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CLINICAL & BASIC RESEARCH

Screening of Five Common Beta Thalassemia Mutations in the Pakistani Population

A basis for prenatal diagnosis

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تحري خمسة أنواع من طفرات ثالاسيميا (ب) في باكستان

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الملخص: الهدف: فقر دم البحر الأبيض المتوسط (ثالاسيميا) من أكثر اضطرابات المورث الأحادي السائد انتشارا في العالم، أعلى انتشار للمرض في «حزام الثالاسيميا» الذي يشمل منطقة البحر الأبيض المتوسط وبعض مناطق الشرق الأوسط وشبه القارة الهندية والأجزاء الجنوبية من الشرق الأقصى وباكستان وجنوب شرق آسيا، نهدف هذه الدراسة إلى اكتشاف التشوهات الجزيئية الشائعة لمتلازمة الثالاسيميا (ب) في باكستان. **الطريقة:** أجريت الدراسة في معهد الدم بجامعة باكي الطبية (كرانسي/باكستان) من أغسطس 2005 إلى نوفمبر 2007. أخذت عينات الدم من (400) مريضا مصابا بالثالاسيميا الكبرى (ب) من مراكز نقل الدم والمختبرات التشخيصية في مختلف مناطق كراتشي يمثلون الأجناس الخمسة **الكبيرة:** البنجابي . الباثان .السندي . البلوشي والأوردو. تم تحليل كل العينات للطفرات الشائعة الخمسة باستعمال تقنية سلسلة انزيم البوليميريز. **النتائج:** أظهرت النتائج خمسة طفرات شائعة وهي IVS 1-5(G→C), Fr 41/42(-CTTT), Fr 8/9 (+G), IVS 1-1 and Del 619. هذه تشمل (90%) من كل مورثات الثالاسيميا (ب) في باكستان. كان مورث ال{IVS 1-5(GRC)} الأكثر شيوعا عند الباكستانيين حيث مثل (44%) عند كل الأجناس الرئيسية . **الخلاصة:** ستساعد نتائج هذه الدراسة على تأسيس برنامج واسع النطاق لتشخيص حالات المرض خلال الحمل في الباكستان.

مفتاح الكلمات: ثالاسيميا (ب)، طفرات . شعب باكستان، أثناء الحمل.

ABSTRACT: ***Objectives:*** Thalassemia is one of the most common autosomal single-gene disorder worldwide. The highest prevalence of the disease is in the "thalassemia belt" which includes the Mediterranean region, parts of the Middle East, the Indian subcontinent, the southern parts of the Far East, Pakistan and South-East Asia. This study aimed to detect the common molecular abnormalities of the beta thalassemia syndrome in Pakistan. ***Methods:*** The study was conducted at the Institute of Hematology, Baqai Medical University, Karachi, Pakistan from August 2004 to November 2007. Blood samples of patients with beta thalassemia major (n = 400) were collected from hospital transfusion centres and diagnostic laboratories in different districts of Karachi representing five major ethnic groups including Punjabi, Pathan, Sindhi, Baluchi and Urdu speaking. All the samples were analysed for five common mutations by using the polymerase chain reaction technique ARMS (amplification of refractory mutation system). ***Results:*** The data revealed five common mutations including IVS 1-5(G→C), Fr 41/42(-CTTT), Fr 8/9 (+G), IVS 1-1 and Del 619. These accounted for 90% of the total beta thalassemia genes in Pakistan. The IVS 1-5(G→C) was found to be the most common beta thalassemia gene in the Pakistani population with a frequency of 44.4% present in all major ethnic groups. ***Conclusion:*** The results of this study will be helpful in the establishment of a large scale prenatal diagnosis programme in Pakistan.

Keywords: Beta thalassemia; Mutations; Pakistani population; Prenatal diagnosis.

Advances in knowledge

1. *This study shows that in the Pakistani population only five mutations are common and these account for 90% of beta thalassemia genes.*

Application to patients care

1. *This study could help in establishing an economical and cost effective large scale prenatal diagnosis programme in Pakistan by focusing on a limited number of mutations since the heterogeneity and complexity of the molecular abnormalities of the beta thalassemia syndrome make antenatal diagnosis difficult and expensive.*
2. *This study will be useful to stop the propagation of the beta thalassemia gene in the homozygous form that causes transfusion dependent beta thalassemia.*

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Thalassemia is the most common genetic disorder world wide.[1,2] More than 200 causative molecular defects have been described to date in the beta-globin gene causing beta thalassemia.[3,4,5] These are not uniformly distributed, but have geographic specificity, and each ethnic group has a few common mutations and a variable number of rare ones. Immigration plays a major role in both the distribution and the extent of mutation variations across the globe.[6,7] Beta thalassemia usually results from mutations that affect transcription, translation, or ribonucleic acid (RNA) stability.[8, 9,10]

Thalassemia is a serious public health problem, demanding the use of a preventive program with newborn screening in specific areas. To date, the highly heterogeneous distribution of molecular defects in the beta-globin gene has hampered the development of a flexible strategy that allows rapid analysis of mutations.[7]

Thalassemia occurs with a particularly high frequency in a broad belt extending from the Mediterranean basin through the Middle East, Indian subcontinent, Burma to South East Asia. [9,11,12,13,]

During the last 8-10 years, the amplification refractory mutation system (ARMS) and restriction fraction length polymorphisms (RFLP) were the principal techniques used for the diagnosis of beta thalassemia.[14] However, Pakistan, with a multiethnic population of around 160 million, represents a vast spectrum of beta thalassemia mutations. We report the application of the ARMS procedure for the detection of five common mutations in five major ethnic groups and its advantages in prenatal diagnosis of beta thalassemia.

Methods

The blood samples from patients with beta thalassemia major (n = 400) were collected in ethylenediaminetetraacetic acid (EDTA). The patients included in this study were all transfusion dependent and had been diagnosed earlier in life as beta thalassemia major with the help of basic haematological parameters, peripheral blood morphology and haemoglobin electrophoresis. Among the 400 beta thalassemia major patients 250 were males and 150 were females with a mean age of 13.5 years. All samples were tested by the modified method of amplification of refractory mutation system (ARMS)[14,15] for the five commonest mutations previously reported in population of the sub-continent.[16,17,18] The five mutated primer sequences which were used during this study were IVSI-1 (G→T), IVSI-5 (G→C), Del 619, Fr 41-42(-TTCT) and Fr 8-9 (+G). For the isolation of DNA, the blood samples were collected in EDTA and DNA was extracted from whole blood by following the kit protocol, using the Gentra Pure Gene kit (Minneapolis, Minnesota, USA).

The ARMS technique was used to characterise and screen the mutations as follows. After DNA extraction, polymerase chain reaction (PCR) reactions were set up in two separate tubes for each sample. The mutant primers, namely IVS 1-1(G→T), IVS 1-5 (G→C), Fr 41/42(-TCTT), Fr 8/9(+G) and Del 619 at the 3' end of the gene were distributed in two separate tubes according to their base pairs. The amplification with primers A and B served as internal controls in all the PCR reaction mixtures giving rise to 861bp fragments.

A total of 20µl final PCR reaction volume was used to measure the PCR condition. The reaction volume was composed of 0.5 micrograms of the DNA template, 10 pmol of each of the five primers (2 control primers, and 1 mutant ARMS primer for the reaction), 2.5 unit Taq DNA polymerase, and 0.2 mM of each deoxyribonucleotide triphosphate (dNTP) in a solution of 10 mM Tris-HC1, 50mM KCl and 1.2mM $MgCl_2$, (Promega, USA). The thermal cycling regimen consisted of 25 cycles; denaturation 94°C for 1 minute, primer annealing at 65°C for 1 minute and extension at 72°C for 1.5 minute with the final extension at 72°C for 3 minutes.

The electrophoresis condition was measured as follows. Fifteen microlitres of the PCR products were removed and mixed with 3 µL of a loading buffer and then loaded on a 2% agarose gel. The gel was set at 100 volts for 1 hour and then stained with ethidium bromide. After staining, the bands became visible under ultraviolet (UV) light. The different mutations were characterised with 100bp or 50 bp DNA ladders.

Approval for this study was obtained from the Ethical Committee of Baqai Medical University.

Table 1: Five commonest beta thalassemia mutations among the five main ethnic groups in Pakistan

Mutation	Punjabi	Pathan	Sindhi	Baluchi	Urdu Speaking	All
IVS-1-5 (G-C)	85 (48.6%)	45 (47.3%)	42 (36.5%)	35 (36.8%)	148 (61.6%)	355 (44.4%)
Fr-8/9 (+G)	30 (17.1%)	18 (18.9%)	40 (34.8%)	07 (7.4%)	22 (9.2%)	117 (14.6%)
Fr-41/42 (-TTCT)	42 (24%)	27 (28.4%)	15 (13%)	26 (27.4%)	30 (12.5%)	140 (17.5%)
IVS-1-1 (G-T)	12 (6.8%)	03 (3.1%)	05 (4.3%)	20 (21%)	20 (8.3%)	60 (7.5%)
Del 619	06 (3.4%)	02 (2.1%)	13 (11.3%)	07 (7.4%)	20 (8.3%)	48 (0.6%)
Total	**175**	**95**	**115**	**95**	**240**	**720 (90%)**

Results

In this study, we used ARMS for the detection of beta-thalassemia mutations, which is one of the most commonly used techniques for diagnosis of this disease. The analyses were carried out on 800 alleles by ARMS technique for the detection of five common mutations IVS-1 nt 5 (G→C), IVS-1 nt 1(G→T), Fr 41/42 (TCTT), Fr 8/9 (+G) and Del 619.

A total of sample of 400 homozygous beta thalassemia patients was analysed and among 800 beta thalassemia genes, 720 were identified with five common mutations [Table 1 and Figures 1a and 1b].

This study confirmed the presence of these five common mutations were: IVS 1-5(G→C), Fr 41/42 (-CTTT), Fr 8/9 (+G), IVS 1-1 and Del 619 comprising 90% of the total beta thalassemia genes in the Pakistani population [Table 1 and Figures 1a and 1b].

Molecular analysis in this study revealed that IVS-1-5 (G→C) was the most common beta thalassemic gene that was present in all major ethnic groups of Pakistan. This mutation comprised around 44.4% of the total beta thalassemia genes in Pakistan with a frequency of 48.6% in Punjabis, 47.3% in Pathans, 36.5% in Sindhis, 36.8% in Baluchis and 61.6% in the Urdu speaking population. Its prevalence in the Urdu speaking population is very high compared to the other four ethnic groups.

Fr 41/42c (-TTCT) was the second most common beta thalassemia gene in this study; it comprised 17.5% of the total beta thalassemia gene with a frequency of 24% in Punjabis, 28.4% in Pathans, 13% in Sindhis, 27.4% in Baluchis and 12.5% in the Urdu speaking population.

The third most common beta thalassemia gene observed during this study was Fr 8/9 (+G). It comprised 14.6% of the total beta thalassemia gene in the Pakistani population with frequency of 17.1% in Punjabis, 18.9% in Pathans, 34.8% in Sindhis, 7.4% in Baluchis and 9.2% in the Urdu speaking population.

Screening of the five common mutations of the beta thalassemia syndrome revealed IVS-1-1 (G→T) as the fourth most common mutation in the Pakistani population. This accounted for 7.5% of total beta thalassemic genes in Pakistan with frequency 6.8% in Punjabis, 3.1% in Pathans, 4.3% in Sindhis, 21% in Baluchis and 8.3% in the Urdu speaking population. These data revealed that the prevalence of IVS 1-1 (G→T) is high in the Baluchi population of Pakistan.

Del 619 was identified as the fifth commonest beta thalassemia gene in this study. It comprised 6% of the total beta thalassemia genes in our population with a frequency of 3.4% in Punjabis, 2.1% in Pathans, 11.3% in Sindhis, 7.4% in Baluchis and 8.3% in the Urdu speaking population. In the Urdu speaking group, the Del 619 mutation was present at high frequency in the Gujurati and Memon communities.

Discussion

Pakistan has a population of around 160 million people. The annual rate of population growth is 3% and almost 40% of the population is below 15 years of age.[16,20] There are five major ethnic groups: Sindhi, Urdu speaking, Punjabi, Baluchi and Pathan. The carrier frequency of beta thalassemia is estimated at around 6% in Pakistani population.[17] The results of the molecular analysis of the beta thalassemia

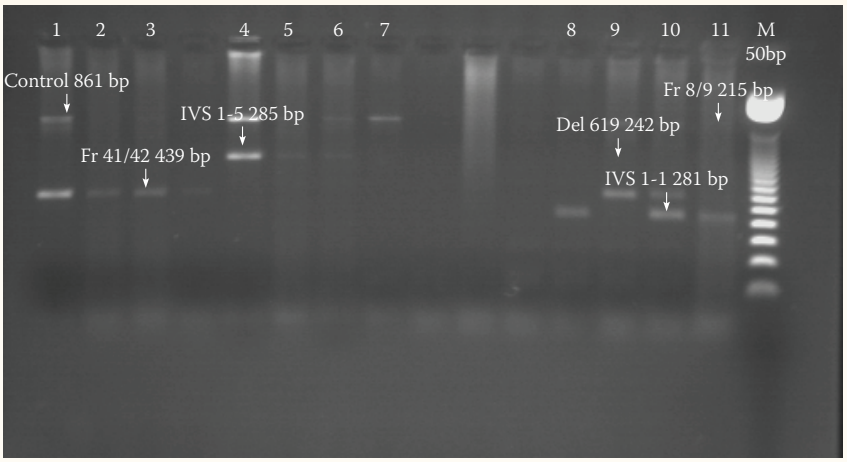

Figure 1a: The analysis of DNA sample with mutation primer IVS 1-5, frame 41/42 IVS 1-1 and frame 8/9Lane 1,2 and 3 are IVS 1-5; Lane 4,5 and 6 are Fr 41/42; Lane 7 shows normal control band ; Lane 8 is del 619, lane 9 and 10 is IVS 1-1 and lane 10 and 11 shows Fr 8/9.

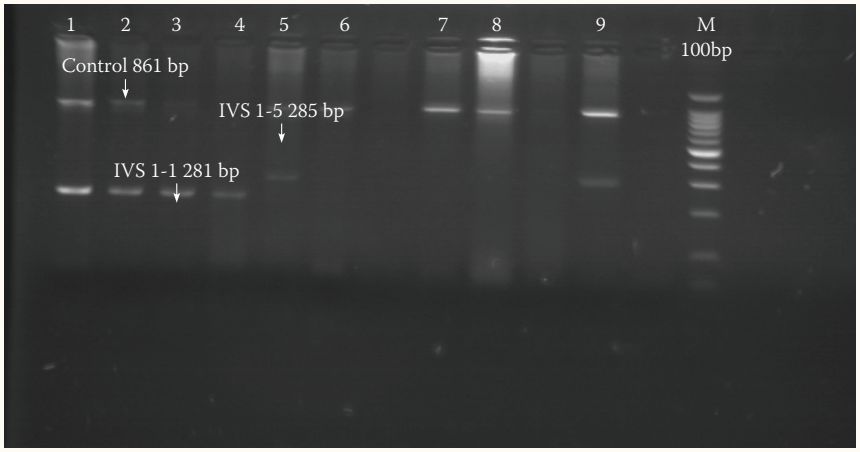

Figure 1b: The analysis of DNA sample with mutation primer IVS 1-5 and IVS 1-1 Lane 1, 2, 3 and 4 are IVS 1-1; Lane5 and 9 are IVS 1-5.

syndrome in these five major ethnic groups revealed five common mutations, which comprised 90% of the total beta thalassemia genes in the Pakistani population. These mutations included IVS-1-5 (G→C), Fr 8/9 (+G), Fr 41/42 (-TTCT), IVS-1-1 (G→T) and Del 619.

Although the five common beta thalassemia mutations reported during this study have already been detected and reported in Pakistani population by Ahmed *et al.* in 1996,[20] the most important difference between the previous study and the present one is that this study detected the frequency of these five common mutations. The data of this study reveal that the frequency of these five mutations is 90% in the Pakistani population while it was reported at around 82% in the previous study. In the light of present study, it is quite clear that these five mutations play a major role in propagation of the beta thalassemia gene in Pakistan as they comprised around 90% of the total beta thalassemia genes in this region. Results of this study showed the strong need for a large scale prevention program based on ante-natal diagnosis by PCR.

The spectrum of beta thalassemia mutations that was identified in the Indian population also showed the same five common mutations IVS-1-5 (G→C), Fr 8/9 (+G), Fr 41/42 (-TTCT), IVS-1-1 (G→T) and Del 619. These accounted for 93.6% of the total beta thalassemia genes in the Indian population.[17] Another exclusive study of molecular genetics of beta thalassemia in the Indian population also showed the same five common mutations i.e. IVS-1-5 (G→C), Fr 8/9 (+G), Fr 41/42 (-TTCT), IVS-1-1 (G→T) and Del 619 that accounted for 91.8% of the total beta thalassemia genes. These mutations were frequently encountered in various regions of India

including Southern, Eastern and Northern states.[18]

Similar patterns of molecular genetics of beta thalassemia syndromes in the Pakistani and Indian populations may have two basic causes. First, Pakistan and India was one state for several hundred years before partition in August 1947. Therefore cross-population and inter-linkage marriages were common. Second, there was large scale migration from India to Pakistan and from Pakistan to India during the Indo-Pak partition in 1947.

The spectrum of molecular genetics of the beta thalassemia syndrome in Pakistan and India also shows the influence of genetic distribution of beta thalassemia from the Mediterranean region. The exact cause of the influence of Mediterranean region in Indian and Pakistani people is unknown; geographical migration and a history of invasions of the subcontinent favour the influence across the borders.[19]

Studies of the molecular spectrum of beta thalassemia syndrome in the UAE (United Arab Emirates) revealed that IVS-1-5 (G→C) was the most frequent mutation in the UAE population.[21, 22]

A study of the molecular spectrum of beta thalassemia in Arab populations (Jordan, Egypt, Syria, Lebanon, Yemen and Saudi Arabia) revealed that the most frequent mutations were IVS-1-5 (G→C), IVS-II-1 (G→A), IVS-1-1, Fr 8/9, Fr 41/42, Cd 15, Cd 16, Cap +1 (A-C), IVS-1-110, IVS-1-3' end (-25 bp) and IVS-1-6.[23]

The close similarity in molecular genetics of the beta thalassemia syndrome in the Arab and Pakistani populations is likely to have arisen because of the influence of trading and invasions of Sindh and Pakistani Punjab by Arabs.

Conclusion

This research will help in the establishment of a large-scale prevention programme based on fetal diagnosis (prenatal) of beta thalassemia by DNA analysis (PCR) in the Pakistani population.

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