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Original Article / Özgün Araştırma

Mutation Analysis of Beta-Thalassemia Major Patients and Their Parents in Diyarbakir Province, Turkey

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Abstract

Objectives: In this study, beta-Thalassemia Major (BTM) diagnosed patients and their parents were subjected to DNA sequencing in order to confirm their diagnosis, improve their treatment and determine the mutation distributions of both patients and their parents.

Methods: A total of 90 people, BTM patients (n=30) and their parents (n=60), were included in the study. For all analyses, two blood samples were taken into EDTA-containing tubes from each of the patients and each of their parents. Complete blood count, hb variant and mutation analysis were studied, respectively.

Results: 8 types of mutation were determined: IVS-I-110 (G->A) 46.67%, codon 8 (-AA) 16.67%, IVS-II-1 (G->A) 11.67%, codon 44 (-C) 10.00%, IVS-II-745 (C->G) 5.00%, IVS-I-1 (G->A) 3.33%, IVS-I-5 (G->T) 3.33% and -30 (T->A) 3.33%. Hb concentrations of 9.2 ± 1.32 g/dL and Hb variant levels of HbA 81.58% ± 11.05, HbF 10.44% ± 11.54 were found in patients with BTM who received transfusion therapy regularly. Typical hemogram count and Hb variants levels were seen in parents.

Conclusion: In our study, a similar distribution was identified throughout Turkey in terms of mutation. Mutations were classified in all the studied people. This study increased the detection percentage of undetermined mutations by the use of DNA sequencing. Thus a multi-centric coordinated study with high capacity will improve detecting these mutations and their effects on the disease.

Keywords: DNA sequence analysis, Thalassemia, Beta-Thalassemia, Mutation

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Diyarbakır İlinde Beta-Talasemi Majörlü Hastalar ve Ebeveynlerinde Mutasyon Analizi

Öz

Amaç: Bu çalışmada beta-Talasemi Majör (BTM) tanısı almış hastalar ve ebeveynlerinde; DNA dizi analizi yöntemiyle tanısının kesinleştirilmesi, tedavisine katkıda bulunulması ve mutasyon dağılımının tespiti amaçlanmıştır.

Yöntemler: Çalışmaya BTM'li hastalar (n=30) ve ebeveynleri (n=60) olmak üzere toplam 90 kişi dâhil edilmiştir. Tüm analizler için hastalar ve ebeveynlerinin her birinden iki adet EDTA ihtiva eden tüplere kan numunesi alınmıştır. Sırasıyla tam kan sayımı, Hb varyant ve mutasyon analizi çalışılmıştır.

Bulgular: IVS-I-110 (G->A) %46.67, codon 8 (-AA) %16.67, IVS-II-1 (G->A) %11.67, codon 44 (-C) %10.00, IVS-II-745 (C->G) %5.00, IVS-I-1 (G->A) %3.33, IVS-I-5 (G->T) %3.33 ve -30 (T->A) %3.33 olmak üzere 8 çeşit mutasyon tespit edildi. Düzenli olarak transfüzyon tedavisi alan BTM'li hastalarda Hb konsantrasyonu 9.2±1.32 g/dL ve Hb varyant düzeyi HbA %81.58±11.05, HbF %10.44±11.54 olarak tespit edilmiştir. Ebeveynlerde ise tipik hemogram sayımı ve Hb varyant düzeyleri görüldü.

Sonuç: Çalışmamızda mutasyon açısından Türkiye geneline benzer bir dağılım tespit edildi. Çalışılan tüm kişilerde mutasyonlar tasnif edildi. Bu çalışma DNA dizi analizi yönteminin kullanımına bağlı olarak saptanamayan mutasyonların belirlenme oranını artırmıştır. Buna bağlı olarak yüksek kapasiteli çok merkezli koordine bir çalışma ile bu mutasyonların ve bunların hastalığa etkisinin belirlenmesini geliştirecektir.

Anahtar kelimeler: DNA dizi analizi, Talasemi, Beta-Talasemi, Mutasyon.

INTRODUCTION

Beta-Thalassemia (BT) is a generally autosomal recessive disorder caused by mutations of the beta-globin gene which is located on the short arm of chromosome 11 resulting in the reduction (β^+) or absence (β^0) of the beta-globin chain synthesis[1,2]. Although BT is a worldwide common disease, it is most frequently seen in the population of the Mediterranean, Middle-East, Transcaucasus, Central Asia, Indian subcontinent, and Far East. Highest incidences were reported from Cyprus (14%), Sardinia (12%) and Southeast Asia. About 1.5% of the world's population is BT carrier[3,4]. BTT frequency in Turkey is approximately 2.1%, while in some regions the frequency increases up to 10%[5]. According to its clinical and hematological severity, BT is categorized in three groups: Beta-Thalassemia Trait (BTT), Beta-Thalassemia Intermediate (BTI) and Beta-Thalassemia Major (BTM). Except for rare dominant forms, BTT is mostly heterozygous whereas BTI and BTM are homozygous or seen in combined heterozygous forms. BT patients are diagnosed based on clinical and laboratorical findings. As for the laboratory diagnosis; complete blood count, qualitative and quantitative Hb analysis [electrophoresis, high performance liquid chromatography (HPLC)] and PCR-based molecular tests for mutation analysis are routinely performed[6-8]. Worldwide, more than 250 different mutations have been detected so far by molecular analysis[9]. In Turkey, more than 40 different BT mutations have been identified showing differences in the distribution of mutation types between different areas of Turkey[10]. Although there are gene therapy studies no definite therapy exists besides bone marrow transplantation in BT so far[11-13].

BT is one of the common genetic diseases in our country and region as well as in the world. Nowadays, DNA sequence analysis is a very sensitive method in determining the diagnosis of BT and the type of mutation. In this study, patients with BTM diagnosed and / or treated in our hospital and their parents were subjected to DNA sequencing in order to confirm the

diagnosis, improve their treatment and determine the mutation distributions of both patients and their parents.

METHODS

Patients (n=30) with diagnosed BTM, who underwent therapy and /or follow-up in the Pediatric Hematology and Oncology Division at Dicle University Faculty of Medicine between January and February 2013 and their parents (n=60) were included in the study. This study was performed in accordance with the Declaration of Helsinki. For our work, we received approval from the ethics committee of the Faculty of Medicine at Dicle University (Decision number: 739/13.11.2012). No distinction was made between age and gender among the individuals included in the study. Blood samples were taken from the patients before transfusion of erythrocyte suspension. Two EDTA- containing tubes (BD Vacutainer® SST™II Advance) of blood samples were taken from the patients and from each of the parents. One of the EDTA tubes was used for complete blood count by the flow cytometry method (Cell Dyn 3700; Abbott Laboratories, Abbott Park, IL, USA) and for measuring Hb variant levels via HPLC (Agilent 1100 series; Agilent Technologies, Waldbronn, Germany). The other EDTA-tubes were stored at -20^{o} C for about 6 months for molecular gene analysis. In our molecular diagnostic laboratory, DNA isolation from EDTA complete blood count samples was carried out using the commercial kit procedure (Macherey-Nagel, Düren, Germany). Samples which underwent DNA-isolation were stored at -20^{o} C. DNA samples of the patients were amplified by the primers from a commercial kit (GML, Switzerland) using GeneAmp PCR System 9700 (Applied Biosystems, USA). Afterwards PCR-product purification was performed with the ExoSAP-IT (GML, Switzerland) reagent. Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was used for sequence reaction. Mutation analysis was carried out by the automated sequence device ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Mutations were detected with SeqScape Software v2.6 program.

Statistical Analysis

Statistical analysis of the data was performed using SPSS 15.0 (SPSS Inc. Chicago, IL, USA) version package program as well as Microsoft Excel 2010 version. Descriptive statistics was applied for the analysis of the data of the study. For quantitative variables mean±standart deviation and median(minimum-maximum) values were used whereas number and percentage ratios were used for qualitative findings.

RESULTS

In our study, patiens with BTM regularly (3-4 week/period) received erythrocyte suspension transfusions. Data gained from BTM patients constituted of data before getting transfusion. Statistical data of the hematological analysis and Hb variant levels obtained from BTM patients and their parents are shown in table I and table II. One parent (mother and father) and one mother were excluded from the Hb variant statistics due to extremely abnormal HbF value and HbA2 value, respectively.

Mutation variants, mechanisms, phenotypic features and frequencies detected by DNA sequence analysis in BTM patients and their parents are shown in table III. In total, according to their frequency, 8 different mutations were detected as follows: IVS-I-110 (G->A) 46.67%, codon 8 (-AA) 16.67%, IVS-II-1 (G->A) 11.67%, codon 44 (-C) 10.00%, IVS-II-745 (C->G) 5.00%, IVS-I-1 (G->A) 3.33%, IVS-I-5 (G->T) 3.33% and -30 T (T->A) 3.33% (table III).

Table I: Statistics of hematological analysis in BTM patients and their parents.

	Statistics	RBC (M/uL)	Hb (g/dL)	MCV (fL)	MCH (pg)
Patient(n=30)	**Mean ± SD[1]**	**3.26± 0.46**	**9.2±1.32**	**78.9±5.47**	**28.3±1.65**
	Median (min-max[2])	3.30 (2.39-4.26)	9.0 (6.8-12.3)	79.4(59.5-86.4)	28.5 (22.7-30.7)
Father(n=30)	**Mean ± SD[1]**	**6.34±0.75**	**13.5±1.16**	**63.8±5.75**	**21.4±1.49**
	Median (min-max[2])	6.39 (3.95-7.97)	13.4 (10.2-16.8)	63.3 (55.8-83.8)	21.2 (19.1-25.9)
Mother(n=30)	**Mean ± SD[1]**	**5.42±0.46**	**11.2±1.05**	**62.6±3.57**	**20.8±1.30**
	Median (min-max[2])	5.38 (4.42-6.57)	11.5 (9.1-13.7)	61.9(54.4-70.4)	20.9 (18.3-24.3)

SD[1]: Standard deviation; min-max[2]: minimum – maximum

Table II: Statistics of Hb variants in BTM patients and their parents.

	Statistics	HbF (%)	HbA (%)	HbA2 (%)
Patient(n=30)	**Mean ± SD[1]**	**10.44 ± 11.54**	**81.58 ± 11.05**	**2.58± 0.32**
	Median (min-max[2])	6.01 (0.91-55.02)	85.92 (39.24-91.51)	2.59 (1.88-3.29)
Father(n=29)	**Mean ± SD[1]**	**1.43 ± 1.60**	**89.34 ± 1.44**	**5.13 ± 0.51**
	Median (min-max[2])	0.94 (0.43-8.63)	89.62 (84.41-91.65)	5.03 (4.23-6.34)
Mother(n=28)	**Mean ± SD[1]**	**1.80 ± 1.90**	**89.21 ± 1.90**	**4.86 ± 0.68**
	Median (min-max[2])	1.09 (0.40-9.28)	89.84 (82.11-91.46)	4.88 (3.44-6.62)

SD[1]: Standard deviation; min-max[2]: minimum – maximum

Table III: Mutation types we detected, their mechanisms, phenotypic features and frequency.

Mutation	Mechanism	Type	Number of the chromosomes	Percent (%)
IVS-I-110 (G->A) (HBB:c.93-21G>A)	RNA processing	β^+	56	46.67
Codon 8 (-AA) (HBB:c.25_26delAA)	Frameshift	β^o	20	16.67
IVS-II-1 (G->A) (HBB:c.315+1G>A)	RNA processing	β^o	14	11.67
Codon 44 (-C) (HBB:c.135delC)	Frameshift	β^o	12	10.00
IVS-II-745 (C->G) (HBB:c.316-106C>G)	RNA processing	β^+	6	5.00
IVS-I1-1 (G->A) (HBB:c.92+1G>A)	RNA processing	β^+	4	3.33
IVS-I-5 (G->T) (HBB:c.92+5G>T)	RNA processing	β^+	4	3.33
-30 (T->A) (HBB:c.-80T>A)	Transcriptional	β^+	4	3.33
Total			**120**	**100**

DISCUSSION

Severity of illness in BT is related to the degree of imbalance between the alpha globin and nonalpha globin chains[14]. BTT patients are usually asymptomatic and may sometimes show mild anemia. However, BTM patients with transfusion dependency demonstrate severe anemia. In BTM, a reduced Hb level <7g/dL and MCH <20 pg levels are observed whereas in BTT there is a decrease in MCV and MCH Levels and an increase in HbA2[4,6].

In our study, regarding hematological analysis, following findings were observed in men and women, respectively: Hb 13.5±1.16 g/dL, MCV 63.8±5.75 fL, MCH 21.4±1.49 pg and Hb 11.2±1.05 g/dL, MCV 62.6±3.57 fL, MCH 20.8±1.30 (table I). There was a MCV and MCH decrease in parents, and a Hb reduction especially in women. Our findings are similar to laboratory findings seen in BTT patients[6]. Concerning hematological analysis in our patients with BTM, Hb 9.20±1.32 g/dL, MCV 78.93±5.46 fL, MCH 28.32±1.65 values were found (table I). Normally, without transfusion, severe hypochromic microcytic anemia is expected in BTM patients. As the patients received erythrocyte suspension transfusions regularly, we observed mild to moderate hypochromic microcytic anemia in our study. For a normal growth and development in patients with BTM, it is recommended to maintain Hb levels at 9.5 g/dL and up through regular transfusion programs[15]. Findings supporting these data were obtained in our study.

According to the type of BT, different Hb variants are found. β^0-Thalassemia: In homozygous forms HbA is absent and total Hb consists of 95-98% HbF and 2-5% HbA2. β^+-thalasssemia: In homozygous or combined β^0/β^+ forms HbA makes up 10-30%, HbF makes up 70-90% and 2-5% HbA2[4]. In BTT patients HbA2 levels usually are 3.5-5.5% and rarely exceed > 6%[1,16].

In our study, we found following Hb variant levels in parents: HbA 89.34% ± 1.44, HbF 1.43%±1.60, HbA2 5.13%±0.51 in men and HbA 89.21%±1.90, HbF 1.80%±1.90, HbA2 4.86%±0.68 in women (table II). An increase of HbA2 is expected in BTT patients. Similarly, we observed elevated HbA2 levels in our work. Hb variant levels were HbA 81.58%±11.05, HbF 10.44%±11.54, HbA2 2.58%±0.32 in our patients with BTM (table II). As our patients regularly got erythroctye suspension transfusions, our Hb variant levels aren't similar to those of BTM patients who don't receive transfusions[4]. Nevertheless, the reduced HbF and elevated HbA levels observed in our patients indicate that the therapy is carried out regularly.

The different mutation types obtained from our work as well as from molecular studies from different regions of Turkey concerning BT are compared in table IV. For mutation analysis we used DNA sequence analysis methods in our work. Other studies used one or two of the Amplification Refractory Mutation System (ARMS), B-Globin Strip Assay or DNA sequence methods. The DNA sequence method enables the detection of all mutations as the entire gene region of interest can be analysed. Whereas methods like AMRS, Strip Assay enable analysing only limited numbers of mutations and/or bases. Mutations reported as mutations not found or known by methods other than DNA sequence analysis reveals the importance of detection by DNA sequence analysis.

The IVS-I-110 (G->A) mutation was the most frequent observed mutation in our study. Except for the Adıyaman centered work, other studies most frequently found the IVS-I-110 (G->A) mutation (table IV). The most often mutations detected in our study are the IVS-I-110 (G->A), codon 8 (-AA), IVS-II-1 (G->A), codon 44 (-C) mutation types, respectively. In other studies, different authors detected following mutation types: İnce et al.[18] IVS-I-110

(G->A), unknown, IVS-I-6 (T->C); Yılmaz[19] IVS-I-110 (G->A), IVS-II-1 (G->A); Ayçiçek et al.[20] IVS-I-110 (G->A), IVS-I-1 (G->A); Genç et al.[21] IVS-I-1 (G->A), codon 17 (A->T), IVS-I-110 (G->A), IVS-II-745 (C->G), codon 8/9 (+G), codon 82/83 (-G); Pehlivan et al.[22] IVS-I-110 (G->A), unknown, IVS-II-1 (G->A); Guzelgul et al.[23] IVS-I-110 (G->A); Güvenç et al.[24] IVS-I-110 (G->A); Ozkinay et al.[25] IVS-I-110 (G->A); Kurtoğlu et al.[26] IVS-I 110 (G->A), IVS-I-6 (T->C); Yalçıntepe[27] IVS-I-110 (G->A), codon 39 (C->T) (table IV). Differences were observed concerning the mutation types between our study and studies of other regions. As for mutation types and frequency our findings were similar to the mutations in Turkey concerning mutation frequencies. Not all mutations could be determined due to methodological differences in some regions. Our studies showed higher similarities with the studies in the Mediterranian region and Aegean than with other regions. Least similarity was found with Edirne and Adiyaman. Due to the difference in the method, it would nevertheless be useful to detect unknown mutations and to increase the study data in order to determine the regional frequency and types of mutations.

Table IV: Mutation types and their frequency in our study and other studies.

Mutation	Our Study	Turkey	Diyarbakir	Siirt	Sanlıurfa	Adiyaman	Gaziantep	Cukurova	Adana	Aegean	Antalya	Edirne
	%	%	%	%	%	%	%	%	%	%	%	%
IVS-I-110(G->A)	**46.67**	**40.88**	**27.8**	**38.89**	**29.1**	**10.7**	**29.1**	**48.08**	**35.14**	**41.7**	**60.8**	**28.6**
Codon 8 (-AA)	**16.67**	4.69	**11.1**	-	9.1	-	5.6	3.85	9.15	7.7	3.1	4.1
IVS-II-1 (G->A)	**11.67**	8.08	8.3	**11.11**	-	7.1	**12.3**	5.77	6.43	7.2	5.2	1
Codon 44 (-C)	**10.00**	1.78	**	7.41	3.5	3.6	3.1	3.85	4.95	1.3	2.1	4.1
IVS-II-745 (C->G)	5.00	6.2	5.5	1.85	1.7	10.7	1.5	0.96	2.22	8.6	-	7.1
IVS-I-1 (G->A)	3.33	5.73	2.8	-	**13.9**	**21.4**	7.7	2.88	8.66	8.9	2.1	8.2
IVS-I-5 (G->T)	3.33	0.28	**	9.26	4.3	-	**	1.92	3.71	2.2	-	-
-30 (T->A)	3.33	4.22	2.8	9.26	-	-	4.6	3.85	7.42	1.0	8.2	-
Unknown	-	2.72	**27.8**	-	9.6	-	**20.9**	5.77	-	-	-	-
Others	-	**23.86**	**13.9**	**22.22**	**18.2**	**42.9**	**14.9**	**23.07**	**22.27**	**21.4**	**18.5**	**46.7**
Total chromosome	120	1064	72	54	230	28	196	132	404	1171	292	98
Method *	3	1,3	1	3	2	1,3	2	1,3	2	3	3	3
References		(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)

**: 1. ARMS, 2. B-Globin Strip Assay, 3. DNA sequence; **. Not studied; - . None*

As BTM patients received regular erythrocyte suspension transfusions, hematological analysis and Hb variant levels weren't congruent with laboratory data seen in BTM patients. But mean Hb levels of 9.20±1.32 g/dL seen in BTM patients suggests that follow-up and therapy were maintained effectively. Hematological values and Hb variant levels typical for BTT patients were also found in parents. Similar mutation frequencies were

observed as seen in Turkey. This study increased the detection percentage of undetermined mutations by the use of DNA sequencing. Thus detection of mutations and determining their effect on disease in multi-centered studies with high capacity will contribute to the patient's diagnose and therapy.

Ethics Committee Approval: Ethics committee of the Faculty of Medicine at Dicle University (Decision number: 739/13.11.2012).

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