Fortuitous description of haemoglobin A2’ \([\delta 16 (A13) \text{Gly} \rightarrow \text{Arg (GGC} \rightarrow \text{CGC})]\) in a Tunisian family: study of the molecular defect and its origin

Détection fortuite de l’hémoglobine A2’ \([\delta 16 (A13) \text{Gly} \rightarrow \text{Arg (GGC} \rightarrow \text{CGC})]\) dans une famille tunisienne : étude du défaut moléculaire et de son origine

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Abstract. The most common inherited haemoglobin disorders encountered in Tunisia are \(\beta\)-thalassemia and sickle cell disease, which result from mutations in the \(\beta\)-globin gene. Few studies focused on \(\delta\)-globin gene variations responsible for \(\delta\)-thalassemia or HbA2 variants. HbA2’ \([\delta 16 (A13) \text{Gly} \rightarrow \text{Arg (GGC} \rightarrow \text{CGC})]\) is a \(\delta\)-chain variant that has been identified in several populations of African origin. We report herein for the first time the description of HbA2’ in the Tunisian population. Identification of HbA2’ in the studied family was carried out by high-performance liquid chromatography and confirmed by sequencing analyses of the whole \(\delta\)-globin gene. Haplotypes of the \(\beta\)-globin gene cluster were constructed by mapping the restriction sites using polymerase chain reaction followed by enzymatic digestion. Compound heterozygosity of HbA2’ with HbO-Arab was identified in the proband. The mother and two other siblings showed heterozygous HbA2’ whereas the father showed heterozygous HbO-Arab. The sum of HbA2 and HbA2’ in all cases was less than 4%, thus excluding \(\beta\)-thalassemia. \(\beta\)-cluster haplotype analysis revealed that this mutation was associated with the F haplotype (-+–+++). The unique origin of this mutation in Africa is likely since the linked \(\beta\)-cluster haplotype is one of the major haplotypes found in African populations.

Key words: HbA2’, HbO-Arab, thalassemia, Tunisia

Résumé. La \(\beta\)-thalassémie et la drépanocytose, dues à des mutations au niveau du gène \(\beta\)-globine, sont les hémoglobinopathies les plus répandues en Tunisie. Bien que le gène \(\beta\)-globine fût bien investigué, peu d’études se sont intéressées aux variations responsables de \(\delta\)-thalassémies ou aux variants de l’HbA2 dans notre pays. L’HbA2’ \([\delta 16 (A13) \text{Gly} \rightarrow \text{Arg (GGC} \rightarrow \text{CGC})]\) ou HbB2 est un variant de la chaîne \(\delta\) qui a été identifié dans plusieurs populations d’origine africaine. Dans cette étude, nous rapportons la première description de l’HbA2’ dans une famille tunisienne. Ce variant était identifié par chromatographie liquide à haute performance et confirmé par le séquençage de la totalité du gène globine \(\delta\). Les haplotypes au niveau du cluster \(\beta\) sont construits par l’exploration des sites polymorphes par PCR-RFLP. Le propositus est hétérozygote composite pour l’HbA2’ et l’HbO-Arab. La mère et le reste de la fratrie sont des porteurs de l’HbA2’, alors que le père est hétérozygote pour l’HbO-Arab. La somme des taux de l’HbA2 et l’HbA2’ est inférieure à 4 %, ce qui exclut une éventuelle présence du trait \(\beta\)-thalassémique. L’analyse des haplotypes montre que ce variant est associé avec l’haplotype africain (-+–++). L’unique origine africaine de ce variant est probable étant donné son association avec l’haplotype le plus répandu chez les populations africaines.

Mots clés : HbA2’, HbO-Arab, thalassémie, Tunisie
The adult human haemoglobins (Hb) include HbA (α2β2) and HbA2 (α2β2). The latter is a tetramer of α and δ globin chains resulting from the low expression of the δ-globin gene. Under normal conditions, HbA2 ranges from 2 to 3% of the total haemoglobins in the post-natal life. In clinical diagnosis, variations in HbA2 levels are used in the screening of β-thalassemia minor (HbA2 > 4%) and α-thalassemia minor (HbA2 < 2%). HbA2 values should also be interpreted together with other findings, such as iron status and erythrocyte indices [1].

Genetic defects in δ-globin gene causing δ-thalassemia or HbA2 variants decrease the HbA2 value (≤ 2% in the heterozygous or ≤ 0.6% in homozygous) but do not affect health [1, 2].

In the Mediterranean region, the carriers of δ-thalassemia defects or HbA2-variants are estimated to be > 1% and about 40 of 70 known alleles have been found in families of this region [2], HbA2’ (or HbB2), which is a clinically silent δ-chain variant resulting from the substitution of Glycine for Arginine at codon 16 (GGC→CGC) of exon 1, is the commonest of the known HbA2 variants [3]. The molecular defect consists of a point mutation on the δ-globin gene (HbD: c.49G>C). HbA2’ has been detected in heterozygous and homozygous states and in combination with other Hb variants and thalassemia [4, 5]. The major clinical significance of HbA2’ is that failure to detect it might lead to underestimation of the total HbA2 and failure to recognize a possible β-thalassemia trait. These data highlight the necessity to consider the sum of HbA2 and HbA2’ levels. Studies of the Restriction fragment length polymorphism (RFLP) haplotypes carried out in a few families led to conflicting hypotheses about the origin of this allele [2, 6, 7].

In the present study, we report for the first time the identification of the HbA2’ co-inherited with HbO-Arab in a Tunisian family. Investigation of the chromosomal background associated with this mutation was conducted in order to highlight its origin in the Tunisian population.

Haematological and biochemical analyses

Haematological parameters and serum ferritin concentration were determined in all family members. Hb identification was carried out by a cation exchange high-performance liquid chromatography (HPLC), using the HbA2/HbA1C Dual Program on Variant™ II Haemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA). Normal and abnormal Hb fractions are eluted from the cartridge based on their ionic interaction with the cartridge material. The system allows an accurate determination of haemoglobins A2, F, and A1C in whole blood samples, without interference from labile A1C, lipaemia, or temperature fluctuations. Blood samples are automatically mixed and diluted in the Variant™ II Sampling Station and injected into an assay specific analytical cartridge to which a buffer gradient of increasing ionic strength is delivered. Then the separated haemoglobins pass through the flow cell of the filter photometer, where changes in the absorbance (415 nm) are measured. Background variations were corrected by using an additional filter at 690 nm and the different haemoglobins were eluted in the following order: HbF, HbA1C, HbA, HbA2, and HbS. A chromatogram is generated for each sample. The printed chromatogram shows all Hb fractions eluted areas of the peaks, values (%) of different Hb components, and retention times (RT). The RT is the time that flows from the sample injection to the apex of the elution peak of normal haemoglobin fractions and common variants. A peak is defined as unknown when it elutes at a RT that is not predefined.

Molecular biology studies

DNA was extracted from peripheral blood leukocytes by a phenol-chloroform procedure. The presence of HbA2’ was confirmed at the molecular level by polymerase chain reaction (PCR), followed by direct sequencing and PCR-RFLP.

Amplification of the δ globin gene exons was carried out using three different primer sets amplifying three different fragments as previously described [8]. The PCR was conducted with an annealing temperature of 60°C for 30 seconds with a total of 30 cycles. The mutation was identified by automated sequence analysis performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using a BigDye-Terminator Sequencing Ready Reaction kit. The mutation was confirmed by PCR-RFLP using HBD1-F and HBD2-R primers [8] and C/6I restriction enzyme, which recognizes and cleaves a sequence created by the GGC→CGC mutation at codon 16 (exon 1) of the HBD gene. In case of mutation, two fragments of 424 and 279bp are generated. Haplotypes of the β-globin gene cluster were constructed by mapping the seven classical restriction sites using a

Methods

Subjects

The study was carried out on a family composed of the father, the mother, and three siblings whose age ranges between 2 and 7 years. The family originates from Ain Drahem, a district in the North-West of Tunisia. Samples were collected after informed consent was obtained in compliance with the local Institutional review board guidelines (Children Hospital, Tunis).
PCR-based approach followed by digestion with the appropriate enzymes: HincII 5’ to the e gene, HindIII in the IVS-II of the Gγ and Aγ genes, HincII 5’ and 3’ in the ψβ gene, AvaII in the IVS-II of the β gene, and BamHI 3’ to the β gene. Haplotypes were numbered according to Orkin and collaborators [9]. Alpha-globin genotyping was performed for the most frequent defects encountered in Tunisia: -α^3.7^, -MedII, and α^Tsaudi^ as previously described [10].

**Results**

Haematological data of the family are summarized in figure 1. The parents (I-1 and I-2) had normal values of Hb and mean corpuscular volume (MCV). Individuals II-1 and II-3 showed mild microcytosis. The proband (II-2) presented a more severe microcytosis. No iron deficiency was noted on serum ferritin measurement. Haemoglobin electrophoresis revealed that individuals I-1 and I-2 were carriers of HbA2’ and HbO-Arab, respectively. II-2 co-inherited HbO-Arab (33.2%) from the father and HbA2’ (1.4%) from the mother, whereas individuals II-1 and II-3 had only HbA2’. Furthermore, the mother and the siblings showed low values of HbA2 ranging between 1 and 1.3%. The sum of HbA2 and HbA2’ in all members is less than 4%, thus excluding a β-thalassemia trait (figure 2). Sequencing of the δ-globin gene showed that individuals I-1, II-1, II-2, and II-3 were heterozygous for HbA2’ (HbD: c.49G>C) mutation (figure 3). RFLP haplotype analyses indicated that HbA2’ was linked to the haplotype F (-+–++++) in the mother and the affected siblings. The α-globin genotype was normal.

**Discussion**

In this study, we report for the first time the fortuitous description of HbA2’ [816 (A13) Gly→Arg (GGC→CGC)], also called HbB2, in the heterozygous state in a family originating from the North-West of Tunisia (figure 1). HbA2’ was identified in the heterozygous state in 4 individuals: the mother and three siblings by using HPLC. HbA2’ is globally the commonest δ-chain variant. It is clinically silent. It was first discovered in 1958 in the Black community of Gulla James Island who are African-Americans living in the low country region of South Carolina and Georgia [11]. Since then, it has been reported with an increasing frequency among Blacks with a
prevalence of 2.16% in Mauritania [12], 12.4% in Dogon (Mali), [13], and a higher frequency reaching 18% in Herero, a Bantu group of 240,000 members living in Namibia, Botswana, and Angola [6].

The substitution of arginine for glycine in HbA2' confers a net positive charge gain to the β-globin chain. In earlier studies, this β-chain variant was identified by standard starch gel and cellulose acetate electrophoresis [6, 12]. This Hb is also detectable by isoelectric focusing, though this method is not widely used as a primary screening technique beyond the neonatal period, when HbA2 levels are normally low. Its main disadvantage is its relatively high cost and requirement for a well-experienced laboratory staff [5, 14]. On micro-column chromatography, HbA2' co-elutes with HbA2 and the total HbA2 level measured would be expected to be accurate, but HbA2' would not be identified.

Of the many techniques of separation and quantification of Hb fractions, HPLC is the most reliable in identifying normal and abnormal Hb variants based on the RT, the percentage of the variant, and the chromatogram profile [3, 5, 7, 15].

Our study was conducted by HPLC using the Dual-Program on Variant™ II Haemoglobin Testing System. The manufacturer defines identification windows of the HbA2 as 2.81 to 3.01 min, the HbS as 3.35 to 3.55 mn, and HbO-Arab at 4.07 min, and recommends a DNA analysis for the positive confirmation of any particular Hb variant. In our studied family, HbA2' was eluted as a small S-window peak at 3.60 mn, whereas HbA2 and HbO-Arab were eluted at 2.95 and 4.00 mn, respectively (figure 2). In earlier reports from Oman, Pakistan, and America, the HbA2' variant was eluted at 4.58 to 4.59 mn [3, 5, 7]. The differences observed in RT could be related to different lots of reagents, manufacturing changes or different separation methods [16]. At the same HPLC RT (3.60min), there is another variant: HbG-San José [β7 (A4) Glu→Gly], which is a β-chain variant that accounts for a significantly greater proportion of the total Hb and therefore, should not be confused with HbA2' [17].

Figure 2. High-performance liquid chromatography results from patients with (A) HbA2' trait and (B) HbA2'/HbOArab. The arrows indicate HbA2' peaks.

Figure 3. Genomic DNA sequence chromatogram showing the HbA2' mutation in heterozygous state: GGC → CGC affecting exon 1 of the HBD gene.
Lys were identified as of today: HbA2-Pasteur-Tunis molecular defects of the Tunisia, few reports have focused on the study of the Despite the high frequency of haemoglobinopathies in trait. HbA2 was less than 4%, thus excluding a total Hb. In our study, the total percentage of HbA2' and the sum of HbA2 and HbA2' is greater than 4% of the be suggestive of compound HbA2'/trait if present. It is noteworthy that those cases may ure to detect it might lead to misdiagnosis of the clinical significance of HbA2' identification is that fail- among the Arab populations [20].

In our study, the family showed co-inheritance of heterozygous HbO-Arab, which is a rare chain variant in Tunisia. This combination resulted in microcytosis without anaemia. In previous studies, HbA2' was associated with a -thalassemia trait [5, 12], -thalassemia trait [7] or with HbS, HbC, and HbG [3, 5]. To the best of our knowledge, this is the first description of HbA2' occurring in conjunction with HbO-Arab. The latter variant is commonly found in the Balkan region and the Middle East. Its frequency with HbD and other -chain variants is estimated to be 0.15% in Tunisia [18]. The HbO-Arab eastern variant has been introduced in Tunisia when Arabs invaded North Africa back in the VII Century [19]. Despite its name, HbO-Arab is rare among the Arab populations [20].

The clinical significance of HbA2' identification is that failure to detect it might lead to misdiagnosis of -thalassemia trait if present. It is noteworthy that those cases may be suggestive of compound HbA2'/ -thalassemia when the sum of HbA2 and HbA2' is greater than 4% of the total Hb. In our study, the total percentage of HbA2' and HbA2 was less than 4%, thus excluding a -thalassemia trait.

Despite the high frequency of haemoglobinopathies in Tunisia, few reports have focused on the study of the molecular defects of the -gene. Only two mutations were identified as of today: HbA2-Pasteur-Tunis [859 (E3) Lys→Asn, AAG→AAC], [21] and the 80 59 (-A), [8]. HbA2' identification for the first time in Tunisia will allow to update the -globin gene defect spectrum. The -cluster haplotype analysis in our study revealed that this mutation was associated with haplotype F (+++++), which is similar to that reported by Spurdle and collaborators, in a family of mixed ancestry of South Africa [6]. This population derived from an admixture of indigenous Khoisan people, immigrant Caucasians, Indonesians, and Bantu-speaking Blacks.

Our findings are different from those of other reports describing HbA2' in association with a unique African haplotype (+++++) (table 1) [4, 6, 7, 11, 22]. The unique origin of this mutation in Africa is likely since the linked -cluster haplotype is one of the two major haplotypes found in all African populations [13]. It is interesting to note that 5' sub-haplotype (+++) is the same in the chromosomes carrying the HbA2'. Hence, a recombination event in the hot spot within the 9.1kb between the ββ and β genes can easily explain its association with two haplotypes.

## Conclusion

Awareness of HbA2' presence will assume an even greater importance in genetic counselling and prenatal diagnosis since it will prevent underestimation of the HbA2 and failure to diagnose -thalassemia trait if present.

## Acknowledgments

We are indebted to the family who participated in the study. This work was supported by a grant from the Ministry of scientific research and technology and competence development.

## Conflict of interest

none of the authors has any conflict of interest to disclose.

## References


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### Table 1. -globin gene cluster haplotypes associated with HbA2'.

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The small fraction of the HbA2’ seen in the HPLC chromatogram could be missed as an artifact from the previous run of a sample containing HbS since they elute in the same window [7]. For this reason, the diagnosis of HbA2’ trait by HPLC is based on a 1 to 2% of Hb fraction identified without a previous run showing HbS or other fractions eluting at the same RT.

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