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Prenatal Diagnosis of Different Polymorphisms of β-globin Gene in Ahvaz

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ABSTRACT

Background: Hemoglobinopathy and thalassemia are prevalent genetic disorders throughout the world. Beta thalassemia is one of these disorders with high prevalence in Iran, especially in Khuzestan province. In this study, the rate of different mutations in β-globin gene for prenatal diagnosis in fetal samples was evaluated.

Materials and methods: In this experimental pilot study, 316 fetal samples (chorionic villus or amniotic fluid) suspicious to hemoglobin disorders were enrolled. Afterwards, DNA was extracted and PCR and DNA sequencing were used for evaluation of different mutations in β-globin gene.

Results: Amongst 316 samples evaluated for prenatal diagnosis, 180 cases (56.8%) were carrying at least one mutated gene of β-thalassemia. In addition, results showed that CD 36-37 (- T) and IVS II-1 (G>A) polymorphisms are the most prevalent polymorphisms of β-thalassemia in Ahvaz city with 13.9% and 10.1% rates, respectively.

Conclusion: Using molecular tests for prenatal diagnosis is considered an efficient approach for reducing the birth of children with hemoglobinopathy and identification of prevalent mutations in each region.

KEY WORDS: Hemoglobinopathy, β-thalassemia, Prenatal diagnosis, Polymorphism

INTRODUCTION

Thalassemia is a common genetic disorder with autosomal recessive inheritance in the world, and is associated with clinical symptoms of hemolytic anemia.[1, 2] This disease has a high incidence in various parts of Iran, such as the Caspian region, Persian Gulf margin and Fars and Isfahan provinces.[3-5] Similar to other genetic disorders of recessive inheritance, the importance of β-thalassemia is due to heterozygote individuals carrying a mutant haplotype without specific clinical symptoms. Following the marriage of two heterozygous individuals for β-thalassemia (thalassemia minor patients), there is 25% chance of homozygous patients, 50% chance of heterozygous birth carrying the disease gene, and 25% chance of birth of a healthy homozygous individual.[6-9] DNA assay can be used for definitive diagnosis of thalassemia. Molecular genetic tests are facilitated because of presumed incidence of a few mutations in any given population. However, molecular genetic methods may not be substituted for biochemical and hematological testing.[6, 10, 11]

Since prenatal diagnosis (PND) is important in many genetic disorders such as hemoglobinopathies,

proper DNA isolation and analysis during fetal period is further emphasized. The first trimester of pregnancy (10-12 weeks) is optimum for DNA extraction from chorionic villi (CVS).[6, 12-14]

MATERIALS AND METHODS

In this study, 316 fetal samples (including amniotic fluid or CVS) from carrier couples for thalassemia or hemoglobinopathy were subject to molecular testing. DNA extractions from these samples were conducted using Bioneer kit (S. Korea). Identification test of the fetus was compared with parent samples to ensure no contamination of the fetal sample with maternal tissues and to properly authenticate the fetus. Due to diversity of common mutations in Khuzestan and the time limit for review of fetal samples, the first step in determining the mutation was sequencing the β-globin gene as two separate segments. The first segment comprising -110 upstream nucleotides of the gene up until the first part of the second intron was amplified and sequenced using forward 5'AACTCCTAAGCCAGTGCCAGAAGA3' and reverse 5'CCCCTTCCTATGACATGAACTTAA3' primers. The second segment of the gene contains the final part of the second intron up to downstream of the gene, amplified and sequenced using primer pair of forward 5'CAATGTATCATGCCTCTTTGCACC3 'and reverse 5'CACTGACCTCCCACATTCCCTTTT3'. PCR mixture contained 100ng patient DNA, 2.5µL 10X PCR buffer, 1.5 mMMg Cl2, 0.2 mM dNTP,0.4 pmol/L of each of the primers, reaching the final concentration of µL using water free from RNase and DNase. PCR program was as follows: 3 minutes in 95°C, 30 temperature cycles consisting of 30 seconds in 95°C, 30 seconds in 59°C, 30 seconds in 72°C and finally 5 minutes of incubation at 72°C. After sequencing, for final confirmation and ensuring the absence of gene deletion or amplification, RFLP Linkage and ARMS were performed in the same PCR conditions, with the primers and enzymes used listed in Tables 1 and 2. RFLP has also been used in hemoglobinopathy for diagnosis of Hb Sand HbD.[23] In all cases, negative control containing all the materials except for patient DNA was used to ensure lack of contamination. In addition, reverse dot blot (RDB) kit (Vienna lab. Austria) was used to detect mutations or deletions not detectable using current sequencing and PCR methods, such as -619 bp Del mutation.

RESULTS

There were a total of 316 fetal samples. It should be noted that all the samples came from families native to Khuzestan Province. PND indicated 11.7% incidence of sickle cell (HbS) mutation, 1.2% incidence of concomitant HbS and β-thalassemia mutation, 0.3% incidence of HbD mutation and 56.8% incidence of at least one of β-thalassemia mutations. The fetus was healthy in 23.4% of the patients, and 6.6% of the patients were excluded from the study for various reasons. The results were indicative of 13.9% and 10.1% incidence of at least CD 36-37 (-T) and IVS II-1 (G>A) polymorphisms in the population under study, respectively. These mutations had the highest incidence in samples subject to PND. Our findings also suggested 0.5% incidence of combined HbS and β-thalassemiain patients. Table 3 shows the number and incidence of each mutation in the population under study.

Table 1.Primers Used in ARMS Method to Evaluate β-globin Gene [23]

(Fragment Length) bp	Second Primer	(sequence) 5'>3'	First Primer
684	**A**	TCACTTAGACCTCACCCTGTGGAGCCTCAT	–88 (C>T) mutant
		CACTTAGACCTCACCCTGTGGAGCCACCCCA	–88 (C>T) normal
520	**A**	ACACCATGGTGCACCTGACTCCTGAGCAGG	CD8 (–AA) mutant
		ACACCATGGTGCACCTGACTCCTGAGCAGA	CD8 (–AA) normal
225	**B**	CCTTGCCCCACAGGGCAGTAACGGCACACC	CD8/9 (+G) mutant
		CCTTGCCCCACAGGGCAGTAACGGCACACT	CD8/9 (+G) normal
439	**B**	GAGTGGACAGATCCCCAAAGGACTCAACCT	CD41/42 (–TCTT) mutant

		GAGTGGACAGATCCCCAAAGGACTCAAAGA	CD41/42 (–TCTT) normal
281	**B**	TTAAACCTGTCTTGTAACCTTGATACCGAT	IVS1-1 (G>A) mutant
		TTAAACCTGTCTTGTAACCTTGATACCCAC	IVS1-1 (G>A) normal
285	**B**	CTCCTTAAACCTGTCTTGTAACCTTGTTAG	IVS1-5 (G>C) mutant
		CTCCTTAAACCTGTCTTGTAACCTTGTTAC	IVS1-5 (G>C) normal
286	**B**	TCTCCTTAAACCTGTCTTGTAACCTTCATG	IVS1-6 (T>C) mutant
449	**A**	AGTTGGTGGTGAGGCCCTGGGCAGGTTGGT	IVS1-6 (T>C) normal
419	**B**	ACCAGCAGCCTAAGGGTGGGAAAATAGAGT	IVS1-110 (G>A) mutant
		ACCAGCAGCCTAAGGGTGGGAAAATACACC	IVS1-110 (G>A) normal
634	**B**	AAGAAAACATCAAGGGTCCCATAGACTGAT	IVS2-1 (G>A) mutant
		AAGAAAACATCAAGGGTCCCATAGACTGAC	IVS2-1 (G>A) normal
738	**D**	TCATATTGCTAATAGCAGCTACAATCGAGG	IVS2-745 (C>G) mutant
		TCATATTGCTAATAGCAGCTACAATCGAGC	IVS2-745 (C>G) normal
		CCCCTTCCTATGACATGAACTTAA	A
		ACCTCACCCTGTGGAGCCAC	B
		TTCGTCTGTTTCCCATTCTAAACT	C
		GAGTCAAGGCTGAGAGATGCAGGA	D

Table 2. Linkage RFLP of β-globin Gene [23]

RFLP Product Size in case of lack of enzymatic excision site	RFLP Product Size in case of presence of enzymatic excision site	PCR Product Size	Enzymes and Primer sequence
760	315	760	Hind II/ε
	445		5'TCTCTGTTTGATGACAAATTC
			5'AGTCATTGGTCAAGGCTGACC
326	235	326	Hind III/Gγ
	91		5'AGTGCTGCAAGAAGAACAACTACC
			5'CTCTGCATCATGGGCAGTGAGCTC
635	327	635	Hind III/Aγ
	308		5'ATGCTGCTAATGCTTCATTAC
			5'TCATGTGTGATCTCTCAGCAG
795	691	795	Hind II/5'ψβ
	104		5'TCCTATCCATTACTGTTCCTTGAA
			5'ATTGTCTTATTCTAGAGACGATTT
795	440	795	Ava II/ψβ
	355		Hind II/5' ψβ RFLP Primer
913	479	913	Hind II/3' ψβ
	434		5'GTACTCATACTTTAAGTCCTAACT
			5'TAAGCAAGATTATTTCTGGTCTCT
411	330	1200	Rsa I/β
694	81		5'AGACATAATTTATTAGCATGCATG
695	694		5'CCCCTTCCTATGACATGAACTTAA
320	213	474	Hinf I/β
154	107		5'GGAGGTTAAAGTTTTGCTATGCTGTAT
	154		5'GGGCCTATGATAGGGTAAT
328	228	328	Ava II/β
	100		5'GTGGTCTACCCTTGGACCCAGAGG
			5'TTCGTCTGTTTCCCATTCTAAACT

Table 3. Evaluation of Mutations, Their Number and Incidence in β-globin Gene, and the Prevalence of β-globin Gene in the Studied Population

B-globin gene mutation	number	Incidence rate
Alpha thalassemia carriers	21	6.6
-28(A>C)	2	0.6
-88(C>A)	3	0.9
-88(C>A)/IVS II-1 (G>A)	3	0.9
+20 with IVS II 745 (	1	0.3
CD 22-24	2	0.6
CD 30 (G>C)	1	0.3
CD 36-37 (- T)	33	10.4
CD 36-37 (- T)/ CD 36-37 (- T)	9	2.8
CD 36-37 (- T)/ CD 82-83 (-G)	1	0.3
CD 36-37 (- T)/IVS I-110 (G>A)	1	0.3
CD 45[-T]	1	0.3
CD 5[-CT]	5	1.6
CD 6(HbS)	28	8.9
CD 6(HbS)/ CD 6(HbS)	9	2.8
CD 6(HbS)/IVS I (-25nt)	1	0.3
CD 8 (-AA)	8	2.5
CD 8 (-AA)/ CD 8 (-AA)	5	1.6
CD 8-9 (+G)	3	0.9
CD 8-9 (+G)/ CD 8-9 (+G)	1	0.3
CD8-9 (+G)/ CD 82-83 (-G)	1	0.3
CD 82-83 (-G)	1	0.3
CD 82-83 (-G)/ CD 82-83 (-G)	1	0.3
CD 39 (C>T)	2	0.6
CD 39 (C>T)/ CD 39 (C>T)	1	0.3
CD 39 (C>T)/IVSI-6 (T>C)	1	0.3
CD 44(-C)	2	0.6
CD 44(-C)/-80	1	0.3
CD 44(-C)/ CD 22-24	1	0.3
CD 44(-C)/ CD 44(-C)	1	0.3
CD 8 (-AA)/IVS I-110 (G>A)	1	0.3
HbD	1	0.3
initCD(T>C)	3	0.9
IVS I (-25nt)	7	2.2
IVS I (-25nt)/ CD 6 (A>T)(HbS)	1	0.3
IVS I (-25nt)/ CD 82-83 (-G)	1	0.3
IVS I (-25nt)/IVS I (-25nt)	1	0.3
IVS I-1 (G>A)	6	1.9
IVS I-1 (G>A)/ CD 6(HbS)	1	0.3
IVS I-1 (G>A)/IVS I-1 (G>A)	1	0.3
IVS I-1 (G>A)/IVSI-128(T>G)	1	0.3
IVS I-110 (G>A)	11	3.5
IVS I-110 (G>A)/ CD 36-37 (- T)	1	0.3
IVS I-110 (G>A)/ CD 8 (-AA)	1	.3
IVS I-110 (G>A)/IVS I-110 (G>A)	5	1.6
IVS I-5 (G>C)	7	2.2
IVS I-5 (G>C)/-619bp del	1	0.3
IVS I-5 (G>C)/IVS I-5 (G>C)	4	1.3
IVS II-1 (G>A)	20	6.3
IVS II-1 (G>A)/ CD 8-9 (+G)	1	0.3
IVS II-1 (G>A)/IVS I-110 (G>A)	1	0.3
IVS II-1 (G>A)/IVS II-1 (G>A)	7	2.2
IVSI- 17 pb	1	0.3
IVSI-128(T>G)	2	0.6
IVSI-128(T>G)/IVSI-128(T>G)	1	0.3
IVSI-5(G>C)/ CD 6(HbS)	1	0.3
IVSI-6 (T>C)	3	0.9
IVSI-6 (T>C)/IVSI-6 (T>C)	1	0.3
Normal	74	23.4
Total	316	100.0

DISCUSSION

Since hemoglobinopathy disorders cause social and economic problems in healthcare system of our country, detection of the type of β-globin gene mutations in PND samples is important due to correlation between gene polymorphism and clinical symptoms. However, the relationship between genotype and phenotype in patients with hemoglobinopathy is not straightforward.[6, 16, 17] Indeed, detection of homozygous genotypes can partially reveal the clinical status of patients in terms of thalassemia major, thalassemia intermedia, sickle cell syndrome and other disease cases.[6,17] In heterozygous mutant forms, despite the absence of specific severe clinical findings, identifying the mutations involved can help assess disease risk in newborns. In fact, premarital screening of the couples and PND in the first trimester of pregnancy can help evaluate the risk of disease in newborn.[17] In this study, it was found that about 75% of newborns carry at least a mutant β-globin gene. 18% of PND samples possessed two β-thalassemia genes, indicating β-thalassemia major or intermedia in fetus, whose mothers were subject to abortion procedure. In another study conducted in 2007, it was found that 10.3% of pregnant women under study had fetuses affected by hemoglobinopathy, and the fetuses were eliminated by abortion. In this study, β-thalassemia was found to be the most prevalent hemoglobinopathy disorder with 55% incidence.[17] Due to significant increase in cases of β-thalassemia in Iran in recent years, lack of remarkable outcome from genetic counseling as well as public media in reducing this incidence has been noted by Rahim et al.[16] Other studies have indicated notable effect of various governmental and non-governmental organizations in public notification during premarital screening programs in reducing the incidence of these hereditary disorders in Cyprus, Italy, Canada and United Kingdom.[18-22]

In this study, CD 36-37 (-T) and IVS II-1 (G> A) were the most prevalent β-thalassemia mutations with respective incidence of 13.9% and 10.4%. These findings are in agreement with another study conducted in Khuzestan Province.[16] Maleki et al., have only indicatedIVS II-1 (G> A) as a prevalent mutation in Iran.[15] IVSI-6 (T> C) mutation

(Portuguese variant), a common Mediterranean mutation, has been recognized as the most common marker of mild β-thalassemia.[23] The highest incidence of this mutation has been found in Khuzestan and Shiraz provinces.[24] However, the incidence of this mutation in our study was 1.2%.In the study of Maleki et al., the prevalence of this mutation in Tehran in patients with β-thalassemia was 2.6%.[15] In another study, IVS I-5 (G> C) mutation has been cited as the second most common β-thalassemia mutation in Iran, with higher prevalence in southern Iran than in northern region.[25] However, in this study, we found 4.1% prevalence of this marker in PND samples.

Genetic counseling and prenatal molecular diagnosis are effective in reducing the risk of hemoglobin disorders. In this regard, preventive strategies are significant, especially in parts of Iran with high prevalence of hemoglobin disorders like Ahvaz. In addition, incidence survey of common and rare mutations in these regions can help identify the carriers of hemoglobinopathy gene to prevent the birth of patients with acute symptoms of the disease.

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