Red blood cell phenotypes in α-thalassemias in the Spanish population

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ABSTRACT

Background and Objective. α-thalassemia is very common on all thalassemic geographical regions. The present work aimed at analyzing the relationship between the degree of microcytosis and hematological parameters and the type of α-thalassemic mutation.

Design and Methods. Five hundred and thirty-six subjects with 4 kinds of α-thalassemia were examined using established techniques that determined all hematological parameters, and globin synthesis and molecular biological techniques to study the DNA of globin genes by Southern blotting.

Results. Adult carriers of α+-thalassemia (–α/αα) present very few hematological alterations. In a statistical comparison with normal individuals (αα/αα), significant differences were found between the hemocytometric data and the MCV and MCH of heterozygous α+ thalassemia and the heterozygous α° or homozygous α+ genotype. Hb H disease was detected in 15 patients, presenting a severe degree of anemia, a significant increase in RDW and globin chain synthesis with an α/β ratio of 0.5±0.1.

Interpretation and Conclusions. These data provide reference values for geographical areas where α+ thalassemia is common. These hematocytometric data, together with hemoglobin analysis, could be useful as a future reference data for new patients diagnosed with α-thalassemia.
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Key words: gene mapping, α-thalassemia, globin genes, α-thalassemia indices

α-thalassemia is the most prevalent isolated genetic disorder in the human population. Its geographic distribution is highly variable and the highest levels are found in populations with a high incidence of malaria, where it can reach an alarming level of 80%.[1]

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The α globin gene cluster is located on the distal portion of the short arm of chromosome 16 in band 13.3 and is comprised of 2 functional α genes, a ζ gene, 3 pseudogenes (Ψα1, Ψα2, Ψζ1) and a θ1 gene of undetermined function. These are arranged in the following order: 5'- ζ2, Ψζ1, Ψα2, Ψα1, α2, α1, θ1 - 3'.[2]

Normal subjects have 4 α genes (αα/αα), 2 on each chromosome.[3] There are four kinds of α-thalassemia due to deletions of the structural α-globin genes. In one of these cases only one gene is deleted (α+ thalassemia), another kind is caused by deletion of 2 genes (–α/–α) or (––/αα), the loss of 3 genes corresponds to hemolytic anemia by Hb H disease (––/–α), and the loss of four α genes produces Hb Bart hydrops fetalis syndrome.[3]

Homozygotes for α+ thalassemia (–α/–α) and the heterozygous form of α° thalassemia produce a moderate clinical picture with mild microcytic anemia. Loss of two genes from the same chromosome (––/) causes the total suppression of α chain synthesis by this chromosome and for this reason is called α° thalassemia.[2] These deletions can suppress from 5.2 to more than 100 Kb of DNA, and can result in the elimination of all the genes which comprise the α globin cluster.[3,4]

However, a carrier of silent α-thalassemia (heterozygous α+ thalassemia) usually presents slight hematological changes in adult hemoglobin (Hb), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) in the lower range of normality.[5]

These phenotypes can be demonstrated by *in vitro* synthesis of globin chains in the reticulocytes which reveals a decrease in α chain synthesis. However, there is a great deal of overlapping of the data which corresponds to the different genotypes. More accurate results are obtained using DNA analysis.[5,6]

In this work we present the phenotypic study of 536 patients in which the hemocytometric parame-

ters, hemoglobin study, reticulocytes and Hb H inclusion bodies are defined and analyzed from a perspective of genotype analysis of the α genes using molecular biology techniques.

Materials and Methods

Five hundred and thirty-six subjects with either microcytosis with a MCV < 80 fL in adults or < 75 fL in children or direct family members of these patients presenting a normal MCV were studied within a screening program for microcytosis. All of these had a normal serum iron level, transferrin, transferrin saturation index, serum ferritin concentration and a Hb A_2 lower than 3.5%, ruling out iron deficiency anemia or heterozygous β-thalassemia, respectively.

Hemoglobin electrophoresis was carried out using the conventional technique in alkaline medium except in patients with Hb H in which it was done in neutral buffer at pH=7 for half an hour.

The conventional hematological study was carried out in an STKS Coulter (Coulter Electronics Inc., Hialeah, Florida, USA). In all patients, reticulocytes (Symex R-2000) and hemoglobin H inclusion bodies (brilliant blue staining) were simultaneously determined. Also, in 120 patients *in vitro* globin chain synthesis was determined in reticulocytes by Clegg's method.[7]

A molecular biology analysis was carried out in all 536 patients. DNA extracted from peripheral leucocytes with phenol chloroform (according to the method described by Old[8]) was digested with Bam HI and Bgl II restriction enzymes and hybridized with α (1.5 kb Pst) and ζ (1.8 kb Sac I) probes, respectively.[9] The fragments obtained were visualized by autoradiography. In some cases where the fragments were normal with the previously mentioned enzymes, these were studied with enzymes Nco I and Hph I and hybridized with an α probe.[10,11]

Hematological data and the age and sex of all subjects were recorded (n=536). The age of subjects ranged from 2 to 81 years.

The sample was divided into 3 different groups for the statistical analysis: 1) male and female children from 2 to 16 years; 2) females ≥ 16; 3) males ≥ 16.

Statistical analysis

Data was analyzed using statistical packages. For each parameter studied the mean (X), standard deviation (SD), maximum value, minimum value, range, coefficient of variation (VC) and mean standard deviation (MSD) were calculated.

The distribution of each variable was studied and in the case of non-Gaussian distributions, such as the reticulocytes, non-parametric tests were used (Mann-Whitney) for subsequent comparative analyses. When more than two groups of parametric variables were compared, analysis of variance was used followed by Newman-Keul's test. Frequency distribution was analyzed by using the Kolmogorov-Smirnov method.

Results

On the basis of the results of the molecular biological genotype analysis each individual was allocated to one of two groups: either a control group or subjects without α-thalassemia or a thalassemic group which was divided into the four following subgroups:

1. subjects who had lost one α gene. This group was comprised of 315 individuals with the $\alpha^{3.7}$ haplotype (154 men, 133 women and 28 children), 5 with the $\alpha^{4.2}$ haplotype (4 men, 1 women) and 15 with the non-deletion carrier state α-thalassemia (8 men and 7 women);
2. 114 subjects who had lost 2 α genes from different chromosomes; 112 with $-\alpha^{3.7}/-\alpha^{3.7}$ deletions (42 men, 55 women and 15 children), one child was double heterozygote for the $-\alpha^{3.7}/-\alpha^{4.2}$ mutation and one child was homozygote for the mutation of the initiation codon of the α_1 gene ($\alpha\alpha^{Nco}/\alpha\alpha^{Nco}$);
3. 72 patients who had lost 2 α genes from the same chromosome (heterozygous α° thalassemia, $--/$) in 34 men, 30 women and 8 children;
4. 15 patients with Hb H disease ($--/-\alpha$) comprised of 5 men, 4 women and 6 children.

Hemocytometric data of the controls (men, women and children), together with data of the 4 thalassemic subtypes, are recorded in Table 1.

When analyzed according to their status (male, female or child), no statistically significant differences were found for any hematological parameter in each thalassemic group, except for a low level of Hb and PCV in the female and children groups.

A molecular biology analysis of the genotype revealed significant differences for the Hb study (p<0.001) between all the groups except between ($\alpha\alpha/\alpha\alpha$) and ($-\alpha/\alpha\alpha$) (p>0.05); and between ($-\alpha/-\alpha$) and ($--/\alpha\alpha$) (p>0.05). Similarly, significant differences were found for the MCV and MCH studies (p<0.001) between all the groups except the ($--/\alpha\alpha$) and ($--/-\alpha$) genotypes (p>0.05).

When analyzed according to thalassemic group, significant differences were found for reticulocyte count by using the Mann-Whitney test, between the cases with Hb H disease and the rest of the thalassemic-group (p<0.01); but no significant differences were found for reticulocyte count between ($-\alpha/-\alpha$) and ($--/\alpha\alpha$) (p>0.05), ($-\alpha/\alpha\alpha$) and ($-\alpha/-\alpha$) (p>0.05). Similarly, significant differences were found for RDW by Mann-Whitney test, for Hb H disease and the rest of the thalassemic groups (p<0.001).

Table 1. Hematological data.

	Sex	αα/αα	-α/αα	-α/-α	--/αα	--/-α
RBC (x10^{12}/L)	Male	5.19±0.31	5.55±0.46	5.74±0.48	6.48±0.46	5.51±0.67
	Female	4.49±0.37	5.09±0.48	5.43±0.41	5.83±0.42	5.17±0.73
	Children	4.75±0.33	5.21±0.43	5.98±0.37	5.88±0.20	5.81±0.50
Hb (g/L)	Male	154±12.8	143±14.2	135±13.7	133±12.7	110±12.8
	Female	135±8.3	129±12.0	128±10.2	124±6.4	95±8.1
	Children	138±7.7	135±12.7	131±8.3	124±10.2	101±9.0
PCV (L/L)	Male	0.439±0.04	0.422±0.04	0.406±0.04	0.403±0.04	0.329±0.02
	Female	0.394±0.03	0.384±0.03	0.382±0.06	0.384±0.02	0.255±0.07
	Children	0.405±0.08	0.403±0.02	0.402±0.02	0.372±0.04	0.331±0.03
MCV (fL)	Male	86.3±5.1	76.0±5.0	70.2±3.77	68.2±3.9	61.3±7.1
	Female	87.7±6.0	75.9±5.68	69.2±2.47	65.9±3.09	61.1±1.38
	Children	84.3±4.31	74.2±4.42	67.7±1.90	63.9±4.4	61.7±5.40
MCH (pg)	Male	29.4±1.95	26.1±2.78	23.3±1.49	21.3±1.08	19.6±2.5
	Female	30.5±2.1	25.8±2.62	23.9±8.28	21.5±1.68	18.5±1.0
	Children	29.1±2.35	26.7±2.70	22.2±0.20	22.18±1.5	19.5±1.2
Reticulocytes (%)	Male	1.5±0.3	1.8±0.7	1.2±1.1	1.6±1.6	4.70±4.3
	Female	1.6±0.4	1.5±1.01	1.9±1.2	1.2±0.4	5.0±4.1
	Children	1.4±0.2	1.5±0.8	1.9±0.9	2.3±0.3	5.1±4.1
α/β ratio	Adults	1.16±0.24	0.92±0.10	0.74±0.10	0.70±0.08	0.50±0.09
Inclusion Bodies	Adults	–	–	±	++	+++
No. of cases	Male	62	166	42	34	5
	Female	48	141	55	30	4
	Children	40	28	17	8	6

Discussion

α-thalassemia was previously considered to be a rare disorder in Spain but with the introduction of more reliable diagnostic techniques we find that this is not the case. A study carried out in the Madrid region using direct analysis of the α and ζ genes in 760 blood samples from neonate umbilical cords revealed an incidence of 0.0184 of α-thalassemia, 0.005 of α triplications (ααα) and a zero incidence of ζ triplications, although isolated cases with this latter mutation have been described in our country.[4,12] In this study, 85% of mutations correspond to the -α$^{3.7}$/αα, 2 -α$^{4.2}$/αα. Two newborns presented heterozygotic α° thalassemia --/αα. We did not find any case of homozygous α° thalassemia or Hb Bart's hydrops fetalis, a disease which has not yet been described in our country.[4]

In a screening program for microcytosis proposed by the *Spanish Group of Erythropatology*, we studied a total of 536 subjects with α-thalassemia from 1992 to 1996 included. Results show that the most prevalent form of α-thalassemia is due to the loss of 3.7 kb of DNA which affects an α gene and produces a single functional hybrid gene comprised of the 5' end of the α$_2$ gene bound to the 3' end of the α$_1$ gene. This was the most prevalent mutation type in the aforementioned epidemiological study and is also the most commonly found deletion in studies of the world population including the countries of the Mediterranean basin.[13-18] This deletion is observed both in the heterozygote and homozygote form.

The adult heterozygous carrier of α$^+$ thalassemia (-α/αα) presents few hematological alterations. However, it can produce a significantly lower MCV and MCH in children than in normal controls of the same age.

Due to synthesis of globin chains in the reticulocytes, a decrease in the α chains can be detected in subjects with a lost α gene, but this value cannot distinguish between these individuals and normal controls. However, this technique could be very valuable to differentiate between microcytosis produced by α-thalassemia from that caused by silent β-thalassemia.[19] In subjects who were heterozygote for silent β-thalassemia, this coefficient is always greater than 1 whereas in subjects with α-thalassemia it is always lower than 1. The α/β ratio is closely correlated with the number of deleted genes, with decreases in this ratio corresponding to an increased number of α genes deleted (Table 1, Figure 1).

Significant differences exist between the hemocytometric data and the MCV and MCH of heterozygote α$^+$ thalassemic subjects and patients with deletion of 2 α genes. However, the homozygotic condition for α$^+$ thalassemia and the heterozygotic condition for α° thalassemia prove to be similar with overlapping Hb, PCV and globin chain synthesis values. However, there is a significant difference
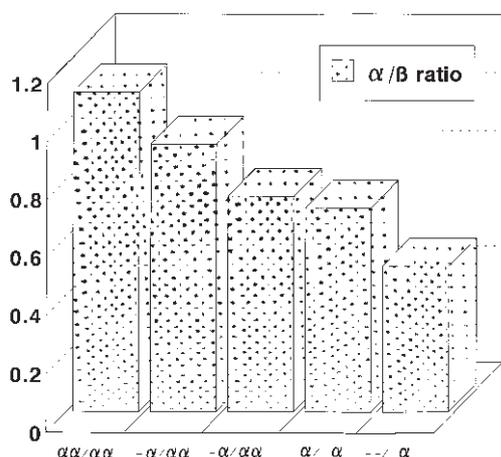
Figure 1. Globin chain synthesis.

in the Hb H inclusion bodies, between these patient groups. Whereas Hb H inclusion bodies are rarely observed in homozygotic α^+ thalassemia, in heterozygous α° thalassemia all patients present inclusion bodies although these are usually present in a very low number.

The cause of α° thalassemia is variable and can be due to molecular lesions which are characteristic of the Hispanic ethnic group ($--^{MA}$, $--^{CANT}$, $--^{SPAN}$)[4,20,21,22] or others previously described in populations all over the world such as $--^{SEA}$, $--^{CAL}$ etc.[23,24] The most prevalent lesion in our medium is a long deletion which involves more than 100 Kb of DNA, suppressing the hypervariable regions of 3'HVR and 5'HVR which we have called deletion $--^{BR}$.[21] One interesting result in our study was the absence of subjects with the $--^{MED}$ mutation, although it has been described previously in our country.[17]

Hb H disease was detected in 15 patients; they presented a more severe degree of anemia than other subgroups although none of the patients needed blood transfusions. Also, a marked increase in RDW was observed in comparison to other subgroups in which it was similar to the values recorded in normal controls. In the globin chain synthesis analysis the α/β ratio is 0.5 ± 0.1, which shows a clear and significant difference from the other α-thalassemia groups.

This is a multicentric study which compiles data from several Spanish hospitals. The importance of this study lies in the fact that all patients were studied by molecular biology techniques. These hematocytometric data, together with hemoglobin analy-

sis, could be useful in the future as reference data for new patients diagnosed with α-thalassemia.

The finding of microcytic red cells is a common finding in clinical practice and, as recently shown in this journal,[25] only the molecular approach allows for the precise diagnosis of α-thalassemia.

References

1. Kattamis AC, Camaschella C, Sivera P, Surrey S, Fortina P. Human α-thalassemia syndromes: Detection of molecular defects. Am J Hematol 1996; 53:81-91.
2. Higgs DR, Vickers MA, Wilkie AO, Pretorius IM, Jarman AP, Weatherall DJ. A review of the molecular genetics of the human α-globin gene cluster. Blood 1989; 73:1081-104.
3. Higgs DR. α-thalassemia. In: Higgs DR, Weatherall DJ, eds. Bailliére Clin Haematol: the hemoglobinopathies. London:WB Saunders, 1993; 6:117-50.
4. Villegas A, Sánchez J, Carreño DL, et al. Molecular characterization of a new family with α-thalassemia-1 ($--^{MA}$ mutation). Am J Hematol. 1995; 49:294-8.
5. Williams TN, Maitland K, Ganczkowski M, et al. Red blood cell phenotypes in the α^+ thalassemias from early childhood to maturity. Br J Haematol 1996; 95:266-72.
6. Foglietta E, Deidda G, Graziani B, Modiano G, Bianco I. Detection of α-globin gene disorders by a simple PCR methodology. Haematologica 1996; 81; 387-96.
7. Clegg JB. Hemoglobin synthesis. In: Weatherall DJ, ed. The thalassemias. Methods in Haematology. Oxford: Churchill Livingstone, 1983. p. 54-73.
8. Old JM, Higgs DR. Gene analysis. In: Weatherall DJ, ed. The thalassemia methods in haematology. Oxford: Churchill Livingstone, 1983. p. 74-102.

9. Proudfoot NJ, Gil A, Maniatis T. The structure of the human ζ-globin gene a closely linked, nearly identical pseudogene. Cell 1982; 31:553-63.

10. Orkin SH, Goff SC, Hechtman RL. Mutation in an intervening sequence splice junction in man. Proc Natl Acad Sci USA 1981; 78:5041-5.

11. Pirastu M, Saglio G, Chang JC, Cao A, Kan YW. Initiation codon mutation as a cause of α-thalassemia. J Biol Chem 1984; 259:2315-7.

12. Fei YJ, Kutlar F, Harris II HF, et al. A search for anomalies in the δ, α, β and γ globin gene arrangements in normal black, Italian, Turkish and Spanish newborns. Hemoglobin 1989; 13:45-65.

13. Pirastu M, Lee KY, Dozy AM, et al. α-thalassemia in two mediterranean populations. Blood 1982; 60:509-12.

14. Paglietti E, Galanello R, Moi P, Pirastu M, Cao A. Molecular pathology of haemoglobin H disease in Sardinians. Br J Haematol 1986; 63:485-96.

15. Velati C, Sampietro M, Biassoni M, et al. α-thalassemia in an Italian population. Br J Haematol 1986; 63:497-501.

16. Nieves MA, De Pablos JM, Garrido F. Alpha thalassemia in a Southern Spanish population. Br J Haematol 1990; 73:282.

17. Villegas A, Sánchez J, Sal del Río E. α globin genotypes in a Spanish population. Hemoglobin 1992; 16:427-9.

18. Bianco I, Graziani B, Foglietta E, et al. Molecular basis of α-thalassemia in Latium (Italy). Second Meeting of the European Haematology Association. Br J Haematol 1996; 93(suppl 2):23.

19. Bianco I, Cappabianca MP, Foglietta E, et al. Silent thalassemias: genotypes and phenotypes. Haematologica 1997; 82:269-80.

20. González Redondo JM, Gilsanz F, Ricard P. Characterization of a new α-thalassemia-1 deletion in a Spanish family. Hemoglobin 1989; 13:103-16.

21. Villegas A, Calero F, Vickers MA, Ayyub H, Higgs DR. α-thalassemia in two Spanish families. Eur J Haematol 1990; 44:109-14.

22. Villegas A, Sánchez J, Ricard P, et al. Characterization of a new α-thalassemia-1 mutation in a Spanish family. Hemoglobin 1994; 18:29-37.

23. Winichagoon P, Higgs DR, Goodbourn SE, Clegg JB, Weatherall DJ, Wasi P. The molecular basis of α-thalassemia in Thailand. EMBO J 1984; 3:1813-8.

24. Fortina P, Dianzani I, Serra A, et al. A newly characterized α-thalassemia-1 deletion removes the entire α-like globin gene cluster in an Italian family. Br J Haematol 1991; 78:529-34.

25. 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.