mother |
|-----------------------------|---------|--------|--------|
| Age (years)                 | 15      | 16     | 33     |
| Hb (g/dL)                   | 15.9 (12–16) | 13.4 (11–15) | 11.8 (11–15) |
| Htc (%)                     | 46.9 (40.1–51.0) | 40.0 (34.1–44.9) | 36.6 (34.1–44.9) |
| RBC (10¹²/L)                | 5.76 (4.00–5.5) | 4.76 (3.5–5.0) | 4.78 (3.5–5.0) |
| MCV (fl)                    | 81.4 (80–100) | 84.0 (80–100) | 76.6 (80–100) |
| MCH (pg)                    | 27.6 (27–34) | 28.2 (27–34) | 24.7 (27–34) |
| Reticulocytes (%)           | 1.13     | 0.86    | 0.84    |
| Ferritin (µg/L)             | 16 (22–320) | 8 (10–290) | 5 (10–290) |
| WBC (10⁹/L)                 | 7.90 (6.00–10) | 6.82 (4.00–10) | 7.35 (4.00–10) |
| PLT (10⁹/L)                 | 278 (150–400) | 204 (150–400) | 217 (150–400) |
| Iron (µg/dL)                | 133 (65–175) | 68 (50–170) | 68 (50–170) |
| TIBC (µg/dL)                | 419      | 447     | 354     |

Hb: hemoglobin; RBC: red blood cell; MCH: mean corpuscular Hb; MCV: mean corpuscular volume; TIBC: total iron binding capacity. The levels which are out of the reference range are marked bold character.
his blood sample for analysis. The hematological and biochemical parameters for the patient and his family were shown in Table 1. The results of two different HPLC analyzers were presented in Table 2. The patient’s blood specimen was studied by two analyzers. It was thought that there was no need to study sister’s and mother’s samples additionally.

In addition, iron status and ferritin levels were studied for all subjects. Iron status was in reference range for the patient and his parents, but all ferritin levels were lower than the reference range.

In blood count analysis, although the patient’s and his sister’s Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) were in the reference range, his mother’s data showed low levels according to reference ranges.

However, erythrocytosis and polycythemia covers the RBC number plus hemoglobin and hematocrit values, a patient has only low ferritin level and hardly exceeding RBC level. In the differential diagnosis of erythrocytosis and polycythemia, WBC and Platelet levels were high in polycythemia. In this study, those parameters were detected in reference range for the patient and his parents consistent with erythrocytosis. As a limitation of the study, any other secondary causes of the increase in RBC number of patient have not been investigated.

It was concluded that the patient’s mother may have iron deficiency or other causes of anemia concomitantly with a variant Hb. It should be investigated by further analysis to obtain a definitive cause of anemia.

Molecular genetic analysis of the patient was performed in by Genetic Diseases Diagnosis Center (Antalya, Turkey) and Intergen Genetic Diagnosis Center (Ankara, Turkey). Genetic analysis of the patient’s family was also performed Genetic Diseases Diagnosis Center (Antalya, Turkey). In the molecular genetic diagnosis stage, firstly, DNA sequence analysis (Miseq-Illumina, Florida, USA) was performed for

| Parameters | Patient | Sister | Mother |
|------------|---------|--------|--------|
| Analyzer I |         |        |        |
| Hb A1 (%)  | 71.9    | 72.0   | 71.7   |
| Hb A2 (%)  | 1.9'    | 2.2'   | 1.5    |
| Hb F (%)   | 0.2     | 1.1    | 0.3    |
| Hb X (%)   | 18.1 (RT 3.93) | 17.8 (RT 3.96) | 18.3 (RT 3.96) |
| Analyzer II|         |        |        |
| Hb A1 (%)  | 70.2    | –      | –      |
| Hb A2 (%)  | 2.2'    | –      | –      |
| Hb F (%)   | 0.2     | –      | –      |
| Hb X (%)   | 17.8 (RRT 0.91) | –      | –      |

RT: Retention time; RRT: Relative retention time.
*HbA2 represents the sum of HbA2 (1.3–1.7%) and HbX2. HbX2 can be seen on the diagrams of separations of Variant II instrument at S-windows (RT: 4.44; 0.6%) and the peak of Primus instrument at RRT: 5.835; 0.5%.

Figure 3: Sequence analysis of alpha genes of the patient.
the beta-globin gene mutations. Beta gene mutations were not detected in the investigated gene regions and it was considered that the patient might have an alpha chain mutation. DNA Sequence analyzes of alpha genes A1 and A2 (Miseq-Illumina, Florida, USA) were performed and an abnormal Hb was detected (Figure 3). This change in the HbA2 gene was at codon85 GAC>AAC (Asp>Asn) in the heterozygous state, known as Hb G-Norfolk [HbA2:c256G>A p.Asp85Asn] based on HbVar database [2]. Meanwhile, the genetic diagnoses of the mother and sister were performed and the same variant was also detected.

**Discussion**

Ager, Lehmann, and Vella discovered and reported new abnormal Hb of a family in Norfolk possessing in 1958 [6]. In 1963, Huntsman et al. conducted a screening study to determine the incidence of this variant in the County of Norfolk. Then, 1,000 unrelated inhabitants were investigated. Huntsman et al. found the other two variant Hb in two unrelated families and they named them as Hb G-Norfolk and Hb D-Norfolk respectively [7]. Firstly, Hb G-Norfolk [HbA2:c256G>A p.Asp85Asn] was as molecular described in by Lorkin et al. in three unrelated English families without any clinical results in 1975 [8]. In the same year, in another study conducted by Cohen-Solal, Hb G-Norfolk was detected in French patient with acute lymphoblastic leukemia [9]. Cohen-Solal also identified this variant in two other family members who were not affected by leukemia.

In this study, we presented Hb G-Norfolk firstly observed in Turkey. This abnormal variant has slightly increased oxygen affinity due to substitution at 85th residue of the α chain (F6) [9]. This substitution is located just below the helix of the heme pocket. Therefore, the increase in oxygen affinity of this Hb is very mild. Since all the cases reported so far are heterozygous, the clinical consequences of this variant Hb in a homozygous state are still unknown.

In this study, our case was also heterozygous and did not have any clinical or laboratory results except erythrocytosis or cyanosis should be investigated by sequence analysis to corroborate alpha and/or beta-globin gene mutations to avoid misdiagnosis and misinterpretation.

The importance of identification of hemoglobin variants is to prevent the risk of encountering with other variants and forming heterozygous compound forms which may appear with clinical findings.

**Acknowledgments:** The authors wish to thank to Ugur Kayce and Gulsah Ayata Topuz and Ersin Kınık for their helpful support and Hülya İnan for her excellent technical assistance.

**References**

1. Weatherall DJ. The inherited disorders of hemoglobin are an emerging global health burden. Blood 2010;115:4331–6. https://doi.org/10.1182/blood-2010-01-251348.
2. Globin Gene Server. HbVar: A Database of human hemoglobin variants and thalassemias. Available from: http://globin.bx.psu.edu/cgi-bin/hbvar/counter (Last accessed: August 2019).
3. Altay Ç. Abnormal hemoglobins in Turkey. Turk J Haematol 2002;19:63–74.
4. Gürgey A. Anormal Hemoglobinler. HematoLog 2014;4:134–45.
5. Hartevedt CL, Higgs DR. Alpha-thalassaemia. Orphanet J Rare Dis 2010;5:13.
6. Ager JAM, Lehmann H, Vella F. Haemoglobin Norfolk: a new Haemoglobin found in an English Family. Br Med J 1958;2:539–41. https://doi.org/10.1136/bmj.2.5095.539.
7. Huntsman RGaa, Hall M, Lehmann H, Sukumaran PK. A second and a third abnormal haemoglobin in Norfolk. Haemoglobin G Norfolk
and haemoglobin D Norfolk. Br Med J 1963;1:720–2. https://doi.org/10.1136/bmj.1.5332.720.

8. Larkin PA, Huntsman RG, Ager JA, Lehmann H, Vella F, Darbre PD. Haemoglobin G Norfolk: α 85 (F6) Asp leads to Asn. Biochim Biophys Acta 1975;379:22–7. https://doi.org/10.1016/0005-2795(75)90004-5.

9. Cohen-Solal M, Manesse B, Thillet J, Rosa J. Haemoglobin G Norfolk α 85 (F6) Asp → Asn. Structural characterization by sequenator analysis and functional properties of a new variant with high oxygen affinity. FEBS Lett. 1975;50:163–7. https://doi.org/10.1016/0014-5793(75)80480-7.