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ORIGINAL ARTICLE

Thalassaemia and Haemoglobin Disorders In the Khuzestan Province of Iran

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Abstract

Background and Aim: : In prevalent regions, thalassaemias often coexist with a variety of structural Hb variants, giving rise to complex genotypes and an extremely wide spectrum of clinical and haematological phenotypes. Haematological and biochemical investigations and family studies provide essential clues to the different interactions, and are fundamental to DNA diagnostics of the Hb disorders

Material and Methods: : A careful three tier approach involving: (1) Full blood count (2) Special haematological tests, followed by (3) DNA mutation analysis, provides the most effective way in which primary gene mutations as well as gene-gene interactions that can influence the overall phenotype, can be detected. In Iran, there are many different forms of α and β thalassaemias. Increasingly, different Hb variants are being detected, and their effects per se, or in combination with the thalassaemias, provide additional diagnostic challenges.

Result: We did a step-by-step diagnostic workup on 800 patients of haemoglobinopathies who were referred to the Research center of Thalassemia and Haemoglobinopathies at the Shafa Hospital of Ahwaz, Joundishapour University of Medical Sciences, respectively. We detected 173 patients with Iron Deficiency Anaemia and 627 individuals as Thalassaemic patients by use of different indices. We detected 75 %(472/627) of the β-thalassaemia mutations by using the amplification refractory mutation system (ARMS) technique, 19 %(130/627) of the α-thalassaemia mutations by using the Gap-PCR technique, and 6 %(25/627) of the Hb variants by the Hb electrophoresis technique successfully.

Conclusion: Almost all haemoglobinopathies can be detected with the current PCR-based assays, with the exception of a few rare deletions. The knowledge of α and ß-gene numbers in the α and ß-thalassaemia traits of any population is necessary, as it modifies the phenotype of thalassaemia by altering the ratio of the α and ß -chains of haemoglobin.

Keywords: α and β- thalassaemia, Haemoglobin variants, Iron Deficiency Anaemia (IDA), Gap-Polymerase Chain reaction (Gap-PCR).

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Introduction

Haemoglobinopathies are inherited disorders of globin, which is the protein component of haemoglobin (Hb). Haemoglobinopathies are the commonest genetic defect worldwide, with an estimated 269 million carriers [1]. Iran, a

country 1,648,000 km^2 wide, like many other countries in the region, has a large number of thalassaemia patients [2]. Thalassaemias are the commonest monogenic diseases worldwide. Mutations in genes coding for the globin proteins that alter protein output, produce the thalassaemia syndromes. Mutations in the globin genes that lead to abnormal proteins are called variant Hbs [3]. More than 23 different molecular defects have been identified for α –thalassaemia, till date. In the case of ß-thalassaemia, the presence of over 150 various known mutations, is even more perplexing [4]. Thalassaemia is found in some 60 countries with the highest prevalence in the Mediterranean region, parts of North and West Africa, the Middle East, the Indian subcontinent, Southern Far East and Southeastern Asia, together composing the so-called thalassaemia belt. In western countries, thalassaemia mostly affects individuals whose ancestries are traceable, to high prevalence areas [5-8]. Each population-at-risk, however, has its own spectrum of common mutations, usually from five to ten; a finding that simplifies mutation analysis, and thus, determines the origin of the mutant genes. For the purpose of this study, we studied the mutation of the important globin genes in humans, i.e the 2 β globin genes and the 4 α globin genes.

Material and methods

Sample collection*:* Blood samples of 2mL each, were collected in EDTA vials and in plain vials from 800 patients (4ml per patient) referred to Research Center of Thalassaemia and Haemoglobinopathies (RCTH), which is the only center working on haematology and oncology in the southwest (Khuzestan) region of Iran. The duration of the study was from Feb 2005 to August 2007.

Full Blood Count*:* In all these patients, serum ferritin levels (and if necessary serum iron, iron binding capacity and percentage saturation) were sought. This is recommended, because at times, particularly during pregnancy, it is possible that iron stores will be low or, in the presence of iron deficiency, it is possible that the MCV or MCH are influenced by the iron deficiency. It is also occasionally seen that the HbA_2 level can be falsely lowered by iron deficiency. We attempted to differentiate β–thalassaemia from Iron Deficiency Anaemia with the help of discriminate indices like Mentzer Index [9], England and Fraser Index [10], Srivastava Index [11], Green and King Index [12], Shine and Lal Index [13], red blood cell (RBC) count, red blood cell distribution width index (RDWI) [14], Mean Density of Haemoglobin per Liter of blood (MDHL) and Mean Cell Haemoglobin Density (MCHD) [15]. If iron deficiency is present, it is essential to correct this, and then repeat the full blood count and all other investigations. . We detected 173 patients to have IDA, and the rest to have thalassaemia disorders.

Special Haematological Tests: We screened all patients by the above mentioned tests, and compared all data in order to classify them. It is appropriate to order all tests simultaneously, since they take time to do, and often information can be obtained from the tests that might not have been seen to be relevant, when first starting off the investigation of a haemoglobinopathy. Some of the tests are technically demanding, and so the person ordering the tests should have some knowledge of the laboratory's technical skills, as well as experience in interpreting results. Other special haematological tests performed, particularly when investigating the more uncommon variant Hbs, were tests for oxygen affinity, haemoglobin stability and detection of methaemoglobin. Mass spectrometry has been used to characterise various variant Hbs [17]. The latter approach might be very valuable for population screening, but not for detection involving individual cases. DNA based approaches remain the methods of choice. We compared values of HbA, HbA2, HbF, and Hb variants to confirm our findings, respectively. We found that all of the 627 patients (130 patients suspected to α-thalassaemia and 472 patients suspected to β-thalassaemia) were having thalassaemia. Also, we detected 23 individuals who have HbS, HbD^{panjub}, and two cases were suspected to Hb Lepore [Table/Fig 1]. Later on the entire β-globin gene was sequenced using direct mutation analysis by nucleotide base sequencing to confirm Hb Lepore status. Finally, we have found that in both cases, the presence of Hb Lepore was ruled out. We also compared values of HbA, HbA2, HbF, and Hb variants to confirm our findings, respectively.

Sources of DNA and DNA preparation*:* The main source of DNA is peripheral leucocytes obtained from anticoagulated peripheral blood,,

preferably with ethlenediaminetetraacetic acid (EDTA). Foetal DNA is mainly isolated from chorionic villi obtained through ultrasound-guided transcervical aspiration or ultrasound-guided transabdominal aspiration. Foetal DNA can also be prepared from amniotic fluid cells directly, or after culture. It is prudent to set aside a few milliliters for culture as a back-up, as the DNA yield from amniotic fluid cells is often minimal, but sufficient for PCR-based analysis. Noninvasive methods of prenatal diagnosis utilize DNA from foetal cells in the maternal circulation [20], or free circulating foetal DNA in maternal blood [21]. The noninvasive methods, however, are still under development, and are not offered routinely for the haemoglobinopathies.

Allele-specific priming – ARMS-PCR*:* Primer-specific amplification is based on the principle that a perfectly matched primer is much more efficient at annealing, and directing primer extension than a mismatched primer. The most widely used method is the ARMS [20, 21], in which allele-specific amplification relies on the specificity of the 3' terminal nucleotide. To enhance allele specificity, it is a common practice to deliberately incorporate a second mismatch at position −2 or −3 from the 3' end. The target DNA is amplified in two separate reactions using a common forward primer, and either one of two reverse allele-specific primers, one complementary to the mutant sequence, the other to the normal DNA sequence. The presence of the mutant allele will generate a PCR product in the tube containing the mutation-specific primer, and vice versa. To monitor false-negative results because of failure of the amplification reaction itself, an internal PCR control which amplifies another region of the genome, should be included in the reaction. ARMS-PCR has the advantage that it is theoretically possible to detect any known mutation [20]. Recently, the methodology has been improved by development of a single tube assay [22], where both the mutant and the wild type alleles are detected simultaneously in the same reaction with an internal positive control, referred to as tetra primer ARMS-PCR.

We analyzed all 472 patients who were suspected to have β-thalassaemia by direct mutation analysis of the β-globin gene by ARMS [16],[17], and found various types of mutations in 385 out of them [Table/Fig 2]. We could not detect the mutation in the rest of the 87 patients by this technique, which means that the technique was helpful to detect approximately 75 %(472/627) of the mutations.

zGap- PCR*:* The common *α*-thalassaemia deletions and rearrangements can be routinely detected using gap-PCR [18,19,20]. Gap-PCR is also routinely used to detect Hb Lepore, a variant created by deletion of 7 kb, and results in a functional hybrid delta/beta globin product [7]. In contrast to *β*-thalassaemia in which mutations are predominantly caused by point mutations in the structural genes, *α*-thalassaemia is more often caused by deletions involving one or both of the *α*-globin genes [26]. A small number of point mutations, usually within the *α*2 gene, have been characterized. Rare deletions removing the upstream *α*-globin regulatory element have also been described. The primers for these deletions can be multiplexed in various combinations to capture the deletions that are most likely to be encountered in the different catchment areas. The multiplex set encompassing gap-PCR primers for $/--^{SEA}$,$/--^{MED}$,$/--(\alpha)^{20.5}$,$/--^{FIL}$$/-\alpha^{3.7}$ and$/-\alpha^{4.2}$ is used by many laboratories supplemented by another set for identification of triple *α* complex (/*ααα*), useful in the work-up of thalassaemia intermedia. Gap-PCR primers are also available for the Thai *α*° deletion ($/--^{THAI}$), and can be included in the multiplex panels. Multiplex gap-PCR requires careful optimization, but once optimized, is highly efficient and rapid for the detection of these common *α*° and α^{+}-thalassaemia deletions, particularly when performed in large batches in microtitre plates.

There were 130 patients who were suspected to have α-thalassemia, based on haematological tests in our study. So to confirm that, we analyzed the α-globin gene deletion by Gap-PCR technique [18],[19],[20], and found 6 types of mutations in 98 individuals out of 130.

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The α/β globin protein ratio

Another useful specialised test helpful in confirming a thalassaemia, and to confirm whether it is due to the α or β globin gene problem, is the α/β globin protein ratio method [21]. This requires the incubation of red blood cells with a radioactive tracer such as H^3-leucine. The peaks representing the α and β globin proteins are then quantitated to provide the α/β ratio, which should equal 1.0. A ratio > 1.0 indicates β thalassaemia, while a ratio <1.0 is caused by α thalassaemia. Although a useful

test in some cases, the α/β ratio is no longer routinely available. This has occurred because DNA testing has essentially become the strategy of choice for testing, and so few α/β ratios are now requested for those laboratories who do not have sufficient experience. The requirement for fresh radioactive material is another disincentive for setting up this assay. It should also be noted that the α/β ratio may not be particularly helpful if gene-gene interactions are occurring, for example, when both α and β thalassaemia are co-inherited.

We used the α/β globin protein ratio method to identify mutations in some individuals who couldn't be detected by previously defined methods. We detected 6 individuals, with three of them having values less than 1.0, and by further investigation using molecular analysis techniques confirmed them as β – thalassaemia affected cases with heterozygous patterns. Three out of six cases had values more than 1.0, and were considered as α-thalassaemia cases, which by the help of further investigation using molecular analysis techniques, were confirmed as α – thalassaemia affected cases with heterozygous patterns.

Table/Fig 1. Hb electrophoresis data of 23 out of 513 β-thalassemic patients showing Hb variants.

Case	HbA(%)	HbA2(%)	HbF(%)	Hb D punjab(%)	Hb S(%)	Hb C
1	74.6	2.3	1.4	21.7	---	---
2	74.4	2.3	0.9	---	22.4	---
3	62.7	2.6	0.2	---	34.5	---
4	73.7	3.2	0.0	---	23.1	---
5	95.9	1.7	0.5	---	---	---
6	57.4	2.9	0.3	---	---	---
7	62.7	4.2	0.5	---	32.6	---
9	67.5	3.5	0.8	---	28.2	---
10	44.8	0.5	2.9	---	51.8	---
11	69.1	1.5	1.8	37.6	---	---
12	57.5	2.4	0.6	---	39.5	---
13	59.1	1.7	0.1	---	39.1	---
14	61.1	0.3	0.0	38.6	---	---
15	53.1	1.9	0.1	44.9	---	---
16	67.6	3	1.4	---	28	---
17	66.5	2.5	1.0	---	30	---
18	57.8	3.1	0.4	39.9	---	---
19	56	1.6	0.7	41.7	---	---
20	54.1	2.5	0.7	---	42.7	---
21	60.9	1.9	0.6	---	35.6	---
22	55.4	0.9	0.4	---	43.3	---
23	58	1.3	0.7	40	---	---
24	58.4	2	0.2	---	39.4	---
25	53	2	1	---	---	44

Results

We did a step-by-step diagnosis workup in 800 patients with haemoglobinopathies, who were referred to the Research center of Thalassaemia and Haemoglobinopathies at the Shafa Hospital of Ahwaz, Joundishapour University of Medical Sciences, respectively. We detected 173 patients to have IDA, and the rest to have haemoglobinopathies, with the help of the above techniques. Also, we found that all of the 627 patients (130 patients suspected to α-

Table/Fig 2: Frequencies of beta-thalassemia mutations in study group

mutation	Number of patient	Frequency (%)	genotype
IVS II – 1(G→C)	96	20	β°
CD 36/37(-T)	63	13.5	β°
IVS I -110(G→A)	45	9.5	$β^+$
CD 6(HbS)	36	7.5	$β^S$
CD 8/9(+G)	22	4.5	β°
IVS I-6(T→C)	21	4	$β^+$
CD 8(-AA)	16	3	β°
CD 44(-C)	14	2.5	β°
IVS II-745(C→G)	13	2.4	$β^+$
CD 5(-CT)	12	2.3	β°
IVS I-5(G→C)	11	2.1	β°
CD 39(C→T)	11	2.1	β°
IVS I-(3' end)-25bp	9	1.5	β°
IVS I-1(G→A)	7	1.3	β°
CD 22(GAA→TAA)	5	1	β°
IVS I - 2(T→C)	4	0.8	β°
-88(C → A)	3	0.6	$β^+$
-30(T→A)	2	0.4	$β^+$
IVS I - 130	2	0.4	β°
IVS I - 116	2	0.4	β°
IVS II-2,3(+11,-2)	1	0.2	$β^+$
Unknown	87	19.8	---
Total	472	100	---

Table/Fig 3: Frequencies of alpha-thalassemia mutations in study group

mutation	Number of patient	Frequency (%)
$– α^{3.7}$	82	63
$– α^{5NT}$	5	4
$- -^{MED}$	2	2
$- -^{MEDII}$	1	0.8
$– α^{4.2}$	4	3
$– α^{PA}$	1	0.8
$- -^{MED}/– α^{3.7}$	3	1.4
Unknown	32	25
Total	**130**	**100**

Table/Fig 4: It shows summery of the hematological data and α/β ratio in the study group.

Case	Age	RBC	MCV	MCH	Hb	α/β ratio	Diagnosis
1	29	6.77	72	21.4	14.3	0.51	α-tha
2	25	6.07	69.9	21.3	12.9	0.55	α-tha
3	29	5.86	79	23.3	11.9	1.08	β-thal
4	22	5.07	68.5	22.6	10.4	1.1	β-thal
5	28	5.79	74	24	13.9	0.83	α-tha
6	28	5.53	84.7	28.5	15.7	1.06	β-thal

thalassaemia and 472 patients suspected to β-thalassaemia) were having thalassaemia. Among the rest of the 25 cases, we detected 23 individuals who had HbS, HbD^{punjab}, and the remaining two cases were suspected to have Hb Lepore [Table/Fig 1]. Later on, the entire β-globin gene was sequenced using direct mutation analysis by nucleotide base sequencing to confirm Hb Lepore status. Finally, we have found that the presence of Hb Lepore in both cases was ruled out. The most predominant β-thalassaemia mutation that we found, was IVS II – 1(G→C) (20 %), followed by many other less frequently known mutations for the Southern part of Iran, listed in [Table/Fig 2]. There were 130 patients who were suspected to have α-thalassaemia, based on haematological tests in our study. So, for confirming that, we analyzed the α-globin gene deletion by Gap-PCR technique, and found 6 types of mutations in 98 individuals out of 130. The most predominant one was $– α^{3.7}$ (62 %) followed by $- -^{MED}$, $– α^{5NT}$, $- -^{MEDII}$, $– α^{4.2}$, $– α^{PA}$, and $- -^{MED}/– α^{3.7}$ (HbH) for the Southern part of Iran, listed in

[Table/Fig 3]. We detected 6 individuals, with three of them showing values less than 1.0, and by further investigation using molecular analysis techniques, confirmed them as β – thalassaemia affected cases with heterozygous patterns. Three out of six cases were showing values more than 1.0, and were considered as α-thalassaemia cases, which with the help of further investigations using molecular analysis techniques, were confirmed as α – thalassaemia affected cases with heterozygous patterns [Table/Fig 4].

Discussion

Among all the haemoglobinopathies, Alpha and Beta thalassaemias are the commonest. Almost all haemoglobinopathies can be detected with the current PCR-based assays, with the exception of a few rare deletions. The complex mutational spectrum of the haemoglobinopathies, especially relevant in a multi-ethnic community, requires a method with the capacity to scan the *β* (and/or *α*) globin genes rapidly and accurately for all mutations. The commonest type of β –thalassaemia seen in Iran, is $-\alpha^{3.7}$ deletion. Hadavi et al[22] has reported the prevalence of $-\alpha^{3.7}$deletion in the population of Iran as 30.2%.Our data showed the prevalence of- $\alpha-^{3.7}$ deletion in 20% in the population of the Southwest (Khuzestan) region in Iran. In our study, we found only two cases of $-\alpha^{4.2}$ deletion and few cases of any other deletions reported so far. Hadavi et al., [22] have reported $-\alpha^{4.2}$ deletion in Iranian subjects with a prevalence of 3.5%. Najmabadi et al., [23, 24] has reported the prevalence of IVS-II-I (G —> A) β-thalassemia mutation of 34 % in the population of the Southwest region of Iran. In our study, we found that the most frequent mutations were CD 36/37, IVS II-I, and IVS I-110, followed by other cases of any other mutations reported so far. Our study showed that the most frequent mutations were the single-gene deletion ($-\alpha^{3.7}$) in Iranian subjects, which is also coherent with other studies. The knowledge of α and ß-gene numbers in α and ß-thalasssemia traits in any population, is necessary, as it modifies the phenotype of thalassaemia by altering the ratio of the α and ß - chains of haemoglobin .

Conclusion

The compactness of the globin genes means that haemoglobinopathy detection is largely a PCR-based approach that can utilize direct sequencing analysis. Almost all haemoglobinopathies can be detected with the current PCR-based assays, with the exception of a few rare deletions. However, the molecular diagnostic service is still under development to try and meet the demands of the population it serves. A higher throughput approach will be required to meet practice pressures and the increasing needs set by the antenatal screening programme. This increasing workload dictates increasing automation, which may necessitate the use of automated robotic platforms to prepare samples and reactions, and the use of automated platforms to perform the actual detection. In most populations, the *β*-thalassaemias (and related haemoglobinopathies) are clinically more relevant than the *α*-thalassaemias. The complex mutational spectrum of the haemoglobinopathies, especially relevant in a multi-ethnic community, requires a method with the capacity to scan the *β* (and/or *α*) globin genes rapidly and accurately for all mutations. This aspect is being addressed by the development of arrays. Although they are still in their infancy, the arrays hold great promise, and are amenable for scaling up and automation. In the short term, the current generation of instruments such as the capillary electrophoresis systems, has greatly simplified DNA sequence analysis. The capillary electrophoresis system also lends itself to the multiplexed mini-sequencing methodology, which is highly suitable for screening for the common globin gene mutations.

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