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Research Article

Association between *HBA* locus copy number gains and pathogenic *HBB* gene variants

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Abstract

Objectives: Alpha (α) and beta (β) thalassemia are the most prevalent genetic hematological disorders. The co-occurrence of silent β-thalassemia with excess α-globin gene copies is associated with the thalassemia intermedia phenotype. This study was an investigation of the α-globulin gene dosage and sequence variations in thalassemia patients.

Methods: Multiplex ligation-dependent probe amplification and Sanger sequencing were used to identify the hemoglobin subunit alpha 1 (*HBA1*) and *HBA2* gene alterations in 32 patients. Deletion, duplication, and other findings were analyzed in the index cases and family members.

Results: Four of the 32 cases (12.5%) were found to have gross duplications. Two cases demonstrated α-globin triplication, and 2 had a quadruplicated *HBA1/2* genes. Affected family members revealed genotype-phenotype correlation. In 1 patient, it was observed that quadruplicated *HBA* genes co-occurrence with hemoglobin subunit beta (*HBB*) mutation was inherited from his mother. Notably, the mother did not demonstrate any thalassemia phenotype. Further investigation showed that the mother was carrying a single copy *HBA* gene deletion in the trans allele that explained her clinical condition.

Conclusion: This study examined the effect of increased copies of the *HBA* gene in *HBB* gene pathogenic variant carriers. The results indicated that β-thalassemia mutations with a co-occurrence of increased α-globin gene dosage is not very rare condition. Patients with clinical findings incompatible with their *HBB* genotypes should be investigated for small and gross α-globin gene variants in order to provide genetic counseling and prenatal diagnosis follow-up, as appropriate.

Keywords: Alpha-globin gene quadruplication, co-inheritance of *HBA* and *HBB*, multiplex ligation-dependent probe amplification, thalassemia intermedia

Alpha (α) and beta (β) thalassemia, blood disorders characterized by decreased hemoglobin production, are the most frequently observed genetic disorders in the world. The carrier frequency has been found to be high in populations where malaria is common [1-3]. Cross-national migration from endemic regions has led to spread of the disease [4, 5]. Thalassemia is classified into 2 types according to the defective gene: In α-thalassemia, the hemoglobin subunit alpha (*HBA*) gene cluster is damaged, and in cases of β-thalassemia, there are pathogenic variants in the hemoglobin subunit beta (*HBB*) gene [6]. According to the globin mutation database, 940 point variants have been recorded that can lead to structural alterations, protein truncation, defective splicing, decrease in transcription, or loss of stability in the *HBB* gene [7]. The database also includes recordings of gross variants of *HBA1/2* genes, which are identified in 2% of thalassemia cases [8]. Four alleles of 2 structurally similar genes, *HBA1* and *HBA2* are responsible for α-globin gene expression and are critically important when establishing genotype-phenotype relationships in patients with β-thalassemia. Although the *HBA1* gene

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has 349 reported small sequence variants (also called non-deletion variants) reported in the globin mutation database and the *HBA2* gene has 455, large deletions are responsible for about 85% of patients with α-thalassemia [7]. Four phenotypes have been identified: hemoglobin Barts hydrops, with a loss of all 4 α-globin genes (--/--), which is usually fatal at birth; hemoglobin H (HbH) disease, with 3 missing α-globin genes (--/-α), which generally includes only moderate symptoms and may not require clinical intervention; α-thalassemia trait/carrier, with 2 absent or inactive *HBA* genes (-α/-α or - -/αα), which may result in very mild anemia; and α-thalassemia silent carrier, caused by the deletion of a single *HBA* gene (-α/αα), which is asymptomatic [9-11].

Although non-deletion variants of α-globulin genes are rarely seen in α-thalassemia, the HbH phenotype occurring with the homozygous form of α-thalassemia ($\alpha^{constant\ spring}$ α/-$\alpha^{constant\ spring}$) results in a truncated *HBA2* messenger-type RNA nonsense mutation in *HBA2* and leads to a more severe clinical course than a deletion HbH phenotype and requires transfusions [10-12]. β-thalassemia is classified into 3 types: thalassemia major, thalassemia intermedia, and thalassemia minor. The type is based on the nature of the mutation, protein function (β^0 or β^+), and zygosity, and is reflected in the severity of clinical symptoms. Heterozygous carriers are usually moderately anemic or healthy, though a few exceptions have been reported.

In addition to moderate/mild homozygous variations, compound heterozygotes or an elevated fetal hemoglobin level can influence the clinical picture [13-15]. Co-existing α-thalassemia and β-thalassemia is most frequently reported in Asian countries, which have a high prevalence of carriers of both α- and β-thalassemia [16-19].

Genotype-phenotype discordance has been reported in the literature in β-thalassemia intermedia and β-thalassemia minor cases. Inconsistency in hematological findings and β-thalassemia traits may be observed in circumstances such as iron deficiency, co-inheritance of α- and β-thalassemia variant heterozygosity, co-inheritance of *HBB* variations, and other globin gene variants [13]. Hepatitis, folic acid or vitamin B12 deficiency can also cause upregulation of α-thalassemia genes, leading to difficulties in interpreting genotype. The coupling of α-thalassemia alterations with *HBB* mutations may affect α/β globin ratio [20, 21]. In heterozygous *HBB* variant carriers, the phenotype may become more severe if there are also gains in α globin. On the contrary, in homozygous or compound heterozygous *HBB* variant carriers, the clinic may be milder if there is also a loss in *HBA* genes [22].

This study examined the effect of increased copies of the *HBA* genes in *HBB* gene pathogenic variant carriers.

Materials and Methods

This study was approved by the Istanbul University İstanbul Faculty of Medicine Ethics Committee on January 28, 2021 (no: 52410). The study cohort comprised 32 patients with hematological findings of thalassemia who were referred from the Istanbul Faculty of Medicine Pediatric Hematology Unit to the Istanbul Faculty of Medicine Department of Medical Genetics for genetic testing and counseling. Detailed pedigrees of the patients were recorded, medical findings were reviewed, and peripheral blood samples were collected upon receipt of written, informed consent.

DNA isolation was performed using a nucleic acid extraction kit (MagNA Pure; Roche Diagnostics, Basel, Switzerland). Primers were designed in-house for the *HBA1* (NM_000558.5), *HBA2* (NM_000517.6), and *HBB* (NM_000518.5) genes for Sanger sequencing using a genome analyzer (ABI3500). Deletion and duplication analysis was performed using multiplex ligation-dependent probe amplification (MLPA) (MLPA Probemix P140 *HBA*; MRC-Holland bv, Amsterdam, The Netherlands) and analyzed with Coffalyser.Net MLPA analysis software (MRC-Holland bv, Amsterdam, The Netherlands).

Results

In all, 4 index patients and family members were identified as variant carriers of the *HBB* gene with an increased *HBA* copy number. The mean hematological data was red blood cell count: 5.3x10^6±1.4/mm^3 (range: 3.9-7.2x10^6/mm^3), mean corpuscular volume (MCV): 59.8 fL±7.6 (range: 55.1-71.1 fL), mean corpuscular hemoglobin (MCH): 18.7±2.6 pg (range: 17.1-2.26 pg), Hb: 8.2±0.9 g/dL (range: 7.2-9.2 g/dL), HbA 87.4±7.2% (range: 76.7-92.3%), and HbA2: 4.9±1.7% (range: 2.6-6.7%).

The hematological and molecular findings of the study cases and family members are presented in Figures 1-4. In Family 1, the index case and affected sister were found to have ~81Kb of heterozygous quadruplication in the α-globin re-

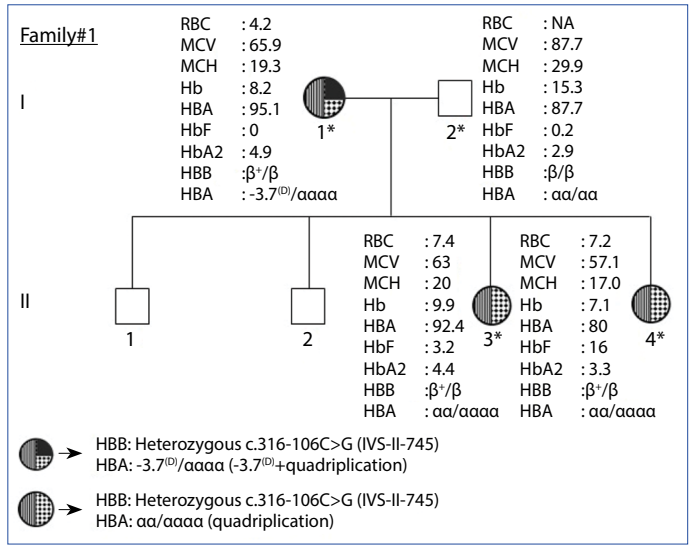

Figure 1. Genetic and hematological results of Family 1.

Hb: Hemoglobin; HBA: Hemoglobin subunit alpha; HbA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; HbF: Fetal hemoglobin; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; RBC: Red blood cell count.

gion (beginning in the HS40 element) with β^+ associated heterozygous c.316-106C>G (IVS-II-745, rs34690599) in the *HBB* gene. In addition to these 2 mutations, their mother had an *HBA* -3.7$^{(D)}$ heterozygous deletion in the other *HBA* allele. The father's results were normal (Fig. 1). In the index case of Family 2, an *HBB* heterozygous c.92+1G>A (IVS-I-1, rs33971440)

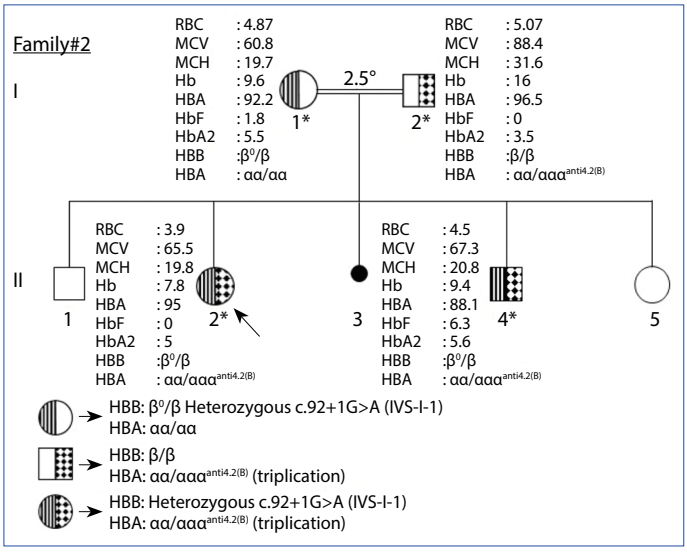

Figure 2. Genetic and hematological results of Family 2.

Hb: Hemoglobin; HBA: Hemoglobin subunit alpha; HbA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; HbF: Fetal hemoglobin; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; RBC: Red blood cell count.

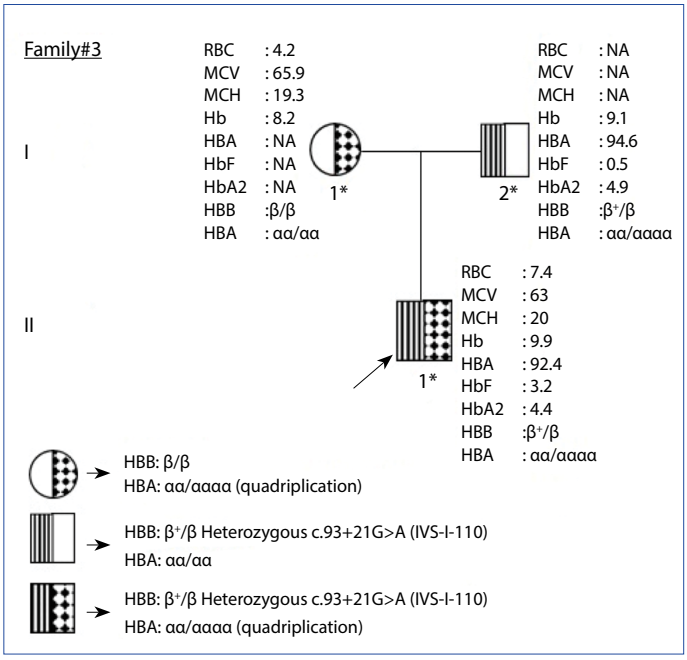

Figure 3. Genetic and hematological results of Family 3.

Hb: Hemoglobin; HBA: Hemoglobin subunit alpha; HbA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; HbF: Fetal hemoglobin; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; RBC: Red blood cell count.

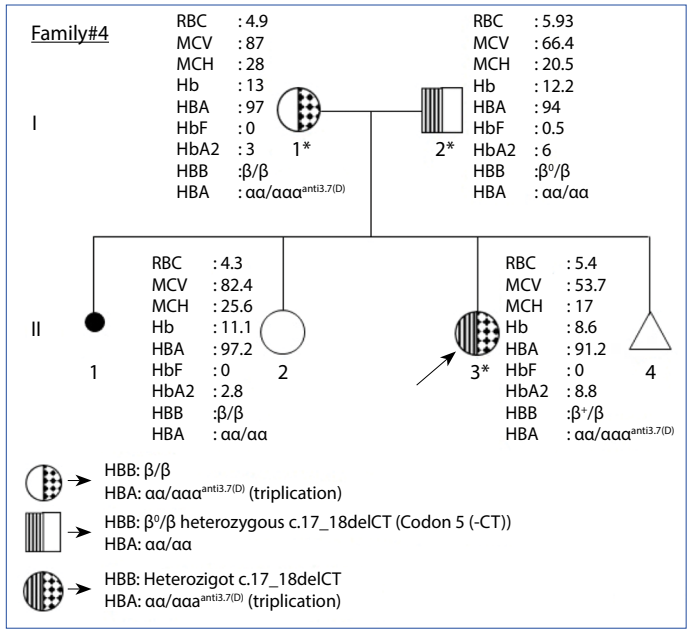

Figure 4. Genetic and hematological results of Family 4.

Hb: Hemoglobin; HBA: Hemoglobin subunit alpha; HbA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; HbF: Fetal hemoglobin; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; RBC: Red blood cell count.

mutation and ααα$^{anti4.2(B)}$ were detected. The affected brother had a similar genotype, while the other siblings had inherited the *HBB* mutation from the mother and *HBA* triplication from the father (Fig. 2). In Family 3, heterozygote c.93-21G>A (IVS-I-110, rs35004220) in the *HBB* gene and ~81Kb of heterozygous quadruplication (beginning in the HS40 region) were detected in the *HBA* locus of the index case. Investigation of the family showed that the patient had inherited both digenic alterations from the father (Fig. 3). The index case of Family 4 carried a heterozygous c.17_18delCT (p.Pro6fs, rs34889882) inherited from the *HBB* gene of his father and ααα$^{anti\ 3.7(D)}$ from his mother (Fig. 4).

Discussion

Co-inheritance of β-thalassemia and α-thalassemia is frequently seen in populations where carriers of both α- and β-hemoglobinopathy are prevalent [16, 23]. Cases with co-inheritance of β-thalassemia with α-globin gene deletion typically are presented with a milder phenotype. This is a result of a decrease in α/β globin ratio that is higher in β-thalassemia cases carrying a normal copy number of *HBA* genes [22, 24]. It has been reported that the coexistence of *HBB* mutations and *HBA1/HBA2* gene triplication or quadruplication results in a more severe phenotype since the ratio of α/β is predicted to deteriorate as the α-globin dose increases [18, 25-33]. In the present study, we found 4 β-thalassemia carrier cases with an α-globin gene copy gain and an unusual phenotype. Each member of the families had an intrafamilial genotype-phenotype correlation. Cebrian et al. [34] reported a milder pheno-

type in heterozygous *HBB* carriers than homozygous carriers among members of a family carrying *HBB* c.93-21G> A (β^+) and *HBA* triplication. In a study of 6 families, Origa et al. [35] reported the need for transfusions in 4 of 14 individuals with a quadruplicated α-globin gene and *HBB* heterozygotes. Other family members had milder phenotypes, despite carrying the same genotype as a result of other genetic factors. Similarly, Farashi et al. [25] observed a thalassemia intermedia phenotype in a patient with heterozygous β-thalassemia found to carry 6 α-globin gene copies. The daughter of this patient had the same *HBB* genotype but 7 copies of α-globin and a clinically similar phenotype to her father.

The fact that our patients with triplication and quadruplication of the α-globin gene and mono-allelic *HBB* mutations had a moderate-to-severe thalassemia intermedia phenotype was consistent with the literature. Our study findings similarly supported reports that α/β globin ratios influence the severity of the disease [17, 22, 36]. In Family 1, the mother's heterozygous *HBB* mutation and *HBA* gene deletion/quadruplication in trans form resulted in a milder phenotype than the index. This case is a good example of why even silent *HBA* alterations should be investigated for segregation to reveal genotype-phenotype variation in the family. Expansion of the segregation may further aid our understanding of the effect of variants in Families 2, 3, and 4, in which *HBB* and *HBA* genes variants were found to inherited bi-parentally. While the parent with the *HBB* mutation alone demonstrated classic β-thalassemia minor, no clinical consequence was revealed in the parents with only α-globin gains. The β^+ *HBB* mutation in our 2 families with triplication and β^0 in the *HBB* mutations in the families with quadruplicated *HBA* prevented comparison of the copy number effect in those families. However, all of the index cases and affected siblings had similar genotypes compatible with thalassemia intermedia. In Family 3, detailed analysis was not possible because the only hematological information for the index patient's father that was available was the molecular results.

Non-allelic homologous recombination is the mechanism for the formation of deletions in α-thalassemia. One product of this recombination is deletion, however, duplication (triplication or quadruplication) is also possible [37, 38]. The frequency of duplication and deletion should be similar according to the mechanism. Though it is thought that there should be more carriers of duplication, since it does not cause mortality and morbidity, as deletions can, α-globin gene deletions have a heterozygous advantage over the malaria parasite, and over time the deletions have become more frequent [1, 39]. This may be why *HBA* deletions are observed more often in *HBB* carriers than duplications. Wu et al. [40] reported that triplications in southern China were 3 times more frequent than quadruplication. Frequent observance of both α- and β-thalassemia mutations in geographical regions where hemoglobinopathies are common leads to the formation of complex thalassemia, resulting in phenotypic findings. Individuals who do not carry the *HBB* mutation but carry α-globin gene duplication are phenotypically normal. Therefore, these duplications are typically only revealed incidentally [35].

HBA studies in Turkey have been performed using restriction enzyme and gap- polymerase chain reaction methods for many years [41, 42]. Target-specific reverse dot blot kits (strip assay) have also been used [43-45]. However, since studies with these widely used kits were limited to targeted regions, only αααanti-3.7 increases from α-globin gains could be investigated. In recent years, the widespread use of MLPA studies has allowed for the identification of a wide spectrum of deletions and duplications in the *HBA* locus [8, 46]. Our study is important in that α-globin dosage increases in *HBB* heterozygous carriers revealed atypical clinical findings.

Children, born to parents one carrying *HBB* and the other *HBA* mutation or multiplication (triplication, quadruplication, etc.), have an increased risk for thalassemia intermedia. Farashi et al. [47] reported that co-inheritance of *HBB* carriers and α-globin gene multiplications was as high as 25% in some parts of Iran. Therefore, prenatal *HBA* analysis should be performed for *HBB* carriers. In this study, *HBA* gains were detected in 4 of 32 families (12.5%) with atypical clinical findings of *HBB* mutation. Co-inheritance of *HBA* gains and *HBB* mutations is not very rare in our population. Low MCV and MCH values in *HBA*-deletion carriers compared with normal individuals suggest the need for *HBA* molecular tests. However, since α-globin dose increases do not prompt a clinical finding in carriers, they pose a risk for the fetal phenotype in prenatal diagnosis and create difficulty in genetic counseling. The study of possible *HBA1/HBA2* gene mutations and dosage increases in β-thalassemia cases with phenotypes that do not match clinical expectations is an auxiliary factor in prenatal diagnosis and genetic counseling to identify the risk of inherited hemoglobinopathy and making informed decisions.

Conclusion

According to the data obtained in this study, *HBA* gene increases can be detected in *HBB* cases showing genotype-phenotype discordance at a rate that cannot be ignored. The detection of carrier families and individuals will contribute to accurate and effective guidance and genetic counseling in prenatal diagnosis or PGD studies.

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Conflict of Interest: The authors declare that there there is no conflict of interest regarding the publication of this article.

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