Prenatal diagnosis of beta-thalassemia in the West Bank and Gaza

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ABSTRACT

Objectives: This study focuses on the genetic aspect of β-thalassemia among 88 at risk couples from the West Bank and Gaza, and the attitude of these couples toward prenatal diagnosis and its outcome as a preventive method.

Methods: We tested 130 prenatal samples for β-thalassemia during the period from January 1999 to July 2005. We performed prenatal diagnosis in these cases using the amplification refractory mutation system, as well as β-globin gene sequencing as a conformational method. We drew a chorionic villus sample (CVS) for 1st trimester pregnant women and amniotic fluid (AF) for those in the 2nd trimester depending on the stage the pregnant woman contacted our lab.

Results: The DNA analysis of 130 prenatal samples revealed 25 (19.2%) cases of β-thalassemia major and 67 (51.5%) cases of β-thalassemia carriers, while the remaining 38 (29.2%) were normal. The 25 affected fetuses were aborted according to the wishes of the parents. In the tested 88 couples, 14 mutations of β-thalassemia were identified. These mutations and their frequencies were: IVSI-110 (22.2%), IVSI-6 (13.6%), Cd37 (12%), IVSI-I (9.7%), IVSII-1 (6.2%), Cd39 (9%), Cd6 (sickle cell mutation) (8.5%), Cd5 (8%), Cd8/9 (2.8%), Cd106/107 (2.8%), -30 promoter (1.1%), -88 promoter (1.1%), IVSI (-1) (2.3%) and IVSI-5 (0.6%). We found that in 77.3% of the couples, both the mother and the father carry the same type of mutation while 22.7% of them carry different mutations. We found 77.9% consanguinity among the couples.

Conclusion: We found very good acceptability for prenatal diagnosis in β-thalassemia afflicted families. All couples with affected fetuses opted for abortion. The spectrum of mutations in the tested couples revealed several similarities to neighboring countries with -88 promoter mutation reported for the first time in our region.

Saudi Med J 2005; Vol. 26 (11): 1771-1776

Beta-thalassemia is an autosomal recessive genetic disorder that results mainly from mutations decreasing (β^+) or eliminating (β^0) the expression of the β-globin gene.[1] The genetic background of β-thalassemia is very heterogeneous with more than 200 mutations identified.[2] The prevalence of the β-thalassemia minor in the West Bank is 3.1% and in Gaza is 4.3%,[3] a frequency similar to other neighboring countries such as Jordan 3-4%,[4,5] Lebanon 2-6%,[1] and 3-4% in central Saudi Arabia.[6,7] Treatment of a thalassemia major patient is considered to be a serious financial burden on the governmental and non governmental institutes that offer the medical services. Patients and their families face hard psychological and social problems with this chronic disease, especially patients that depend on life-long blood transfusion. Moreover, the political instability, military closure, traveling restrictions, poor medical and health service availability and financial restrains are extra

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Received 26th March 2005. Accepted for publication in final form 23rd August 2005 .

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hardships faced by the patients, families and health institutions. Therefore, there is a strong necessity for preventing new births of β-thalassemia. Carrier screening, premarital testing, genetic counseling, and increasing awareness of the risk of consanguineous marriages are effective means followed to prevent β-thalassemia. The prevention of β-thalassemia is advanced by the introduction of prenatal diagnosis, which determines whether the unborn child has thalassemia major, or not. If the fetus is affected, the couple has the option of aborting the baby to prevent the birth of an affected child. Thus, prenatal diagnosis allows the parents who already have an affected child to reduce the socio-economic pressure on them, so that they can better look after the affected child. This also benefits the government by having to provide the facilities to a smaller number of children, leading to improved management. Additionally, the cost of mutation screening for patients and their first-degree relatives, as well as the prenatal diagnosis test, is approximately one-tenth the costs for treating a thalassemia patient. We think that if each thalassemia carrier couple is appropriately counseled and offered the option of prenatal diagnosis, the incidence of thalassemia affected newborns per year will sharply decrease according to the Sardinia experience.[8,9] In this study, we present our experience in prenatal diagnosis of β-thalassemia for couples from the West Bank and Gaza, and we will describe the mutation spectrum of these couples and their fetuses as well as their attitude toward prenatal diagnosis.

Methods.

Study subjects. This study included all the β-thalassemia prenatal diagnoses performed in the Molecular Genetics Lab at Al-Makassed Hospital in Jerusalem between January 1999 and July 2005. During this period, 130 prenatal samples were tested from 88 couples (some of the couples carried out prenatal diagnosis more than once) originating from West Bank and Gaza. Eighty couples requested prenatal diagnosis because they already had a β-thalassemia major child (71 couples), or sickle cell anemia child (6 couples) or β/sickle thalassemia child (3 couples). Eight couples were newly married but known to be β-thalassemia carriers. As soon as the pregnant woman intending to do the prenatal test contacted our lab, she was asked about her last menstrual period (LMP) in order to calculate the date on which the chorionic villus sample (CVS) or amniotic fluid (AF) sample would be drawn. The CVSs were taken between the 9th to 12th week of gestation while AF samples were drawn between the 16th to 18th week of gestation, as the kind of prenatal sample drawn depends on the time the pregnant woman decides to do the test. The CVS or AF sample was used as the DNA source for the prenatal test. Approximately 4 ml of blood samples with EDTA were collected for DNA analysis from the parents (similarly from their affected child if available).

Prenatal diagnosis are carried out only when both parents were thalassemia carriers. However, if only one of the parents is willing to do the prenatal diagnosis and the other parent is normal, no thalassemia prenatal test was performed.

Molecular diagnosis. DNA was extracted from the blood samples and CVSs using the MasterPure™ Genomic DNA Purification Kit (Epicenter Technologies, USA) according to the standard procedure.[10] The AF samples were first cultured for 10 days, and then DNA was extracted from the harvested amniocytes. We used the Amplification Refractory Mutation System (ARMS) as the screening method. The ARMS is an amplification strategy in which a polymerase chain reaction (PCR) primer is designed in such a way that it is able to discriminate among templates that differ by a single nucleotide residue.[11,12] In our lab, we identified 14 mutations: -88, -30, IVSI(-1), Cd 5, Cd 8/9, IVS-I-1, IVS-I-5, IVS-I-110, Cd 39, IVS-II-1,[13] IVS-I-6,[14] and Cd 6 (sickle cell anemia mutation).[15] The primers' sequences used to detect the remaining 2 mutations (namely, Cd 37 and Cd 106/107+G) are designed locally in our laboratory (**Table 1**). The PCR products were generated in 25 ul volume

Table 1 - Sequences of Cd 37 and Cd 106/107 +G primers.

Mutation	Primers' Sequence (5'-3')	Type
Cd 37	ACCTCACCCTGTGGAGCCAC	Common
	TCCCCAAAGGACTCAAAGAACCTCTGGGTT	Mutated
	TCCCCAAAGGACTCAAAGAACCTCTGGGTC	Normal
Cd 106/107 +G	GAGTCAAGGCTGAGAGATGCAGGA	Common
	CCTCTTATCTTCCTCCCACAGCTCCTGGGG	Mutated
	CCTCTTATCTTCCTCCCACAGCTCCTGGGC	Normal

Table 2 - Number and frequency of the different β-thalassemia alleles identified in 88 couples and their fetuses.

Mutation	Couples' alleles		Fetuses' alleles*	
	No.	(%)	No.	(%)
IVSI-110[G>A]	39	(22.2)	34	(29)
IVSI-6[T>C]	24	(13.6)	17	(14.5)
Cd37[G>A]	21	(12)	15	(12.8)
IVSI-1[G>A]	17	(9.7)	9	(7.7)
Cd39[C>T]	16	(9)	8	(6.8)
Cd 6 [A>T]+	15	(8.5)	13	(11.1)
Cd5[-CT]	14	(8)	3	(26)
IVSII-1[G>A]	11	(6.2)	7	(6)
Cd 8/9[+G]	5	(2.8)	2	(1.7)
Cd106/107 (+G)	5	(2.8)	4	(3.4)
IVSI(-1)[G>C]	4	(2.3)	1	(0.85)
-30 promotor[T>A]	2	(1.1)	1	(0.85)
-88 promotor[C>T]	2	(1.1)	1	(0.85)
IVSI-5[G>C]	1	(0.6)	1	(0.85)
Total	**176**		**117**	

*The alleles of the fetuses represent the 25 affected fetuses and the 67 carriers.
+ - Sickle cell anemia mutation

containing 1X TaKaRa PCR buffer (50 mM KCl, 10 mM Tris HCl, pH 8.3, 1.5 mM $MgCl_2$), 0.2 mM dNTPs (TaKaRa Biomedicals, Japan), 0.625 U of recombinant Takara Taq™ DNA polymerase, 0.2 ug of each primer and 0.4 ug of template DNA (for Cd 37) or 0.6 ug DNA [for Cd 106/107 (+G)]. In order to increase specificity of bands, 4% formamide was added to the mixture of Cd 37 and 5% DMSO was added to the mixture of Cd 106/107+G. Amplifications were performed in either a MiniCycler™ (MJ Research, Inc., USA) or (HyBaid OmniGene, UK). The PCR conditions for Cd 37 were as follows: DNA denaturation at 94ºC for 4 minutes, followed by 25 cycles at 93ºC for one minute, 67ºC for one minute then at 72ºC for 1.5 minute, and a final extension step at 72ºC for 3 minute. The PCR conditions for Cd 106/107+G were as follows: DNA denaturation at 98ºC for 2 minutes followed by 25 cycles at 98ºC for 10 seconds, 67ºC for 30 seconds, then 72ºC for 30 seconds and a final extension step at 72ºC for 2 minutes. Each amplified PCR product was analyzed using 2% agarose gel electrophoresis (Tech comp, Ltd., USA) and stained with ethidium bromide. Bands were visualized on an ultraviolet illuminator and photographed with a polaroid camera (Fuji Photo Film Co., Japan). Our strategy for the prenatal diagnosis, at Molecular Genetics Lab, starts by screening the DNA sample of the thalassemic child (if available) or the DNA samples of the couple directly by ARMS. As soon as the mutation is identified, the fetal sample (CVS or AF sample) is tested for the same mutation. The test result for the fetus is confirmed by DNA sequencing. For sequencing, we amplify 2 DNA fragments of the β-globin gene from 166 nucleotides upstream of the cap site to 60 nucleotides downstream of the polyadenylation site.[16] The PCR amplification was performed in a 100 ul reaction volume containing the following mixture: 1X TaKaRa PCR buffer (50mM KCl, 10mM Tris HCl pH 8.3,1.5 mM $MgCl_2$), 0.2 mM dNTPs (TaKaRa Biomedicals, Japan), 2.5 U of recombinant Takara Taq™ DNA polymerase, 0.8 ug of each primer and 0.5 ug of template DNA. The amplification program was as follows: DNA denaturation at 98ºC for 2 minutes followed by 35 cycles at 98ºC for 15 seconds, 62ºC for 45 seconds, then 72ºC for 1 minute and a final extension step at 72ºC for 5 minutes. Direct genomic sequencing was performed after purifying the PCR products using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, USA).[17] Sequencing was performed using ABI PRISM™ Model 3700 version 3.3 DNA sequencer (Perkin Elmer, USA).

Results. Of the 88 couples included in the study, 71 (80.7%) originated from the West Bank and 17 (19.3%) originated from Gaza. The ages of the pregnant women on whom the prenatal tests were carried out ranged from 19-34 years old. The prenatal samples tested were either of CVS, collected between the 9th-12th week of pregnancy, or AF, collected between the 16-18th weeks depending on the stage the pregnant woman contacted our lab. Ninety-six samples (73.8%) out of the 130 prenatal samples were CVS, while the remaining 34 (26.2%) were AF. The CVS has the advantage of being a rapid means of diagnosis as it is drawn in the first trimester of pregnancy. Moreover, in the case of an effected fetus, termination of pregnancy could be carried out at a more religiously acceptable stage of pregnancy. Among the 88 couples, 71 were at risk of β-thalassemia as both the mother and the father were carriers of the β-thalassemia mutation, 6 couples were carriers of the sickle cell anemia mutation, and 3 couples were either sickle cell or β-thalassemia carriers. The β-thalassemia mutations identified among the couples comprise 91.5% of the diseased β-globin gene while the sickle cell anemia mutation comprise 8.5% of the diseased β-globin gene.

In this study, we encountered 14 different mutations including the sickle cell anemia mutation. **Table 2** shows the number and frequency of the mutated alleles among the couples and their fetuses. There is a prominent similarity in the frequencies in both groups. A total of 25 (19.2%) fetuses out of the 130 tested were found to be affected and termination of pregnancy was medically indicated,

67 (51.5%) were found to be carriers and 38 (29.2%) were normal. Seventeen (68%) of the affected fetuses were homozygotes and 8 (32%) were compound heterozygotes (2 different mutations). All couples with affected fetuses opted for abortion as soon as they got the result. The genotype of the 25 affected fetuses is shown in **Table 3**, and the frequency of the mutations in the affected and carrier fetuses is shown in **Table 2**. We found that 68 (77.3%) of the couples were heterozygotes for the same type of mutation. However, only 20 (22.7%) couples carry different mutations. After reviewing the data of these couples, we found that 77.9% of them are either first or second cousins, namely, consanguineous.

Table 3 - Genotype of the 25 affected fetuses.

Genotype	No. of fetuses
Homozygous	
IVSI-110 / IVSI-110	7
Sickle cell / sickle cell	2
IVSI-6 / IVSI-6	3
Cd 37 / Cd 37	2
IVSI-1 / IVSI-1	2
Cd 106/107 / Cd 106/107	1
Total	**17**
Compound heterozygous	
IVSI-6 / Cd 39	2
IVSI-110 / Cd 37	1
IVSI-5 / Cd 106/107	1
IVSI-110 / IVSI-6	1
IVSI-1 / Cd 39	1
Cd 37 / Cd 5	1
IVSI-1 / IVSI-6	1
Total	**8**

Discussion. The thalassemias, one of the most important forms of hemoglobinopathies, are a heterogeneous group of hemoglobin synthesis diseases in which mutations reduce the synthesis or stability of either the α- or β-globin chain, to cause α- or β-thalassemia.[18] There is a characteristic distribution of the thalassemias in a band around the old world: in the Mediterranean, the Middle East, and parts of Africa, India, and Asia. Among Palestinians, thalassemia is one of the major health problems, mainly β-thalassemia with a carrier rate estimated to be 3-4%.[3] Therefore, the issue of thalassemia prevention is one of the most important health measures to be undertaken. This lies mainly on carrier screening, premarital testing, genetic counseling, and prenatal diagnosis on which we focus in this paper.

In general, β-thalassemia mutations are relatively population specific, namely, each ethnic group has its own set of common mutations. Comparison of the thalassemia mutations spectrum reported in the studied 88 couples with other Arab populations revealed several interesting similarities as well as differences. The IVSI-110 is the most common mutation identified in the study (22.2%) (**Table 2**), in accordance with other neighboring countries such as Lebanon in which IVSI-110 is 33%, Syria 24%, Jordan 22%, Egypt 33% and Saudi Arabia 22%.[19] The IVSI-6 is the second most encountered mutation, composing 13.6% of the tested alleles; similarly in Egypt the frequency of IVSI-6 is 13.6%.[19] The Cd 37 is the third most common with a frequency of 12%. Similarly, it has been the third most common in a study in the West Bank region with a ratio of 10.4%.[20] The rest of the mutations range in ratios from 9.7% to 0.6%. The IVSI-5 was the least encountered mutation (0.6%) carried only by one parent in our study, although it is the most common mutation in Oman (62%)[21] and the United Arab Emirates (55%).[22] Interestingly, the -88 promoter mutation, which was not previously reported in our region,[20,23,24] is found in one of the couples from the West Bank. Similarly, the Cd 106/107 (+G), which is not reported in Jordan, Syria and Lebanon, is found in our study in a ratio of 2.8%.

After studying the mutations carried by the families undergoing prenatal diagnosis, we found that 77.3% of the couples are carriers for the same type of mutation, while only 22.7% of them carry different mutations. In accordance, 68% of the affected fetuses were homozygotes and only 32% were compound heterozygotes. These results can be mainly attributed to consanguinity, which is found to be the case among 77.9% of the 88 couples. Consanguinity is an ancient custom and is widely spread among Palestinians. Statistical information supplied by the Palestinian Central Bureau of Statistics indicates that 28.8% of the marriages are among first cousins, and 49.3% of marriages are among members of the same clan.[25] Therefore, efforts are directed to encourage premarital testing. Nowadays there is a law implemented by the Palestinian Legislative Council, which states that premarital screening for thalassemia is a prerequisite for receiving a marriage license from the religious authority. Therefore, couples now have to undergo a series of required blood tests, which include mean corpuscular volume (MCV). In the case of an abnormal MCV that is lower than 76 fL, hemoglobin electrophoresis is requested to decide if couples are carriers or not. However, proper counseling following this initial screening is equally important and cannot be overlooked. If this action is strictly adopted, it is expected to lead to a dramatic decrease in the number of thalassemia major births

in the future. However, for couples already married who have a thalassemic child and plan to have more children, or those couples who insist on marrying even though they are carriers, prenatal diagnosis is the only means to prevent the birth of a thalassemic child.

First trimester prenatal diagnosis using CVS actually has several advantages over a second trimester procedure like amniocentesis, which include: reduced emotional and physical stress in the couples at risk; a less obvious pregnancy and therefore more privacy. Termination of pregnancy if indicated, can be carried out at a safer time both medically and religiously. According to Islamic law, elective abortion in life-threatening diseases is not permissible after 120 days of gestation (around the 17th week), when the soul emerges.[26] Therefore, prenatal diagnosis should be performed as early as possible to give time for the diagnostic processes that should be completed before the 17th gestational week (119 days). It was found that 73.8% of the tested prenatal samples are CVSs, which are drawn much earlier (9-12th week of gestation) than AF (16-18th week of gestation), which comprises 26.2%. This indicates an advantageous direction of the couples towards first trimester prenatal diagnosis in order to have a religiously accepted termination of pregnancy in the case of an affected fetus.

Each family whose fetus was found to be affected opted for terminating the pregnancy as soon as they got the result. Some of these families decided to carry out prenatal diagnosis a second time after the first resulted in an affected fetus. Actually, the acceptability and demand for the prenatal test was reflected by the number of times couples carried out prenatal diagnosis. Four couples underwent the prenatal test 4 times, another 4 couples carried out the test 3 times and 17 had the test twice. Interestingly, 5 pregnancies were for twins: in 2 pregnancies fetuses were found to be normal, in another one both were carriers, and in the remaining 2 pregnancies one of the fetuses was homozygous and the other twin was either normal or heterozygous. The affected fetus in each of the latter pregnancies was successfully aborted to leave only the normal and the carrier fetus.

The 88 couples who were able to contact our lab for prenatal diagnosis do not actually represent all the couples in West Bank and Gaza that are at risk, since the entrance for Jerusalem (where the lab is located) is hindered by many checkpoints. Since March 1993, the Israeli government imposed a general closure denying Palestinians from the West Bank and Gaza entrance to Jerusalem, free movement between the southern and northern part of the West Bank, and access to Jerusalem, thus depriving thousands of Palestinians from accessing medical, educational and economic services. Therefore, at least 5 couples who had an appointment to carry out the prenatal test could not reach the lab at the specific date and thus could not avail this facility. Moreover, some of the couples were given appointment to have a first trimester prenatal diagnosis, but due to closures, were postponed to a second trimester diagnosis.

In conclusion, in the West Bank and Gaza, where β-thalassemia is prevalent and health care resources are limited, prevention is the least expensive and most effective means of dealing with this disease. The prevention of β-thalassemia is actually advanced by the introduction of prenatal diagnosis. Our experience in this regard shows very good acceptability of the inflicted families for prenatal diagnosis and all families with affected fetuses decided to terminate the pregnancy, thus decreasing the number of thalassemia children born per year. The spectrum of mutations among the tested couples has several similarities to other neighboring countries. It is worth mentioning that the mutation -88 promoter is reported for the first time in our population. Moreover, the techniques used in diagnosis were convenient and we were able to diagnose all the specimens we worked on.

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