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Molecular Characterization of Alpha Thalassemia via Multiplex Ligation Dependent Probe Amplification in Konya, Turkey: A Single Center Study

Konya Bölgesinde Multipleks Ligasyon Bağımlı Prob Amplifikasyonu Aracılığıyla Alfa Talaseminin Moleküler Karakterizasyonu: Tek Merkez Çalışması

Emine Goktas[1], Sumeyye Sanal[1], Huseyin Tokgoz[2], Ayse Gul Zamani[1], Mahmut Selman Yildirim[1]

[1]Necmettin Erbakan University, Faculty of Medicine, Department of Medical Genetics, Konya, Türkiye
[2]Necmettin Erbakan University, Faculty of Medicine, Department of Pediatric Hematology, Konya, Türkiye

ÖZET

Amaç: Alfa talasemi, hipokrom mikrositer anemi ile karakterize, klinik fenotipi asemptomatikten lethal anemiye kadar değişkenlik gösteren, otozomal resesif kalıtılan bir hastalıktır. Alfa talasemi, %85 oranında HBA1 veya HBA2 genlerinin delesyonundan, %15 oranında ise nondelesyonel dizi değişimlerinden kaynaklanmaktadır. Biz de çalışmamızda alfa talasemi ön tanılı hastalarda Multipleks Ligasyon-bağımlı Prob Amplifikasyon (MLPA) yöntemiyle yaptığımız delesyon-duplikasyon analizi sonuçlarını sunmayı amaçladık.

Materyal and Metod: Çalışmamıza 2021-2023 yılları arasında alfa talasemi ön tanısıyla Çocuk Hematoloji bölümünde takip edilen ve Tıbbi Genetik polikliniğine yönlendirilen 29 hastanın (16 kız, 13 erkek) MLPA yöntemiyle belirlenen alfa globin kopya sayısı varyasyonları geriye dönük olarak incelendi ve MLPA'da delesyon saptanan hastaların hemogram parametreleri ve hemoglobin elektroforezi bulgularıyla genotip-fenotip korelasyonu yapıldı.

Bulgular: Alfa talasemi ön tanısı ile başvuran hastaların 15 (%51.7)'inde delesyon saptandı. Delesyon saptanan olguların 11'inde(7 kız, 4 erkek) bir α-globin gen kopyasında delesyon saptanırken; 4'ünde(3 kız, 1 erkek) ise iki α-globin gen kopyası delesyona uğramış olarak bulundu. Hastalarda en sık (%36.7) $-\alpha^{3.7}$ delesyonu gözlendi ve tespit edilen diğer delesyonlar arasında $-\alpha^{20.5}$ (%16.7), –αMED-1 (%6.6) delesyonları yer almaktaydı. İki olguda $-\alpha^{3.7}$ delesyonunun farklı formları [$-\alpha^{3,7(A)}/-\alpha^{3,7(D)}$, $-\alpha^{3,7(D)}/-\alpha^{3,7(F)}$] biallelik olarak gözlenirken, bir olguda $-\alpha^{3,7(D)}$ ile birlikte $(-\alpha)^{20,5}$ biallelik delesyonu ve diğer bir vakada ise $-\alpha^{3,7(D)}$ delesyonuna ek olarak dizi analizi ile HBA1 geninde p.Gly60Asp patojenik varyantı saptandı.

Sonuç: Alfa talasemi etiyolojisinden % 85 oranında α-globin genlerinin delesyonu sorumlu olduğundan; MLPA analizi ile moleküler genetik tanı oranı oldukça yüksektir. MLPA analizi normal olan veya saptanan α-globin gen kopya sayısının fenotipik bulguları açıklamadığı alfa talasemi olgularında HBA1 ve HBA2 genlerinin dizi analizi ile incelenmesi gerektiği akılda tutulmalıdır.

Anahtar Kelimeler: Alfa talasemi, delesyon, MLPA, amplifikasyon

ABSTRACT

Objective: Alpha thalassemia is an autosomal recessive hemoglobinopathy characterized by hypochromic microcytic anemia, exhibiting variable clinical phenotypes. Eighty-five percent of cases arise from deletions in the HBA1 or HBA2 genes. The objective of this study is to present the results of Multiplex Ligation Dependent Probe Amplification (MLPA) analysis of patients under the age of 18 with a preliminary diagnosis of alpha thalassemia.

Material and Methods: The present study examined alpha globin copy number variations determined by the MLPA method in patients who were followed up in the Pediatric Hematology department and referred to the Medical Genetics outpatient clinic with a preliminary diagnosis of alpha thalassemia between 2021-2023. We analyzed the hemogram parameters and hemoglobin electrophoresis results of the patients with deletions detected by MLPA and correlated them with their genotypes.

Results: A deletion was identified in 15 (51.7%) of 29 patients with a preliminary diagnosis of alpha thalassaemia. In eleven patients, one α-globin gene copy was deleted, while two α-globin gene copies were deleted in four patients. The most prevalent deletion was $-\alpha^{3.7}$ (36.7%), followed by $-\alpha^{20.5}$ (16.7%) and $-\alpha^{MED-1}$(6.6%). Biallelic observation of different forms of the $-\alpha^{3.7}$ deletion [$-\alpha^{3.7(A)}/-\alpha^{3.7(D)}$, $-\alpha^{3.7(D)}/-\alpha^{3.7(F)}$] was noted in two cases. Additionally, one case showed biallelic $-\alpha^{3.7(D)}$ deletion along with $(-\alpha)^{20.5}$ biallelic deletion, and in another case, besides $-\alpha^{3.7(D)}$ deletion, a pathogenic variant p.Gly60Asp was detected in the HBA1 gene through sequence analysis.

Conclusion: Since 85% of alpha thalassemia etiology is attributed to α-globin gene deletions, the molecular genetic diagnosis rate is considerably high with MLPA analysis. In alpha thalassemia cases where MLPA analysis is normal or identified α-globin gene copy numbers that do not explain the phenotypic findings, sequencing analysis of the HBA1 and HBA2 genes should be considered.

Keywords: Alpha thalassemia, deletion, MLPA, amplification

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Sorumlu Yazar/Corresponding Author: Emine Goktas, Necmettin Erbakan University, Faculty of Medicine, Department of Medical Genetics, Konya, Türkiye
e-mail: emineaktas88@hotmail.com

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INTRODUCTION

Alpha thalassemia is an autosomal recessive hemoglobinopathy characterized by reduced synthesis of the alpha globulin chain, resulting in a disruption of the ratio of alpha to beta globulin chains (1). While the precise prevalence is uncertain, it is known that 5%-20% of the world's population carries one or more α-thalassemia variants and that it is the most common monogenic disease in African, South-East Asian and Middle Eastern countries, particularly in Mediterranean countries (2). The synthesis of human haemoglobin is mediated by an α-globin and β-globin gene cluster located on chromosome 16 and chromosome 11, respectively (3). Four functional α-globin genes (α2α1 / α2α1) are required for normal production of α-globin protein, however deletional (-) or non-deletional (α^T) variations in α-globin genes result in impaired production of this protein. Approximately 130 different molecular defects, mainly large fragment deletions, are known to cause α-thalassaemia (4). While the variation of one of the α-globin gene pairs (α2 α1) is called "heterozygous" (-α/αα) or "homozygous" (-α/-α) α^+-thalassemia; variations of both α-globin genes in a linked pair on the same chromosome 16 is called α^0-thalassemia (--/αα)(1). More than 40 variations of α^0 thalassemia have been identified, the most common being Southeast Asian ($-^{SEA}$/αα), Philippine ($-^{FIL}$/αα), and Mediterranean ($-^{MED}$/αα) (5).

While copy number changes in the α-globin genes and regulatory region sequences are responsible for the disease in 85% of alpha thalassaemia cases, non-deletional inactivations (point mutations) of the HBA1/HBA2 genes are responsible for the aetiology in 15% of cases. Copy number changes in the HBA1 and HBA2 genes can be detected with gene-targeted deletion/duplication analysis methods (Gap PCR, MLPA, chromosomal microarray, etc.), and the MLPA method is widely used for this purpose. Sequence analysis(Sanger or Next Generation Sequencing) of HBA1 and HBA2 can be performed if a common deletion was not identified with MLPA (6)

MLPA is a multiplex PCR technique that uses a single primer pair to amplify approximately 60 targeted probes. By comparing the signal patterns obtained from a sample with a set of reference samples, the number of genomic targets present in the sample of interest can be determined. MLPA can detect 50-70 nt sequence aberrations in a single gene that cannot be detected by fluorescent in situ hybridisation (FISH) (7). In alpha thalassaemia patients, most deletion variants, including common deletions such as the 3.7 kb deletion ($-\alpha^{3.7}$), the 4.2 kb deletion ($-\alpha^{4.2}$), and the Southeast Asian deletion ($--^{SEA}$), can be detected by MLPA (6).

The aim of this study is to detect variations in the HBA1 and/or HBA2 genes using the MLPA method in patients under the age of 18 who have presented to the Medical Genetics Polyclinic with a preliminary diagnosis of alpha thalassaemia, and to evaluate the influence of these variations on blood parameters.

MATERIALS AND METHODS

The study included 29 patients (16 girls, 13 boys, mean age 9±4.5 years) aged 0-18 years who were followed up at the Paediatric Haematology Department and were referred to the Medical Genetics Outpatient Clinic with a pre-diagnosis of alpha thalassemia between 2021-2023. Analyses of alpha globin copy number variation determined by the MLPA method were included. The study was approved by University Non-Drug and Medical Device Research Ethics Committee (number: 2024/4858). Written informed consent was obtained from the patients and their legal guardians. Genomic DNA was extracted from 2 ml of peripheral blood samples collected in EDTA tubes using an automated DNA isolation system (MaelstromTM 4800, Taiwan). Quality control and purity of isolated genomic DNA samples were identified spectrophotometrically using NanoDrop [NanoDrop 2000C; Thermo Fisher Scientific Inc., Wilmington, MA, USA]. Genomic deletions and duplications in the HBA1 and HBA2 genes were detected by MLPA analysis in high quality samples with A260/280 values between 1.8-2.0. The SALSA® MLPA® P140-C1 HBA probemix kit (MRC Holland, Amsterdam, The Netherlands) was tested according to the manufacturer's protocols. Fluorescent fragments obtained after ligation and amplification were separated on an ABI 3500 capillary electrophoresis system (Applied Biosystems™, California, USA) and the sizes of the fragments were determined using the GeneMapper program (Applied BioSystems, USA). The results were analysed using Coffalyser.Net data analysis software. HBA gene sequencing was performed with the next generation sequencing method (NGS, Illumina Miniseq System, San Diego, USA) in a patient whose clinical condition could not be explained by MLPA.

Statistical analysis of our study was performed using the SPSS package (SPSS for Windows, Version 25.0, SPSS Inc., USA). Descriptive statistics such as mean and standard deviation were used to characterise the haematological indices associated with each thalassemia genotype.

RESULTS

A deletion variant in the alpha globulin genes was found in 15 (51.7%) of 29 cases aged 3-18 years who presented with a preliminary diagnosis of alpha thalassaemia. Table 1 presents the distribution of globin deletions/mutations of the patients among the total number of allels. The most prevalent globin deletion was the $-\alpha^{3.7}$ deletion, observed in 36.7% of cases. The second most prevalent deletion was the $--^{20.5}$ deletion, which

Table 1. The distribution of globin deletions/mutations of the patients among the total number of allels.

Alpha globin variant	Affected allel count	%
αα (Normal)	11	36.7
-α3.7	11	36.7
--20.5	5	16.7
--MED	2	6.6
-αcd59	1	3.3
Toplam	**30**	**100**

Table 2. The age and hematological data of patients with alpha thalassemia associated with deletions.

Genotype	n	%	Gender	Age at diagnosis (years)	Hb (g/dL)	MCV (fL)	MCH (pg)	RBC (x1012/L)	RDW	Hb A2 (%)	Hb F (%)
$-\alpha^{3.7}/\alpha\alpha$	5	33.3	3M, 2F	13,4±4,16	12,32±2,54	69,48±8,3	21,52±3,15	5,72±0,81	18,32±3,56	1,6±0,14	0
$-\alpha^{3.7}/-\alpha^{3.7}$	2	13.3	2F	10±1,41	12,6±0,71	66,7±1,84	21,65±1,2	5,81±0,2	14,95±1,48	1,5±0,14	0
$--^{20.5}/\alpha\alpha$	4	26.7	3F, 1M	6,25±2,5	11,38±0,17	62,05±1,54	19,25±0,58	5,92±0,9	16,93±1,73	1,65±0,39	3±4,12
$--^{MED}/\alpha\alpha$	2	13.3	2F	3,5±0,71	9,3±0,42	59,95±1,06	18,35±1,06	5,07±0,4	19,65±4,03	1,6	0,4±0,57
$-\alpha^{3.7}/--^{20.5}$	1	6.7	1M	7	9,8	51,8	16	6,11	20,8	1,1	5,5
$-\alpha^{3.7}/\alpha\alpha^{cd59}$	1	6.7	1F	10	11,2	68,1	21,5	5,22	14,2	1,5	1,1
Total	15	100	---	9±4,5	11,4±1,7	64,5±6,8	20,1±2,4	5,6±0,5	17,5±3	1,5±0,2	1,3±2,6

was identified in 16.7% of cases. The most frequently detected genotype was $-\alpha^{3.7}/\alpha\alpha$ and its frequency was 33.3%. Two cases exhibited biallelic forms of the $-\alpha^{3.7}$ deletion, designated as [$-\alpha^{3.7(A)}/-\alpha^{3.7(D)}$, $-\alpha^{3.7(D)}/-\alpha^{3.7(F)}$]. In another case, $--^{20.5}$ biallelic deletion together with $-\alpha^{3.7(D)}$ was detected. Segregation analysis was performed on cases in which biallelic deletions were detected, and it was confirmed that the detected variants were in the trans position. $--^{MED}$ deletion, which includes 2 α globin genes, was detected in 13.3% of the cases. In another case, since the monoallelic $-\alpha^{3.7(D)}$ deletion did not explain the patient's clinic, HBA1/HBA2 genes were sequenced by next generation sequencing (Illumina Miniseq System, San Diego, USA) and a heterozygous c.179G>A G60D (Codon 59, Hb Adana) pathogenic variant was detected in the HBA1 gene. Other deletion patterns (e.g. $-\alpha^{4.2}$ $--^{SEA}$, $--^{FIL}$) and alpha triplications were not found in our paediatric patients. Figure 1 shows that MLPA images of patients with $--^{20.5}$ deletion, $--\alpha^{MED}$ deletion, biallelic $-\alpha^{3.7}/--^{20.5}$ deletion, and biallelic $-\alpha^{3.7(D)}/-\alpha^{3.7(F)}$ deletion. Table 2 presents the age and hematological data of patients with alpha thalassemia associated with deletions.

DISCUSSION

While the carrier frequency of alpha thalassaemia is between 3% and 4% in Turkey and Italy, countries around the Mediterranean, it is around 60% in eastern Saudi Arabia. Despite the existence of considerable gaps in our current understanding of the prevalence and health burden of alpha thalassaemia, the increasing speed and decreasing cost of genetic testing and other screening methods are facilitating the goal of treatment and future disease prevention (1). Understanding the genotype-phenotype relationships of different globin gene variants, as well as the interactions between multiple mutations when co-inherited, is essential for identifying mutations in carriers and affected patients (8). In

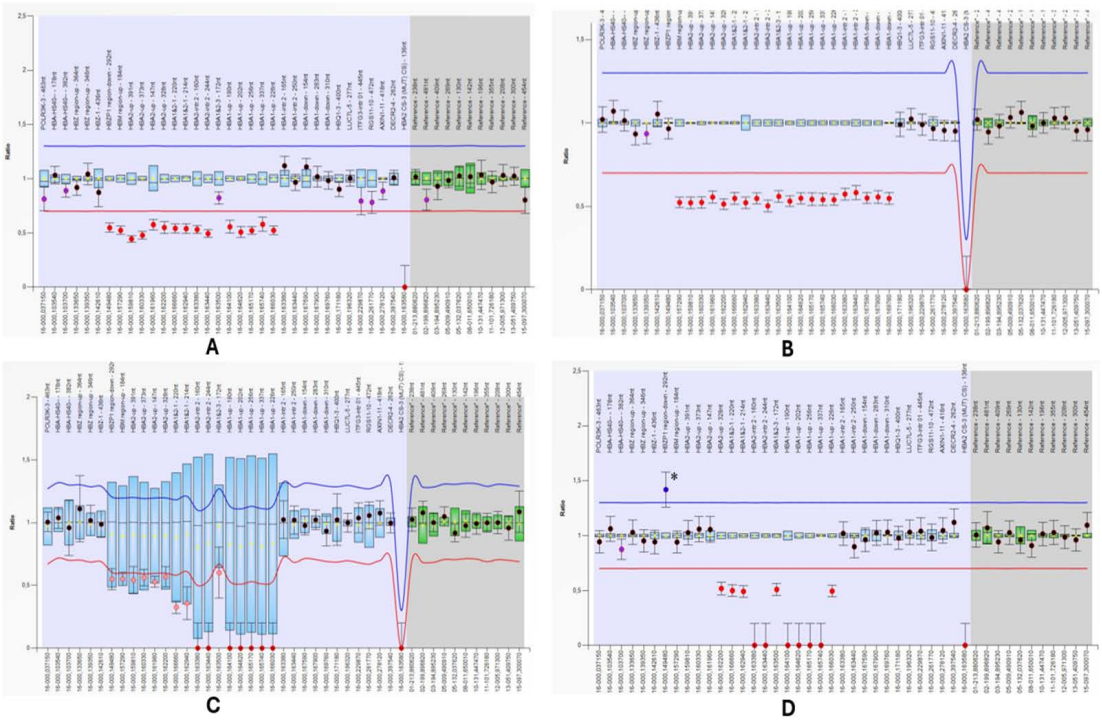

Figure 1. Coffalyser views of $--^{20.5}$ deletion (A); $--\alpha^{MED}$ deletion (B); biallelic $-\alpha^{3.7}/--^{20.5}$ deletion (C); and biallelic $-\alpha^{3.7(D)}/-\alpha^{3.7(F)}$ deletion (D). * In the patient who has biallelic $-\alpha^{3.7(D)}/-\alpha^{3.7(F)}$ deletion, additionally an Asian polymorphism indicating the duplication of the HBZ & HBZP1 locus was detected.

our study, four different alpha globin variants were determined. Three of them were deletions of varied sizes of the alpha globin gene cluster, the remaining one was co-occurrence of copy number variation type with the pathogenic sequence variant -α^{cd59}(c.179G>A, codon 59, G60D, Hb Adana, HbVar ID 87).

α^{+}-thalassemias are caused by deletions or inactivating mutations of one of the α-globin gene pairs (α2 α1) and the patient may be heterozygous or homozygous. -$\alpha^{3.7}$ or -$\alpha^{4.2}$ deletions, resulting from unequal crossing over in meiosis, causes deletional α^{+} thalassemias. According to Farashi at al., the most common α^{+} thalassemia deletion has been reported as the 3.7 kb deletion (-$\alpha^{3.7}$) (3,8). Former studies from at various times indicated that the frequency of -$\alpha^{3.7}$ deletions varies across different regions of Turkey: 52.28% in the Aegean Region, 43.2% in the southern region, 35.3% in the Trakya region among alpha thalassemia patients and also 39% of the hypochromic microcytic anemia patients in Istanbul (8–11). In our study, where data from the pediatric population aged 3-18 years were analyzed in Konya, located in the Central Anatolia region of Turkey, -$\alpha^{3.7}$ deletion was detected in 36.7% of the patients. And this rate is consistent with similar studies covering all age groups reported from Turkey.

It is reported that -$\alpha^{4.2}$, which is responsible for α^{+} thalassemias, occurs less frequently than the -$\alpha^{3.7}$ deletion (9). In two separate studies that shared data from different region of Turkey, the frequency of the -$\alpha^{4.2}$ deletion was reported as 0.95% and 4.2% (11,12) On the other hand, Onay et al (9) stated that they did not observe any -$\alpha^{4.2}$ deletion and similarly, in our study the -$\alpha^{4.2}$ deletion was not detected. The second most prevalent deletion detected in the present study was -$\alpha^{20.5}$, with a frequency of 16.7%. This variation consist of double gene deletion (α1 and α2) and is located in α0 thalassemias. This phenomenon has been rarely reported in Asia, the Middle East, and in Arab countries. However, it was reported to be the second most prevalant deletion in the studies performed by Demir (8) and Onay (9) et al. The --$^{20.5}$ deletion was followed by the --^{MED1} deletions with a frequency of 6.6%, in our study. --^{MED} deletions are a type of -α0 thalassemia. Demir (8) et al. stated the frequency of --^{MED} deletion as 2.6% and Onay (9) et al. indicated the frequency of --^{MED} deletion as 10.53%. One of the most prevalent copy number changes observed in the alpha globin gene cluster is triplication (8) However, we didn't find any triplications in our study.

The analysis of the molecular basis of alpha globin genes indicates that commonly observed variants of alpha thalassemia, previously attributed to the deletion of a single alpha globin gene copy, are in fact the result of unequal crossover and recombination occurrences. These events lead to the fusion of the two alpha globin genes into a single entity (13)

Although hematological parameters obtained from blood analyzers can serve as valuable predictive indicators of the number of deleted alpha genes, definitive diagnosis of α-thalassemia typically requires molecular studies. In previous studies, it has been widely reported that there is a considerable decrease in Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) when comparing patients with two functional alpha globin genes to those with one defective alpha globin gene (14, 15). Demir et al (8) and Barış et al (12) observed that the MCV value was lowest for the -$\alpha^{3.7}$/--^{SEA} genotype and the --^{MED}/αα genotype, respectively. Conversely, they found that the MCV value was highest for the -$\alpha^{3.7}$/αα genotype in their respective studies. While the patient population size is limited for conducting a statistical analysis, we revealed that MCV values were lower in patients with compound heterozygous -$\alpha^{3.7}$/--$^{20.5}$ and --^{MED} /αα deletions compared to those with -$\alpha^{3.7}$/αα genotypes. Additionally, Velasco-Rodríguez and colleagues have shown in their study, which included 129 alpha thalassemia cases with alpha globin gene deletions, that the MCH value is lower in α^{0} individuals compared to α^{+} individuals(14) In our study as well, the MCH concentration in cases with α^{0} alleles (--$^{20.5}$, --^{MED}) was found to be lower than in cases with α^{+} genotype.

The molecular complexity of alpha thalassemia renders diagnosis challenging. MLPA is a suitable method that can be used in the molecular diagnosis of alpha thalassemia. MLPA, a hybridization-based technique, has long been utilized for the detection of deletions and duplications. The MLPA method offers several advantages over alternative approaches, as highlighted in studies conducted by Colosimo et al. (15) However, sequencing methods like Sanger sequencing or NGS are necessary for identifying non-deletion mutations. While studies conducted both globally and within Turkey have primarily reported deletions as the predominant alpha globin mutations, instances of non-deletion mutations have also been documented. Our study demonstrated the effectiveness of the MLPA method in the diagnosis of alpha thalassemia in the pediatric age group and contributed to the literature on the frequency of HBA gene variants in the pediatric patient group.

A limitation of this research is the relatively small number of patients included. This was due to the fact that the study group consisted solely of paediatric patients. Secondly, sequence analysis was not applied to patients whose alpha-thalassemia MLPA was studied, despite the absence of a deletion being detected.

CONCLUSIONS

The molecular basis of alpha thalassemia is a complex phenomenon. Genetic counselling can be challenging in instances where thalassemia trait has been demonstrated clinically and haematologically, yet cannot be confirmed at the molecular level. In particular, molecular genetic diagnosis is of great value in cases of α^{+}-thalassemia and α^{0}-thalassemia. It enables the determination of the disease prognosis, the provision of accurate and effective genetic counselling, the assessment of the carrier risk of future pregnancies, and the dissemination of information about prenatal or preimplantation tests when necessary.

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Address correspondence to: *Emine Goktas, Necmettin Erbakan University, Faculty of Medicine, Department of Medical Genetics, Konya, Türkiye*
e-mail: *emineaktas88@hotmail.com*

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