Original Article

GENOTYPING OF THALASSEMIA IN MICROCYTIC HYPOCHROMIC ANEMIA PATIENTS FROM SOUTHWEST REGION OF IRAN

Fakhar Rahim[1]

ABSTRACT

Objective: To evaluate the frequency of α-gene, ß-gene, and hemoglobin variant numbers in subjects with Microcytic hypochromic anemia.

Methodology: In total out of 850, 340 subjects with microcytic hypochromic anemia [MCV<80fl; MCH<27pg] from Southwest part of Iran, were studied in Research Center of Thalassemia and Hemoglobinopathies (RCTH) which is the only center working on hematology and oncology in Southwest (Khuzestan) region of Iran. These include 325 individuals: 171 with Beta-thalassemia trait, 88 with Alpha-thalassemia trait, 13 with thalassemia major, 11 with hemoglobin variants (HbS, HbC, and HbD ^{Punjab}) and 42 with iron-deficiency anemia. The rest 15 patients diagnosed with no definite etiology.

Results: Genotyping for $-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{PA}$, $--^{5NT}$ and $---^{MED}$ was done with gap-PCR. The overall frequency of $--^{3.7}$ deletion in 325 individuals is 20%. Genotyping for 23 most known ß-gene mutations was done with direct mutation analysis by Amplification Refractory Mutation System (ARMS). The most frequent mutations were CD 36/37, IVS II-I, and IVS I-110 with 9.7%, 11.7%, and 3.5% respected frequencies in 340 patients. There was statistically significant difference between Beta-thalassemia trait and Beta-thalassemia Major in case of MCV (p- value = 0.25) and MCH (P–value =0.23) indices, and also MCH index between Beta-thalassemia trait and Hb Variants (P-value = 0.04).

Conclusion: The α-gene and ß-gene mutation is quite common in the Southwest part of Iran. Molecular genotyping of α-thalassemia and ß-thalassemia help to diagnose unexplained microcytosis, and thus prevent unnecessary iron supplementation.

KEY WORDS: Alpha-gene deletions, Beta-gene mutations, Evaluation of anemia, Microcytic hypochromic anemia, Southwest part of Iran.

Pak J Med Sci January - March 2008 Vol. 24 No. 1 23-28

1. Fakher Rahim,
M.Sc Bioinformatics,
Research Center of Thalassemia & Hemoglobinopathies,
Physiology Research Center,
Ahwaz Jondishapur University of Medical Sciences,
Ahwaz – Iran.

Correspondence

Fakher Rahim
E-mail: fakherraheem@yahoo.com

* Received for Publication: September 17, 2007

* Revision Accepted: November 3, 2007

INTRODUCTION

Iran, a country 1,648,000 km wide, has a large number of thalassemia patients like many other countries in the region.[1] Iranian population is a mixture of different ethnic groups, frequency and distribution of ß-globin mutations in various regions of the country need to be clarified. In Iran, with a remarkable prevalence of α and ß- globin mutation, the increased like-

lihood of co-inheritance of α- and ß-thalassemia may result in a large variety of phenotypes.[2] ß-thalassemia is very rare in Iran. The gene frequency of ß-thalassemia, however, is high and varies considerably from area to area, having its highest rate of more than 10% around the Caspian Sea and Persian Gulf. Unlike ß-thalassemia trait and iron deficiency, no simple biochemical test can detect α-thalassemia. There is a paucity of data on α and ß -genotyping in Iran. The key to successful detection and characterization of the hemoglobinopathies, particularly the thalassemias, is the initial hematological data. The clue for a thalassemia comes with a low mean corpuscular volume (MCV) or mean corpuscular hemoglobin (MCH). Although iron deficiency is the other explanation for a low MCV or MCH, it is likely that this finding will point to thalassemia in regions of countries with at-risk ethnic populations.

There are several causes of the anemia produced by different abnormal hemoglobins. In general the anemia is both dyserythropoietic as well as hemolytic. In some, like HbS the cause is apparent as lowering of oxygen tension leads to a process known as 'sickling' of the red cells making them more susceptible to destruction by the spleen. Red cell indices are taken to be the differentiating factor in anemias due to thalassemias and from iron deficiency and are very necessary as both occur in the same areas. Relative increase in red cell count in thalassemias gives reduced MCH and MCV values. Microcytic hypochromic anemia is a common hematological abnormality in clinical practice and usually is caused by iron deficiency and thalassemia trait.

The degree of microcytosis and type of mutation in thalassemias had shown wide variation between ranges of MCV. There is limited data reported in the literature for prevalence of α-gene deletion in patients with microcytosis.[3,4] Carriers of α-gene deletion have mild microcytosis with or without anemia. Although anemia is absent or unremarkable, it is important to diagnose ß-thalassemia in order to diagnose the cause of microcytosis and to avoid repeated expensive analysis and/or prolonged iron therapy. The co-existence of α-deletions in ß-thalassemia patients modifies the phenotype. So far no biochemical diagnostic test is available for detection of α-thalassemia carriers. Globin chain synthesis studies are time-consuming and expensive, and require radioactive amino acids for analysis. Molecular methods, like Southern blot hybridization, and sequencing, are conventionally used for diagnosis of α-thalassemia. However, molecular diagnosis by PCR has proven to be less time-consuming and less expensive, with a more definite outcome at clinical setup.[5,6] The most widely used method in detection ß-thalassemia' mutation is the Amplification Refractory Mutation System (ARMS).[7,8] Unlike the ß-thalassemias, deletions are a common cause of α-thalassemia. The common α-thalassemia deletions and rearrangements can be routinely detected using gap-PCR.[9-11] The present study was aimed to identify the prevalence of thalassemia mutation and hemoglobin variants in unexplained microcytic anemia cases and its utility in clinical practice, with special reference to genetic counseling.

METHODOLOGY

Blood samples of 2mL each were collected in EDTA vials and in plain vials from 850 patients (4ml per patient) referred to Research Center of Thalassemia and Hemoglobinopathies (RCTH) which is the only center working on hematology and oncology in Southwest (Khuzestan) region of Iran, between January 2004 and April 2007. Written consent was obtained from all the patients before collecting the blood, which is a part of ethical clearance from the institute. Red cell indices were measured on an automated cell counter (Sysmex Kx-21, Japan). Hb A2 and Hb F were measured in the Hb Variant [Bio-rad USA] system by using the thalassemia short program. The serum iron and total iron binding capacity (TIBC) were measured by using kits (Span Diagnostics Ltd.) and percent saturation was calculated

Table-I: Prevalence of α–genotype in various á-thalassemia subgroups of microcytic hypochromic anemia

Group	*Alpha - genotypes*	*Numbers (% prevalence)*	*Homozygote*	*Heterozygote*	*Compound heterozygote*
1	αα /-α$^{3.7}$	64(72.7)	4	60	0
2	αα /-α$^{4.2}$	1(1.1)	0	1	0
3	αα /-α^{PA}	1(1.1)	0	1	0
4	αα /- -^{MED}	6(6.8)	0	6	0
5	αα /-α^{5NT}	3(3.4)	1	2	0
6	*HbD and* αα /-α$^{4.2}$	1(1.1)	0	0	1
7	*HbD and* αα /-α$^{3.7}$	1(1.1)	0	0	1
8	*HbC and* αα /-α$^{3.7}$	1(1.1)	0	0	1
9	*Unknown*	10(13.9)	4	6	0
10	*Total (% prevalence)*	88(100)	9(10.2)	76(86.3)	3(3.5)

accordingly. The patients with iron saturation <16% were considered iron-deficient.

Following the initial evaluation, 340 samples were found as microcytic [MCV < 80 fl] hypochromic [MCH< 27 pg mL–1] anemia and subjected to a thalassemia study. This included 171 with Beta-thalassemia trait, 88 with Alpha-thalassemia trait, 13 with thalassemia major, 11 with hemoglobin variants (HbS, HbC, and HbD), 42 with iron-deficiency anemia, and 15 patients with no definite etiology, listed in (Table-I). For á and ß genotyping, genomic DNA was prepared from peripheral blood by the standard phenol chloroform extraction method. The protocol followed for the detection of –α$^{3.7}$ deletion, -α$^{4.2}$ deletion was followed as described by Agarwal et al.[6] For detection of other deletions Liu et al,[12] method was followed. The amplified products were electrophoresed on 1.5% agarose gel (Sigma) and stained with ethidium bromide. We analyzed all 88 patients who were suspected of having α-thalassemia by direct mutation analysis of α-globin gene by gap-PCR and found the type of mutation, which are listed in (Table-II).

We analyzed all 171 patients who were suspected of having ß -thalassemia by direct mutation analysis of ß -globin gene by Amplification Refractory Mutation System (ARMS) and found the type of mutation, which are listed in (Table-III). Documentation of the results was done on the gel documentation system (Alpha Innotech Corporation. USA). Statistical analysis was carried out by Statistical

Table-II: Hematological parameters in different groups with microcytic hypochromic anemia

Group (No. of cases)	*Hb mean ± SD (Range)*	*MCV mean ± SD (Range)*	*MCH mean ± SD (Range)*
Beta-thalassemia Trait(171)	9.53 ± 1.43 (5.60 – 12.10)	62.9 ± 5.3 (49 – 78.90)	20.03 ± 1.80 (15 – 26)
Beta-thalassemia Major(13)	7.5 ± 1.34 (5.20 – 9.28)	71.6 ± 5.20 (63 – 79)	22.9 ± 2.1 (20.9 – 25.6)
Iron deficiency(42)	7.75 ± 2.05 (4.3 – 12.95)	69.35 ± 6.95 (52.0 – 77.8)	17.52 ± 2.84 (12.10 – 23.12)
Alpha-thalassemia Trait(88)	11.1 ± 1.25 (9.20 – 12.25)	73.6 ± 4.67 (60 – 79)	23.9 ± 1.82 (19 – 26.20)
Hb Variants(11) *	12.1 ± 2.63 (7.8 – 15.20)	73.9 ± 4.4 (66 – 79)	23.5 ± 1.65 (21 – 26.5)

* includes Hemoglobin S(HbS), Hemoglobin C(HbC), and Hemoglobin D ^{Punjab} (HbD $^{Punja)}$

Package for Social Sciences version 11.5 and an independent-sample *t* test was used for comparison of hematological parameters. Genotypic and allelic frequencies were also calculated.

RESULTS

The initial analysis of hematological parameters was done on 850 samples. Of these, 340 samples displayed microcytosis [MCV < 80 fl] and hypochromia [MCH < 27 pg]. In 283 samples, hemoglobinopathy was found. Of the remaining 57 samples, 42 showed iron-deficiency anemia, which diagnosed by percentage of saturation technique. However, no definite etiology was confirmed in 15 patients. All 340 samples were analyzed for α-gene number by GAP-PCR. The frequency of $-\alpha^{3.7}$ deletion in various groups is shown in Table-II. Among the 340 patients with microcytic hypochromic anemia, 60 patients had heterozygous $-\alpha^{3.7}$ deletion [$-\alpha^{3.7}$ / αα] whereas 4 patients had homozygous -α3.7 deletion [$-\alpha^{3.7}/-\alpha^{3.7}$]. The carrier status in these 340 patients was 20%. Allele frequency for $-\alpha^{3.7}$ deletions is calculated as 0.10 (70 in 680 chromosomes). All 340 samples were analyzed for ß-gene number by Amplification Refractory Mutation System (ARMS). The frequencies of different detected ß-gene mutations are shown in Table-III. The most frequent mutations were CD 36/37, IVS II-I, and IVS I-110 with 9.7%, 11.7%, and 3.5% related frequencies in 340 patients. Hematological parameters of patients in different groups are shown in Table-I. There was statistically significant difference between Beta-thalassemia trait and Beta-thalassemia Major in case of MCV (p- value = 0.25) and

Table-III: Prevalence of ß–thalassemia in various ß-thalassemia subgroups of microcytic hypochromic anemia

Group	*Beta - genotypes*	*Numbers (% prevalence)*	*Homozygote*	*Heterozygote*	*Compound* heterozygote
1	CD 36/37	33(17)	3	30	0
2	IVS II-I	40(20.6)	3	37	0
3	$ß^S$ (CD6 GAG ⟶ GTG)	7(3.6)	0	7	0
4	HbD^{Punjab} : ß(CD121 G ⟶ C)	3(1.5)	0	3	0
5	IVS I-6	5(2.5)	5	0	0
6	CD 8(-AA)	7(3.6)	1	6	0
7	IVS I - 25	7(3.6)	0	7	0
8	- 88 (C ⟶ A)	3(1.5)	0	3	0
9	CD 8/9	3(1.5)	0	3	0
10	IVS I - 110	12(6.1)	1	11	0
11	IVS II - 745	7(3.6)	0	7	0
12	IVS I - 5	5(2.5)	0	5	0
13	CD 83 (-G)	1(0.5)	0	1	0
14	CD 44	8(4.1)	0	8	0
15	IVS II – 2,3(+11,2)	1(0.5)	0	1	0
16	CD 5	3(1.5)	0	3	0
17	IVS I – I	3(1.5)	0	3	0
18	CD 39	5(2.5)	0	5	0
19	IVS I – 130	1(0.5)	0	1	0
20	CD44/CD36/37	1(0.5)	0	0	1
21	HbC/IVS II - I	1(0.5)	0	0	1
22	IVS I – 130/IVS I - 25	1(0.5)	0	0	1
23	IVS II – I / IVS II - 745	1(0.5)	0	0	1
24	Unknown	36(20.8)	10	26	0
25	Total (% prevalence)	194(100)	23(11.8)	167(86)	4(2.2)

MCH (P –value =0.23) indices, and also MCH index between Beta-thalassemia trait and Hb Variants (P-value = 0.04). There was no statistically significant difference in red-cell indices between the rests of hemoglobinopathies.

DISCUSSION

Alpha and Beta-thalassemia are the commonest single-gene hemoglobin disorder in the world.[13-15] The commonest type of ß- thalassemia seen in Iran is $-\alpha^{3.7}$ deletion. At our hospital the overall frequencies of ß-thalassemia and ß-thalassemia among microcytic, hypochromic anemia patients are 57% and 25.8%, respectively. Our study based on non-tribal is 20%, which is comparable to other studies. Hadavi et al[16] has reported the prevalence of $-\alpha^{3.7}$ deletion of 30.2% in the population of Iran (87 million). Our data showed the prevalence of $-\alpha^{3.7}$ deletion of 20% in the population of Southwest (Khuzestan) region in Iran (4.3 million). In our study also we found only two cases of $-\alpha^{4.2}$ deletion and few cases of any other deletions reported so far. Hadavi et al[16] have reported $-\alpha^{4.2}$ deletion in Iranian subjects with a prevalence of 3.5%. Najmabadi et al[17,18] has reported the prevalence of IVS-II-I (G —> A) beta-thalassemia mutation of 34 % in the population of Iran. In our study showed most frequent mutations were CD 36/37, IVS II-I, and IVS I-110 with 9.7%, 11.7%, and 3.5% followed by other cases of any other mutations reported so far. Hematological parameters in patients with α-thalassemia were compared with iron-deficiency anemia and ß-thalassemia. Individuals with the single-gene deletion ($-\alpha^{3.7}$) have lower levels of hemoglobin, MCV and MCH than normal controls. The carriers of á-thalassemia and Hb Variants have a mild microcytic hypochromic anemia.

However, their MCV and MCH are better than those of patients with iron-deficiency anemia. MCH is a better discriminator than other red-cell indices in the diagnosis of α-thalassemia, which is usually less than 26 pg. Since there is no definitive hematological marker that can give the diagnosis of α-thalassemia, molecular analysis remains the only diagnostic approach in microcytic, hypochromic patients. Our findings are in concordance with previous reports where microcytosis was explained on the basis of α-gene number.[3,19-22]

The identification of α and ß-thalassemia carrier status is important to prevent erroneous and expensive investigations to define the etiology of anemia and unnecessary prolonged iron supplementation. The knowledge of α and ß-gene number in α and ß-thalassemia traits in any population is necessary, as it modifies the phenotype of thalassemia by altering the ratio of α and ß -chains of hemoglobin. Thus screening for thalassemia should be considered during genetic counseling of high-risk couples of thalassemia for prenatal diagnosis.

ACKNOWLEDGEMENT

This work was supported by the director of Shafa hospital and department of hematology and oncology. The author also wants to thank the patients and their families.

REFERENCES

1. Haghshenas M, Zamani J. [Thalassemia]. 1st ed. Shiraz University of Medical Sciences Publishing Center: Shiraz; 1997. [Book in Persian]
2. Najmabadi H, Ghamari A, Sahebjam F, Kariminejad R, Hadavi V, Khatibi T. Fourteen-year experience of prenatal diagnosis of thalassemia in Iran. Comm Gen 2006;9:93-7.
3. Ko TM, Hwa HL, Liu CW, Li SF, Chu JY, Cheung YP. Prevalence study and molecular characterization of alpha thalassemia in Filipinos. Annals of Hematology 1999;78:355-7.
4. Bergeron J, Weng X, Robin L, Olney HJ, Soulieres D. Prevalence of alpha-globin gene deletions among patients with unexplained microcytosis in a north-American population. Hemoglobin 2005;29:51-60.
5. Nadkarni AH, Gorakshakar AC, Mohanty D, Colah RB. Alpha genotyping in a heterogeneous Indian population. Am J Hematol 1996;53:149-50.
6. Agarwal S, Sarwai S, Nigam N, Singhal P. Rapid detection of α+ thalassemia deletion & ß- globin gene triplication by GAP-PCR in Indian subjects. Indian J Med Res 2002;116:155-61.

7. Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 1989;17:2503-16.
8. Old JM, Varawalla NY, Weatherall DJ. Rapid detection and prenatal diagnosis of ß-thalassemia: Studies in Indian and Cypriot populations in the UK. Lancet 1990;336:834-7.
9. Chong SS, Boehm CD, Higgs DR, Cutting GR. Single-tube multiplex-PCR screen for common deletional determinants of alpha-thalassemia. Blood 2000;95:360-2.
10. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of alpha-thalassemia deletions and alpha-globin gene triplication by multiplex polymerase chain reactions. Br J Haematol 2000;108:295-9.
11. Tan JA, Tay JS, Lin LI, Kham SK, Chia JN, Chin TM, et al. The amplification refractory mutation system (ARMS): A rapid and direct prenatal diagnostic technique for beta-thalassemia in Singapore. Prenat Diagn 1994;14:1077-82.
12. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of á-thalassemia deletions and _-globin gene triplication by multiplex polymerase Chain reactions. Br J Haematol 2000;108:295-9.
13. Weatherall DJ, Clegg TB. The thalassemia syndromes, 4th ed. Oxford: Blackwell Science 2001;267.
14. Baysal E, Huisman THJ. Detection of common deletional á-thalassemia –2 determinants by PCR. Am J Hematol 1994;46:208-13.
15. Shaji RV, Eunice SE, Baidya S, Srivastava A, Chandy M. Determination of the breakpoint and molecular diagnosis of a common -thalassemia –1 deletion in the Indian population. Br J Haematol 2003;123:942-7.
16. Hadavi V, Taromchi AH, Malekpour M, Gholami B, Law HY, Almadani N, et al. Elucidating the spectrum of á-thalassemia mutations in Iran. Haematologica 2007;92:992-3.
17. Najmabadi H, Karimi-Nejad R, Sahebjam S, Pourfarzad F, Teimourian S, Sahebjam F, et al. The beta-thalassemia mutation spectrum in the Iranian population. Hemoglobin 2001;25:285-96.
18. Najmabadi H, Pourfathollah AA, Neishabury M, Sahebjam F, Krugluger W, Oberkanins C. Rare and unexpected utations among Iranian b-thalassemia patients and prenatal samples discovered by reverse-hybridization and DNA sequencing. Haematologica 2002;87:1113-4.
19. Bergeron J, Weng X, Robin L, Olney HJ, Soulieres D. Prevalence of alpha-globin gene deletions among patients with unexplained microcytosis in a north-American population. Hemoglobin 2005;29:51-60.
20. Sivera P, Roetto A, Mazza U, Camaschella C. Feasibility of molecular diagnosis of α- thalassemia in the evaluation of microcytosis. Haematologica 1997;82:592-3.
21. Mach-Pascual S, Darbelly R, Pilotto PA, Beris P. Investigation of microcytosis: A comprehensive approach. Eur J Haematol 1997;57:54-61.
22. Borges E, Wenning MRSC, Kimura EM, Gervasio SA, Costa FF, Sonati MF. High prevalence of α-thalassemia among individuals with microcytosis and hypochromia without anemia. Braz J Med Biol Res 2001;34:759-62.