SHORT COMMUNICATION

Characterization of Two Deep Intronic Variants on the β -Globin Gene with Inconsistent Interpretations of Clinical Significance

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Characterization of Two Deep Intronic Variants on the β -Globin Gene with Inconsistent Interpretations of Clinical Significance

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Abstract

Sequence variants located in the introns of the β -globin gene may affect the mRNA processing and cause β -thalassemia (β -thal). Sequence variants that change one of the invariant dinucleotides at the exon, and intron boundaries may have fatal consequences for normal mRNA splicing. Intronic variants located far from obvious regulatory sequences can be more difficult to evaluate. There is a potential for misinterpretation of such sequence variants. Hence, thorough evaluation of patient data together with critical use of databases and *in silico* prediction tools are important. Here, we describe two rare sequence variants in the second intron of the β -globin gene, *HBB*: c.316-70C>G and *HBB*: c.316-125A>G (NM_000518.4), both previously reported as variants causing β -thal, and later as benign sequence variants. Due to the limited number of published cases and inconsistent interpretations, the significance of these sequence variants has been unclear. We have identified these two sequence variants in multiple individuals, alone and in a variety of combinations with other δ - and β -globin defects, and we find no influence of the sequence variants on the phenotype.

KEYWORDS β -Globin gene; β -thalassemia (β -thal); deep intronic variants; interpretations; sequence variants

Several sequence variants located in the introns of the β -globin gene are known to affect mRNA processing and cause β -thalassemia (β -thal) [1]. Sequence variants that change one of the invariant dinucleotides at the exon-intron boundaries or alter the flanking regions of canonical splice sites, completely abolish or reduce normal mRNA splicing to variable degrees, respectively [1,2]. Deep intronic variants, on the other hand, may be located far from obvious regulatory sequences and the functional significance can be more difficult to evaluate. They may activate cryptic splice sites that are used during RNA processing, leading to abnormal mRNA splicing

[1]. Deep intronic variants may also affect binding of specific splicing factors such as SR proteins, which are important splicing factors in both constitutive and alternative mRNA splicing [3]. Different *in silico* prediction tools are available for sequence variant interpretation. Most of these tools tend to have low specificity and the significance of the computational evidence should not be overestimated [4]. To establish the functional significance of deep intronic variants, molecular studies combined with a careful evaluation of the phenotype of multiple patients with that variant is the most reliable approach. It is important to share rare sequence variants together with hematological data in variant databases, such as HbVar, to aid correct classification and facilitate genetic counseling of presumed couples at risk.

Here, we describe two deep intronic variants both previously reported to cause β -thal and later as benign sequence variants. *HBB*: c.316-70C>G (NM_000518.4) was first reported as a β^+ - or β^0 -thal variant in the HbVar database in 2010 [2]. In a review article by Henderson *et al.* [5], the same sequence variant was described as a β^+ variant in a patient with a split Hb A₂ peak, due to heterozygosity for the δ variant Hb A₂' [*HBD*: c.49G>C (NM_000519.3)]. The total value of Hb A₂ was 4.4%, but the hematological parameters were normal, except from a slightly decreased mean corpuscular hemoglobin (Hb) (MCH) of 26.4 pg [5]. *In silico* prediction tools integrated in the Alamut Visual version 2.4 (Interactive Biosoftware, Rouen, France) suggest activation of a cryptic acceptor site, reducing normal mRNA splicing. According to American College of Medical Genetics and Genomics standards and guidelines for the interpretation of sequence variants [4], such predictions should be cautiously interpreted and never be used as 'evidence of pathogenicity.' We have found the *HBB*: c.316-70C>G sequence variant in 15 individuals in a variety of combinations with other α -, β - or δ -globin gene defects (Table 1). Normal hematological parameters were found in five patients with the combination of *HBB*: c.316-70C>G and the δ variant Hb A₂'. In addition, the sum of Hb A₂ and Hb A₂' resulted in a normal value, indicating no sign of β -thal. In combination with Hb S [*HBB*: c.20A>T (NM_000518.4)] and Hb A₂', with and without carrier state of α -thal (heterozygous $-\alpha^{3.7}$ deletion), we found no change in the expected level of Hb S. If the sequence variant *HBB*: c.316-70C>G was a thalassemic variant, we would expect an increased Hb S fraction. Furthermore, in combination with Hb C [*HBB*: c.19G>A (NM_000518.4)] and Hb A₂', the *HBB*: c.316-70C>G variant did not show a thalassemic phenotype and the level of Hb C was 38.3%, as would be expected in a regular carrier of Hb C. To investigate whether the *HBB*: c.316-70C>G variant was in *cis* or *trans* with Hb C, molecular cloning of the *HBB* gene from patient 11 confirmed that the two sequence variants were in *trans* (data not shown). Two individuals were found to be carriers of the *HBB*: c.316-70C>G variant together with the hereditary persistence of fetal Hb (HPFH-2) Ghanaian deletion (NG_000007.3: g.54867_139178del84312) and Hb A₂'. Despite the absence of any abnormalities on the α -globin genes, patient 14 showed unexplained microcytosis. In this patient, iron deficiency could not be excluded, due to slightly elevated zinc protoporphyrin (ZPP) (data not shown). Furthermore, the regulatory region of the α -globin genes was not investigated; hence, the

microcytosis might also be due to extremely rare forms of α -thal. Elevated ZPP was also found in patient 8 (data not shown), suggesting iron deficiency as an explanation for the hematological profile, showing microcytic anemia. We also found *HBB*: c.316-70C>G in combination with a β -thal variant [*HBB*: c.95+5G>C (NM_000518.4)] in patients 12 and 13, and the hematological parameters were those of normal carriers (Table 1). Thus, in contrast to some of the previously published data [2,5], we consider the *HBB*: c.316-70C>G as a benign sequence variant without thalassemic effect. Vinciguerra *et al.* [6] drew the same conclusion in 2016, presenting similar data for four patients. As Vinciguerra *et al.* [6] proposed, we found that the sequence variant, *HBB*: c.316-70C>G, was associated with Hb A₂' in most cases, suggesting that these two mutations are in linkage. In addition, our data suggest another haplotype, namely *HBB*: c.316-70C>G in linkage with *HBB*: c.92+5G>C without the Hb A₂' allele. Further studies need to be done to establish whether *HBB*: c.316-70C>G occurs in *cis* or *trans* with *HBB*: c.92+5G>C.

The second sequence variant we investigated in the current study, *HBB*: c.316-125A>G (NM_00058.4), was first identified in the homozygous state in a Moroccan 8-year-old boy who presented with a β -thal intermedia (β -TI) phenotype [7]. His mother was a carrier of the same sequence variant and showed hematological changes consistent with β -thal trait [7]. In contrast to these data, the *HBB*: c.316-125A>G variant was described in the heterozygous state in two cases by Vinciguerra *et al.* [8] in 2012, both showing normal hematology and normal levels of Hb A₂. The latter corresponds with our findings in six individuals heterozygous for the *HBB*: c.316-125A>G variant. Five of them presented with normal hematology (Table 2). Patient 18 showed microcytosis (MCH 21.4 pg) and erythrocytosis [red blood cell (RBC) count $7.45 \times 10^{12}/L$] with no other abnormalities in the globin genes than the *HBB*: c.316-125A>G variant. The ZPP was elevated (data not shown); hence, the hematological changes might be due to long-lasting iron deficiency. The coinheritance of *HBB*: c.316-125A>G in *trans* with Hb D-Punjab [*HBB*: c.364G>C (NM_000518.4)] seen in patient 21 (confirmed with molecular cloning of the β -globin gene, data not shown) neither reduced the fraction of Hb D-Punjab below the expected level, nor caused a thalassemic phenotype (Table 1). Even though *in silico* analysis (Alamut Visual version 2.4) predicts alteration in the binding site of the SRp40 protein, we did not see any thalassemic effect, and we considered this as a benign sequence variant.

These two deep intronic variant examples point out the importance of critical use of databases in variant interpretation. Both *HBB*: c.316-70C>G and *HBB*: c.316-125A>G are registered in databases as thalassemic variants based on personal communications or on a single case report, respectively. Thorough evaluation of data presented in published papers is pivotal when deciding whether a sequence variant is pathogenic or not. Shared data on benign globin sequence variants is useful for the clinical laboratory's interpretation of DNA sequencing results. A database dedicated to compiling data on such presumably benign variants would add on to our armamentarium of sequence interpretation tools. A natural harbor for such a resource might be the HbVar database [2],

IthaGenes [9] and the LOVD database [10].

In conclusion, we have identified the *HBB*: c.316-70C>G and *HBB*: c.316-125A>G sequence variants in multiple unrelated individuals, alone and in a variety of combinations with other δ - and β -globin gene defects. Due to limited numbers of previously published cases and inconsistent interpretations, the significance of these sequence variants has been unclear. Based on our data, these sequence variants do not appear to possess thalassemic effects and we propose these variants be classified as benign sequence variants.

The Norwegian Regional Ethics Committee (#2015/2352) approved the study. Informed consent was obtained from all the included subjects.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Table 2. Hematological profiles associated with heterozygous *HBB*: c.316-125A>G.

Case	Sex-Age	Hb (g/dL)	MCH (pg)	RBC ($10^{12}/L$)	Hb A ₂ (%)	Hb X (%)	Additional Findings and Remarks
16	F-47	13.5	30.9	4.37	2.9	NA	no additional findings ^{a-d}
17	M-14	12.9	26.7	4.84	2.4	NA	no additional findings ^{a-d}
18	F-19	15.9	21.4	7.45	1.7	NA	iron deficiency ^{a-g}
19	F-35	11.9	29.6	4.05	2.8	NA	no additional findings ^{a-d}
20	M-45	15.3	28.5	5.35	2.9	NA	no additional findings ^{a-d}
21	M-54	15.6	29.6	5.29	2.5	38.3	Heterozygous <i>HBB</i> : c.364G>C ^{a-d,h}

Hb: hemoglobin; MCH: mean corpuscular Hb; RBC: Red blood cell count; NA: not applicable. Reference intervals: Hb: males 11.7-15.3 g/dL, females 13.4-17.0 g/dL, MCH: 27.0-33.0 pg, RBC: males $3.9-5.2 \times 10^{12}/L$, females $4.3-5.7 \times 10^{12}/L$. Age specific reference intervals (case #17): MCH: 25.0-33.0 pg.

^a Hb F was within reference limits.

^b Hb A₂' was not detected.

^c α -Thalassemia gap-PCR.

^d Sanger sequencing of NM_000518.4 (*HBB*).

^e Sanger sequencing of NM_000517.4 (*HBA2*) and NM_000558.3 (*HBA1*).

^f Copy number variation analysis (MLPA or real-time PCR) of the β -globin gene cluster.

^g Zinc protoporphyrin (ZPP) analysis.

^h Found in *trans* with *HBB*: c.316-70C>G, confirmed with molecular cloning of *HBB*.

Table 1. Hematological profiles associated with heterozygous *HBB*: c.316-70C>G, several in linkage with Hb A₂'.

Case	Sex-Age	Hb (g/dL)	MCH (pg)	RBC ($10^{12}/L$)	Hb A ₂ (%)	Hb F (%)	Hb A ₂ ' (%)	Hb X (%)	Additional Findings and Remarks
1	F-33	11.5	32.2	3.56	1.3	ref. limits ^a	1.1 ^b	NA	no additional findings ^{d,e}
2	M-60	13.1	33.0	3.97	1.6	ref. limits ^a	1.2 ^b	NA	no additional findings ^{d,e}
3	M-32	15.8	29.4	5.40	1.3	ref. limits ^a	1.2 ^b	NA	no additional findings ^{d,e}
4	F-39	12.0	31.7	3.77	1.3	ref. limits ^a	1.5 ^b	NA	no additional findings ^{d,e}
5	F-31	11.5	33.0	3.49	1.4	ref. limits ^a	1.1 ^b	NA	no additional findings ^{d,e}
6	F-40	11.4	27.9	4.10	1.6	ref. limits ^a	1.5 ^b	34.0	heterozygous <i>HBB</i> : c.20A>T/ $-\alpha$ ^{3,7d,e}
7	F-29	12.7	24.8	4.88	1.4	ref. limits ^a	1.3 ^b	35.0	heterozygous <i>HBB</i> : c.20A>T/ $-\alpha$ ^{3,7d,e}
8	F-19	9.1	18.7	4.93	1.2	ref. limits ^a	1.2 ^b	31.0	heterozygous <i>HBB</i> : c.20A>T/ $-\alpha$ ^{3,7d,e} ; iron deficiency ^{f,g}
9	F-8	10.9	27.0	4.03	1.9	ref. limits ^a	1.6 ^b	39.0	heterozygous <i>HBB</i> : c.20A>T ^{d,e}
10	F-31	12.1	26.9	4.47	1.7	ref. limits ^a	1.4 ^b	39.0	heterozygous <i>HBB</i> : c.20A>T ^{d,e}
11	M-44	15.4	30.0	5.20	1.6	ref. limits ^a	see ^c	34.0	heterozygous <i>HBB</i> : c.19G>A ^{d,e,h}
12	M-73	10.8	19.5	5.51	4.5	ref. limits ^a	NA	NA	heterozygous <i>HBB</i> : c.92+5G>C (β^+) ^{d,e}
13	F-28	10.3	22.2	4.62	4.9	ref. limits ^a	NA	NA	heterozygous <i>HBB</i> : c.92+5G>C (β^+) ^{d,e}
14	F-23	11.8	23.3	5.01	NA	21.3	1.4 ^b	NA	heterozygous HPFH-2 Ghanaian deletion ^{d,e,f,i}
15	F13	12.2	29.1	4.19	NA	30.4	1.8 ^b	NA	heterozygous HPFH-2 Ghanaian deletion ^{d,e,i}

Hb: hemoglobin; MCH: mean corpuscular Hb; RBC: red blood cell count; ref. limits: within reference limits; NA: not applicable.
Reference intervals: Hb: males 11.7-15.3 g/dL, females 13.4-17.0 g/dL, MCH: 27.0-33.0 pg, RBC: males $3.9\text{--}5.2 \times 10^{12}/L$, females

$4.3\text{-}5.7 \times 10^{12}/\text{L}$. Age specific reference intervals (case #9): Hb:11.0-15.5 g/dL, MCH: 25.0-33.0 pg (also case #15), RBC: $3.9\text{-}5.3 \times 10^{12}/\text{L}$.

^a Within reference limits.

^b Hb A₂' (*HBD*: c.49G>C) were confirmed with DNA sequencing of NM_000519.3 (*HBD*).

^c Hb A₂' not quantifiable due to Hb C interference.

^d α -Thalassemia gap-PCR.

^e Sanger sequencing of NM_000518.4 (*HBB*).

^f Sanger sequencing of NM_000517.4 (*HBA2*) and NM_000558.3 (*HBA1*).

^g Zinc protoporphyrin (ZPP) analysis.

^h Found in *trans* with *HBB*: c.316-70C>G, confirmed with molecular cloning of *HBB*.

ⁱ Copy number variation (CNV) analysis [multiplex ligation-dependent probe amplification (MLPA) or real-time PCR] of the β -globin gene cluster.