Two new β-thalassemia deletions compromising prenatal diagnosis in an Italian and a Turkish couple seeking prevention

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ABSTRACT

When the molecular background of couples requesting prevention is unclear, family analysis and tools to define rare mutations are essential. We report two novel deletion defects observed in an Italian and in a Turkish couple. The first proband presented with microcytic hypochromic parameters without iron deficiency, a normal HbA_2 and an elevated HbF (10.6%). His father presented with a similar phenotype and his wife was heterozygous for the common Mediterranean codon 39 (HBB:c.118C>T) mutation. Having excluded point mutations and common deletions, Multiplex Ligation-dependent Probe Amplification was performed revealing an unknown $G\gamma(A\gamma\delta\beta)^0$-thalassemia defect spanning from the Aγ gene to downstream of the β-globin gene provisionally named *Leiden 69.5 kb deletion*. In the second case, the wife presented with a mild thalassemic picture, normal HbA_2, elevated HbF (18.5%) and a β/α globin chain synthesis ratio of 0.62, without iron deficiency or any known β-thalassemia defect, while the husband was a simple carrier of the common Mediterranean IVS-I-110 (HBB:c.93-21 G>A) mutation. A new large deletion involving the β-gene and part of the δ-gene was identified by Multiplex Ligation-dependent Probe Amplification provisionally named "Leiden 7.4 kb".

Key words: thalassemia, prevention, Multiplex Ligation-dependent Probe Amplification.

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Introduction

Assessment of couples for the risk of transmitting β-thalassemia major is usually achieved through basic hematology diagnostics and molecular analysis. As the majority of β-thalassemias is caused by point mutations, direct sequencing analysis is the first method to be applied, and if negative, gap-PCR can be used to investigate the presence of known deletions. Rare cases with large deletions in the β-globin gene cluster have been associated with microcytic hypochromic red cell parameters, along with normal HbA_2 and elevated or normal HbF, and such cases may be difficult to define at the molecular level or even be overlooked. The interaction of β^0-thalassemia point mutations with *elevated HbF* β-globin gene deletions might result in mild or intermediate phenotypes. However, combinations with deletions involving both γ-globin genes may result in severe compound heterozygosity due to the absence of the compensatory effect of fetal hemoglobin. Prenatal diagnosis might lead to a false adverse result, when the fetus has inherited a point mutation from one parent and a deletion allele with an intact – but inactive – β-globin gene from the other parent. We report 2 cases of novel deletions identified by MLPA in at-risk couples requesting prevention, which were studied in collaboration with different reference centers.

Design and Methods

Propositus A

An Italian male who was requesting prevention for β-thalassemia due to a diagnosis of β-thalassemia minor codon 39 (HBB:c.118C>T) mutation in his wife (Figure 1A).

Propositus B

A Turkish woman requesting prenatal diagnosis, with a history of an inconclusive result from a previous pregnancy which was consequently terminated. Her husband was diagnosed as a carrier of the IVS-I-110 (HBB:c.93-21G>A) β-thalassemia point

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mutation Figure 1B).

In both probands, who presented with elevated HbF level, no point mutations or known deletions were found. Case A was studied in Rome, Genoa and Leiden; case B in Istanbul, Cagliari, Athens and Leiden.

Hematologic data were obtained on several different occasions using the locally available standard protocols. Separation of the Hb fractions and measurement of the HbA_2 and HbF levels were obtained from different automatic high performance liquid chromatographic (HPLC) devices.[1] Globin chain synthesis was performed in Cagliari using a modified procedure based upon Kan *et al.*[2] Genomic DNA was isolated from peripheral leukocytes using either automatic or manual high salt extraction methods based on Miller *et al.*[3] Molecular analysis for common deletions and the α-triplication was performed at different centers using multiplex PCR methods.[4,5] Point mutation analysis of the β-globin gene cluster was performed by direct sequencing in different locations and on different apparatuses. Multiplex Ligation-dependent Probe Amplification (MLPA) was carried out in Leiden as previously described.[6,7]

Results

Case A

Propositus (II-2), presented with microcytic hypochromic red cell parameters (Hb 12.4 g/dL; RBC 6.07×10^{12}/L; MCV 63 fL; MCH 20.5 pg) without iron deficiency (ferritin 154 ng/mL), with normal HbA_2 (3.2%) and elevated HbF (10.6%) levels, compatible with a δβ-thalassemia deletion. His father presented with a similar phenotype but they were both negative for the common δβ-deletions such as the Black, Sicilian, Macedonian-Turkish, Asian-Indian, Filipino, Chinese, HPFH-I or HPFH-V. Sequencing of the β-globin genes revealed no point mutations. The Aγ-globin promoter sequence showed heterozygosity for the common 4 nt deletion (HBG2:c.-278_-275delAGCA), a mutation described in a mild Spanish codon 39 (HBB:c.118C>T) homozygous case.[8] Finally, MLPA of the β-globin gene cluster revealed a deletion of at

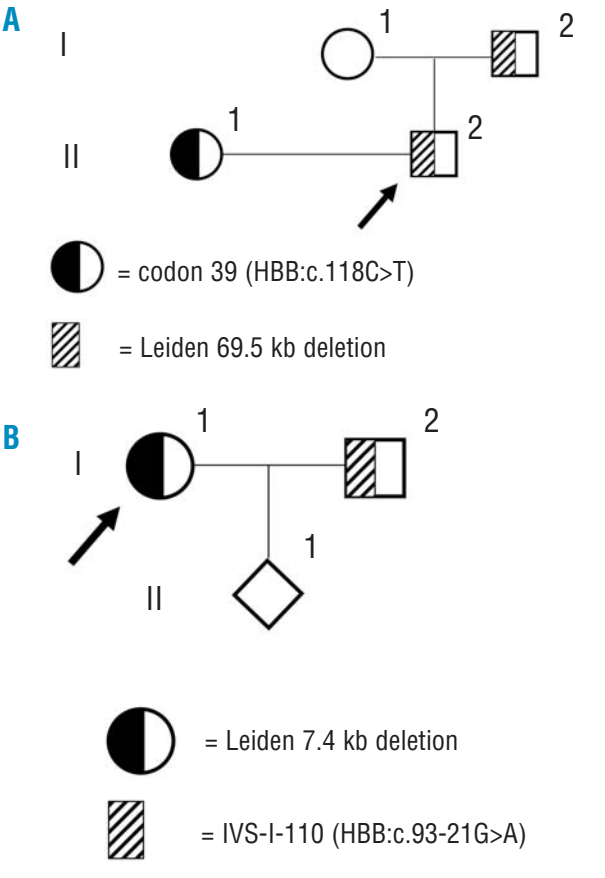

Figure 1. Pedigrees of the Italian (1A) and Turkish (1B) families. Both probands carry an unknown deletion in the β-globin gene cluster, while their partners are diagnosed with a known point mutation.

Table 1. Overview of the hematologic, biochemical and molecular data.

Individuals	I-1	I-2	II-1	II-2*	I-1**	I-2
Age/Gender	60/F	68/M	32/F	34/M	30/F	44/M
Hb (g/dL)	13.6	13.3	10.8	12.4	12.4	13.1
PCV (l/L)	0.41	0.43	0.34	0.38	0.37	0.39
RBC ($x10^{12}$/L)	4.68	6.47	5.22	6.07	5.21	6.43
MCV (fL)	88	67	64	63	70.8	64.4
MCH (pg)	29.2	20.6	20.6	20.5	23.8	20.41
Ferritin (ng/mL)	n.d.	202	56	154	5.52	n.d
ZPP (mmol/mol)	n.d	n.d.	n.d.	n.d.	88	n.d
HPLC	A-A2	A-F-A2	A-A2	A-F-A2	A-F-A2	A-A2
HbA_2 %	2.7	2.9	6.1	3.2	2.7	5.2
HbF %	0.5	4.2	0.5	10.6	18.5	<1
β/α ratio	n.d.	n.d.	n.d.	n.d.	0.62	n.d.
DNA β	n.d.	69.5 kb del	Cd39 (C→T)	69.5 kb del	7.4 kb del	IVS1-110 (G→A)

** = Casus propositus A ; ** = Casus propositus B ; n.d.: not determined.*

least 69.5 kb spanning from the Aγ-globin gene to ~37 kb downstream of the β-globin gene. We have provisionally named this event *Leiden 69.5 kb* deletion (Figure 2A left and 2B). His wife was carrier of the codon 39 (HBB:c.118C>T) mutation. Her parents were not available for analysis.

Case B

The propositus (I-1) presented with mild anemic hematologic parameters, normal HbA_2, elevated HbF (18.5%) and a β/α-globin chain ratio of 0.62, with low ferritin and normal ZPP. No point mutations or known deletions in the β-globin gene were found. The same phenotype was present in her mother (*data not shown*). The 13.4 kb Sicilian δβ-deletion and the Turkish δβ-inversion/deletion were excluded by gap-PCR. A new large deletion involving the β-globin gene and part of the δ-globin gene was detected by MLPA. The deletion is at least 7.4 kb in length, with a maximum of 22 kb. The 5' break point is located within or upstream of the δ-globin gene and the 3' break point is located around a ~6.1 kb L1-repeat at the 3' end of the β-globin gene cluster. We have provisionally named the new deletion "Leiden 7.4 kb" (Figure 2A right and Figure 2B). The husband was found to be a carrier of the common IVS-I-110 (HBB:c.93-21G>A) mutation and presented with the expected red cell parameters and elevated HbA_2 level. All hematologic, biochemical and molecular data are summarized in Table 1.

Discussion

Deletion defects affecting the β-globin gene cluster, associated with a high HbF expression in adult life, are usually subdivided in two categories. This distinction is purely clinical and based on the observation of the hematologic indices of the specific cases. Those cases with (near) normal hematologic indices are defined as HPFH, those presenting with abnormal indices as δβ-thalassemia.

In fact, all deletion defects involving the β-globin gene should be considered as β-thalassemia determinants with a variable phenotype. The variability depends upon the *rescue effect* from the β-thalassemia minor phenotype associated with the presence of elevated HbF production. However, this normalization has to be evaluated with caution. The normocytic or macrocytic F-cells contribute to a false impression of normalized red cell indices and furthermore, since HbF is a poor carrier of oxygen to the tissues, chronic hypoxia remains. Some erythrocytosis is usually triggered, generating a so called *normal* (non-anemic, normocytic-normochromic) state but still in the absence of β-globin gene expression. Moreover, polymorphisms on the promoters of the Gγ- and Aγ-globin genes, the only conditions to be appropriately categorized as HPFH, may be present and modulate the HbF expression of the partially deleted allele, introducing more phenotype variability. In the end it will be the β/α and γ/α synthetic ratio that will differentiate thalassemia from HPFH.

In case A, MLPA showed that the 5' break point is located in an area between the two γ-globin genes, which is common to several HPFH and $Gγ(Aγδβ)^0$-thalassemia deletion defects, such as the Indian (32.6 kb) and the Turkish (30 kb) HPFH, as well as the Indian (8.5 kb), the Black (35.7 kb), the Belgian (50 kb) and the Italian (52kb) $Gγ(Aγδβ)^0$-thalassemia defects.

The new deletion was associated with the expected microcytic hypochromic parameters of a $Gγ(Aγδβ)^0$-tha-

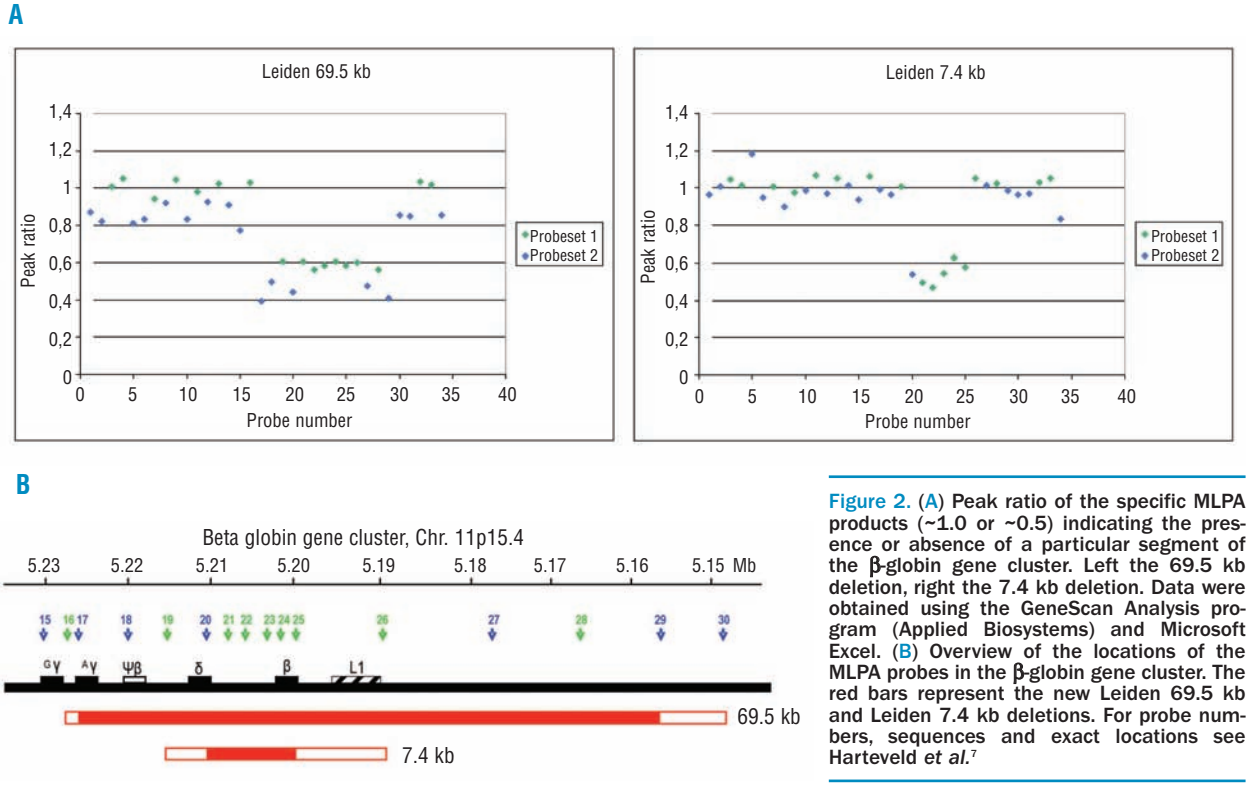

Figure 2. (A) **Peak ratio of the specific MLPA products (~1.0 or ~0.5) indicating the presence or absence of a particular segment of the β-globin gene cluster. Left the 69.5 kb deletion, right the 7.4 kb deletion. Data were obtained using the GeneScan Analysis program (Applied Biosystems) and Microsoft Excel. (B) Overview of the locations of the MLPA probes in the β-globin gene cluster. The red bars represent the new Leiden 69.5 kb and Leiden 7.4 kb deletions. For probe numbers, sequences and exact locations see Harteveld *et al.*[7]**

lassemia defect because the HbF expression, in the absence of the Aγ-globin gene, was limited to 10.6% in the proband, and is unexpectedly even lower (4.2%) in his father. The heterozygous state for the 4nt deletion polymorphism in the Aγ-globin gene promoter (HBG2:c.-278_-275delAGCA) indicates that this region of the Aγ gene is still present. This polymorphism has been described in association with a mild β^0/β^0 patient with high HbF expression,[8] but cannot account for a higher HbF expression of the deleted allele, since the Aγ-globin gene is partially deleted. Since the Xmn-I polymorphism was absent, the 10% HbF expression could be directly associated with the deletion, or alternatively be increased by some Aγ-globin gene expression in trans if the -4nt deletion polymorphism is not on the deleted allele. An attempt to justify the difference in HbF expression between father and son by the absence of this polymorphism in the father was unsuccessful, since the proband and both his parents were all carriers of the -4nt deletion.

In conclusion, heterozygosity for the -4nt deletion polymorphism indicates the Aγ-globin gene promoter as break point boundary. None of the known deletions matches the 3' break point at approximately 69.5 kb downstream. We are in the process of narrowing down the gaps to define the break points precisely in order to design specific primers. For the time being, we may assume with sufficient confidence that we are dealing with a new $G\gamma(A\gamma\delta\beta)^0$-thalassemia deletion defect, that in combination with a β^0-thalassemia defect, as in this couple with the codon 39 (HBB:c.118C>T), would probably cause a thalassemia intermedia phenotype with a limited transfusion dependency.

In case B, the deletion extends from at least the 5' end of the 3rd exon of the δ-globin gene to the 3' end of the 3rd exon of the β-globin gene and is possibly extending to the L1 repeat area. The deletion reduces the level of the HbA_2 to normal and elevates the HbF expression of both Gγ and Aγ genes in cis, as one would expect from a classic δβ-thalassemia deletion, as confirmed by the 0.60 β/α globin chain synthesis ratio. However, this deletion does not match with any of those previously reported.

In this couple, analysis of a prenatal diagnosis could distinguish between affected and unaffected. If heterozygosity for the point mutation of the father is found in the fetus, then this implicates inheritance of the normal allele from the mother and thus a carrier status. Conversely, IVS-I-110 homozygosity (hemizygosity) would mean an affected fetus compound heterozygous for deletion and point mutation. Unfortunately, since the extent of the deletion was not known at the time of a first prenatal diagnosis, this couple, chose precautional abortion since the fetus was defined as having the IVS-I-110 mutation.

Unless rare and new deletions can be defined by break point gap-PCR (including the two described in this report), the important issue when performing prenatal diagnosis for combinations of point mutation and unknown deletions is to define whether the β-globin gene is absent by using MLPA, or present but non-functional by using MLPA and globin chain synthesis. In the first case, the only unfavorable result would be homozygosity (in fact, hemizygosity) for the point mutation. In the second case, an apparent favorable heterozygosity could cause a misdiagnosis. Finally, the prediction of phenotype remains a complex matter. It cannot be guaranteed that a child with a combination $\beta^0/ G\gamma(A\gamma\delta\beta)^0$-thalassemia will be healthy and able to survive without hematologic complications and without blood transfusions.

Authorship and Disclosures

MP characterized the deletions and contributed to the paper; AA, MPC and GI examined case A locally and contributed to the paper; NB examined case B in Turkey and contributed to the paper; JT-S and EK examined case B in Greece and contributed to the paper: RGG and TT examined case B in Italy and contributed to the paper; CH designed the methodology for deletion analyses and contributed to the paper; PCG wrote the paper and coordinated this study.

The authors reported no potential conflicts of interest.

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