Alpha Thalassaemia and extended alpha globin genes in Sri Lanka

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A R T I C L E   I N F O

A B S T R A C T

The α-globin genes were studied in nine families with unexplained hypochromic anaemia and in 167 patients with HbE β thalassaemia in Sri Lanka. As well as the common deletion forms of α^+ thalassaemia three families from an ethnic minority were found to carry a novel form of α^0 thalassaemia, one family carried a previously reported form of α^0 thalassaemia, $--^{THAI}$, and five families had different forms of non-deletional thalassaemia. The patients with HbE β thalassaemia who had co-inherited α thalassaemia all showed an extremely mild phenotype and reduced levels of HbF and there was a highly significant paucity of α^+ thalassaemia in these patients compared with the normal population. Extended α gene arrangements, including $\alpha\alpha\alpha$, $\alpha\alpha\alpha\alpha$ and $\alpha\alpha\alpha\alpha\alpha$, occurred at a low frequency and were commoner in the more severe phenotypes of HbE β thalassaemia. As well as emphasising the ameliorating effect of α thalassaemia on HbE β thalassaemia the finding of a novel form of α^0 thalassaemia in an ethnic minority, together with an unexpected diversity of forms of non-deletion α thalassaemia in Sri Lanka, further emphasises the critical importance of micro-mapping populations for determining the frequency of clinically important forms of the disease.

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Introduction

Because the milder forms of α thalassaemia have come under intense selection due to their protective effect against malaria, the α thalassaemias are amongst the commonest monogenic diseases [1]. They result from deletions or mutations involving the linked pair of α globin genes on chromosome 16 ($\alpha\alpha/\alpha\alpha$) [2,3]. The deletion forms are divided into the α^+ thalassaemias, in which one of the pair of α globin genes is deleted ($-\alpha/\alpha\alpha$), and the α^0 thalassaemias in which both of the pair of α globin genes are deleted ($--/\alpha\alpha$). Much less commonly one of the α globin genes may be inactivated due to a mutation, a condition designated non-deletion α thalassaemia ($\alpha^{ND}\alpha/\alpha\alpha$). The compound heterozygous state for α^+ and α^0 thalassaemia results in HbH disease, a thalassaemic disorder of intermediate severity, while the homozygous state for α^0 thalassaemia results in stillbirth and the Hb Bart's hydrops syndrome.

While the mild deletion forms of α^+ thalassaemia occur at an extremely high frequency right across the tropical belt, stretching from Africa to the Middle East and throughout south and southeast Asia, α^0 thalassaemia only occurs at a high frequency in southeast Asia and in some Mediterranean island populations. With the exception of Hb Constant Spring, which is found in 1.5–11% of the population of Thailand [4], most of the non-deletion forms of α thalassaemia occur at a low frequency and are distributed sporadically across the tropical zone. Although the deletion forms of α^+ thalassaemia are not associated with any clinical disability, there is increasing evidence that they have an ameliorating effect if co-inherited with HbE β thalassaemia, the commonest form of severe thalassaemia throughout Asia [5].

The deletion forms of α thalassaemia are the result of unequal crossing over between the linked α globin genes resulting in one chromosome with a missing α gene and the opposite of the pair with additional α genes ($\alpha\alpha\alpha/\alpha\alpha$ or $\alpha\alpha\alpha\alpha/\alpha\alpha$). These latter genotypes do not appear to have come under selection and are found at a low level in many populations in the tropical belt [6].

In a previous publication we described the approximate frequency of the deletion forms of α^+ thalassaemia in Sri Lanka together with the occurrence of extended α gene arrangements in the population [7]. The extension of these studies in this report describes the first identification of α^0 thalassaemia and the remarkable diversity of non-deletion α thalassaemia in the Sri Lankan population together with further extended α gene arrangements; these findings include a previously unidentified lesion of the α globin genes. In addition, the α globin genes of 167 patients with HbE β thalassaemia have

been analysed and the effects of the co-inheritance of α thalassaemia with this condition have been further defined with respect both to the haemoglobin constitution and the clinical phenotype.

Patients and methods

Patients

The patients with different forms of α thalassaemia that form the basis of this study were referred to the National Thalassaemia Centre, General Hospital, Kurunegala, Sri Lanka, with various degrees of hypochromic anaemia. The α globin genes were also assessed in a group of over 167 patients with HbE β thalassaemia who had been followed for over 15 years at the Centre. The haematological findings, methods for classification of phenotypic severity, and the description of some of the genetic and environmental modifiers responsible for variable severity have been reported previously [8].

Ethical approval

Ethical approval for this research programme on thalassaemia was obtained from the Ethical Committee of the College of Paediatricians of Sri Lanka and the Oxford Tropical Research Ethical Committee.

Methods

Blood samples were collected into EDTA and standard haematological data were obtained (Coulter MD8; Coulter Electronics, Luton, UK). The methods used for demonstration of HbH inclusions and haemoglobin analysis by HPLC were as previously described [6].

Whole blood samples were frozen and transported to Oxford for analysis. Southern blot and sequence analysis for the α globin gene arrangements were performed as previously described [7]. Multiple ligation polymerase amplication (MLPA) (Service XS™, Leiden, The Netherlands) was performed as per manufacturer's instruction. Known deletions were confirmed using a GAP PCR method [9,10]. A new deletion identified by MLPA was further characterised by Gap PCR using a newly designed primer pair (SL forward: 5′-GGGGTGCCCGGA GGCTCTAT-3′ and SL reverse: 5′-TCGGGTCACTGCAACCTCTGC-3′) that spanned the deletion and could be amplified under denaturing conditions to produce a 454 bp product along with a primer within the deletion as a normal control (SL reverse 2: 5′-TCGGGTCACTGCAACCT CTGC-3′) to amplify an 830 bp product. Sequence analysis was performed on the amplicon spanning the deletion using the amplification primers and confirmed with different primer sets.

Statistical analysis was carried out using SPSS 16 (SPSS Inc.). $p < 0.05$ was considered statistically significant.

Results

Studies of the patients referred with refractory hypochromic anaemias yielded a surprisingly large variety of different forms of α thalassaemia, both deletion and non-deletion.

Deletion forms of α thalassaemia

As well as the previously identified deletion forms of α^+ thalassaemia, $-^{3.7}\alpha$ and $-^{4.2}\alpha$, two deletional forms of α^0 thalassaemia, one of which has not been described previously, were encountered. The pedigrees of families with the novel form of α^0 thalassaemia, all of whom were from the Muslim population, are shown in Fig. 1 and the associated haematological findings in Table 1. The propositus in pedigree 1 was a 25-year-old female who presented with pallor. Her haemoglobin level was 8.9 g/dl and her blood film showed marked microcytosis and hypochromia which were reflected in her red cell indices. Her red cells also showed

typical HbH inclusions after incubation with brilliant cresyl blue and haemoglobin analysis revealed HbH. Molecular analysis showed that she was heterozygous for both the common deletional form of α^+ thalassaemia, $-\alpha^{3.7}$, and a previously unreported deletional form of α^0 thalassaemia.

The results of an analysis of this novel deletion are summarised in Fig. 2. Southern Blot analysis using an α-probe on a *Bam-HI* digest and a ζ-probe on a *Bgl-II* digest appeared like homo- or hemizygous single α-globin gene deletions. MLPA analysis revealed a deletion of probes 11–15 on one chromosome. The 5′ breakpoint of the deletion was located between probe 10 and probe 11, a gap of 3.8 kb, and the 3′ breakpoint was located between probe 15 and probe 16, a gap of 3.1 kb. Primers flanking the putative deletion and within these regions of uncertainty were used in a multiplex amplification and any amplicons were sequenced. These revealed a novel deletion, $--^{SL}$, removing 12,819 bp that included both in-tandem adult α globin genes. New primers (SL forward and reverse, 1 and 2) were designed to flank the break points and allowed the amplification of a single product and a primer within the deletion for a larger control band to detect a normal α-globin gene arrangement.

Further studies of the complex pedigree of the propositus showed that her son was a carrier for the $--^{SL}$ deletion and that her husband was a carrier for the common deletion form of α^+ thalassaemia ($-\alpha^{3.7}$). It was also found that she was distantly related to another family in which there were two further individuals with HbH disease due to the interaction of α^+ thalassaemia and $--^{SL}$ (Fig. 1). This branch of the family also had two heterozygotes for α^+ thalassaemia and a carrier of $--^{SL}$.

Two other Muslim families were found with the Sri Lankan form of α^0 thalassaemia. In pedigree 2 (Fig. 1) the propositus was a 5-year-old male who presented with a respiratory infection and was found to have a hypochromic microcytic anaemia with a haemoglobin value of 8.4 g/dl. After incubation of his red cells with brilliant cresyl blue numerous HbH bodies were seen and haemoglobin analysis showed HbH. Molecular analysis disclosed that he was heterozygous for both an α^+ deletion form of α thalassaemia, $-\alpha^{4.2}$, and $--^{SL}$. Very similar haematological findings were observed in his mother who also had HbH disease with the same genotype; his father was a carrier for $--^{SL}$. There was a history of a previous stillbirth in this family with the clinical picture of hydrops fetalis. The third pedigree carrying the Sri Lankan form of α^0 thalassaemia consisted of a mother with HbH disease with the genotype $-\alpha^{3.7}/--^{SL}$. Her two children showed a mild hypochromic microcytic blood picture and were found to be heterozygous for $--^{SL}$.

A further patient with a deletion form of α^0 thalassaemia was encountered (pedigree 4, Table 1). The propositus was a 9-year-old child who presented with a haemoglobin level of 7.7 g/dl and a hypochromic microcytic blood film with a normal serum ferritin level. Her red cells showed numerous HbH inclusions and HbH was found on electrophoresis. Southern blot analysis revealed the $-\alpha^{3.7}$ deletion on one chromosome and an α^0 thalassaemia deletion on the other. Further studies using MLPA analysis revealed a deletion removing a single copy of probes 9 through to 16, similar to the previously reported Thai deletion, $--^{THAI}$. GAP PCR and sequence analysis using published primers for this deletion revealed that it was identical to the previously reported findings in the Thai variety of α^0 thalassaemia [11].

Non-deletion α thalassaemia

Five families were found with different forms of non-deletional α thalassaemias. The main haematological findings in the propositii are shown in Table 1.

The propositus in pedigree 5 was an otherwise healthy 1-year-old girl who was found to have a haemoglobin level of 9.3 g/dl with hypochromic microcytic red cells and a normal serum ferritin level. No haemoglobin H inclusions were found. Southern blot analysis
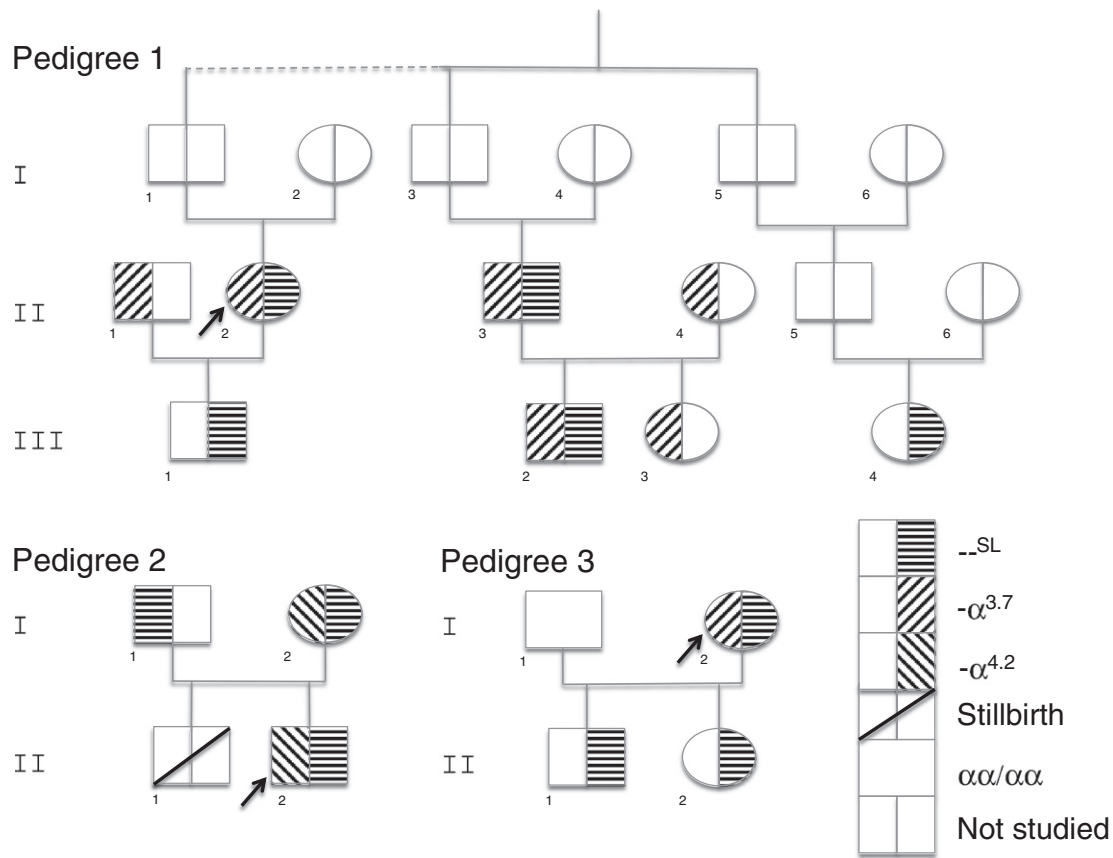
Fig. 1. Family pedigrees of the new form of α^0 thalassaemia, ($--^{SL}$). The propositii are indicated by arrows. The broken line in pedigree 1 denotes uncertainties about the precise relationships in the different branches of this family.

showed that the child and her father were heterozygous for the $-\alpha^{3.7}$ deletion. Sequence analysis showed that the child was also heterozygous for a splice acceptor mutation, IVS1-117 (G-A), in the $\alpha1$ globin gene; the child's mother was also heterozygous for this mutation. This is a rare form of α thalassaemia which has been found in isolated cases in several racial groups [12].

The propositus in pedigree 6 was an otherwise healthy 8-year-old girl with a haemoglobin level of 12.7 g/dl who was found by chance to have a hypochromic microcytic blood picture; no HbH inclusions could be found. Molecular analysis showed a T-G change at codon 14 of the $\alpha1$ globin gene, a mutation that has previously been described and named Hb Evanston [13]. This child was heterozygous

Table 1
Haematological data of the pedigrees with the new deletion ($--^{SL}$) and from the propositii of cases with previously described mutations.

| Pedigree	Case	Hb (g/dL)	mcv (μm³)	mch (pg)	HbA$_2$ (%)	HbF (%)	HbH inclusions	Genotype
1	II.1	15	77.8	25.9	2.4	0	−	$-\alpha^{3.7}/\alpha\alpha$
	II.2	8.9	50.8	14.2	1.7	0	+	$-\alpha^{3.7}/--^{SL}$
	II.3	9.7	65.4	19.1	2.0	0.8	+	$-\alpha^{3.7}/--^{SL}$
	II.4	12.3	73.8	24.8	2.5	0.5	−	$-\alpha^{3.7}/\alpha\alpha$
	III.1	10.2	54.7	17.2	3.1	0	−	$--^{SL}/\alpha\alpha$
	III.2	10.2	60.8	17.7	1	1.1	+	$-\alpha^{3.7}/--^{SL}$
	III.3	12.6	76.4	26.2	1.6	0	−	$-\alpha^{3.7}/\alpha\alpha$
	III.4	12.3	73.8	24.8	1.2	0	−	$--^{SL}/\alpha\alpha$
2	I.1	12.1	69.3	20.4	2.7	0.5	−	$--^{SL}/\alpha\alpha$
	I.2	7.8	52.5	15.6	2.2	0.5	+	$-\alpha^{4.2}/--^{SL}$
	II.1	8.4	44.7	14.5	1.7	0	+	$-\alpha^{4.2}/--^{SL}$
3	I.1	15.1	87.1	30.1	2.4	1.5	−	$\alpha\alpha/\alpha\alpha$
	I.2	8.9	46.2	12.9	1.6	0.5	+	$-\alpha^{3.7}/--^{SL}$
	II.1	13.7	63.5	20.4	2	3.1	−	$--^{SL}/\alpha\alpha$
	II.2	11.2	63.5	20.8	2	1.5	−	$--^{SL}/\alpha\alpha$
4	Propositus	7.7	57.4	15.6	2.5	0.5	+	$-\alpha^{3.7}/--^{Thai}$
5	Propositus	9.3	56.2	18.1	1.5	12.2	−	$-\alpha^{3.7}/\alpha\alpha^{IVSI-117(G>A)}$
6	Propositus	12.7	68.3	23.2	1.2	0	−	$\alpha\alpha^{Hb\ Evanston}/\alpha\alpha$
7	Propositus	6.7	60.6	16.6	0.8	0	+	$\alpha^{AATAAA>AATAAG}\alpha/\alpha^{AATAAA>AATAAG}\alpha$
8	Propositus	4.7	62.4	19.5	1.6	0.9	+	$\alpha^{AATAAA>AATAAG}\alpha/\alpha^{AATAAA>AATAAG}\alpha$
9	Propositus	8.6	68.9	22.5	2.2	1.9	−	$-\alpha^{3.7}/\alpha\alpha^{Koya\ Dora}$
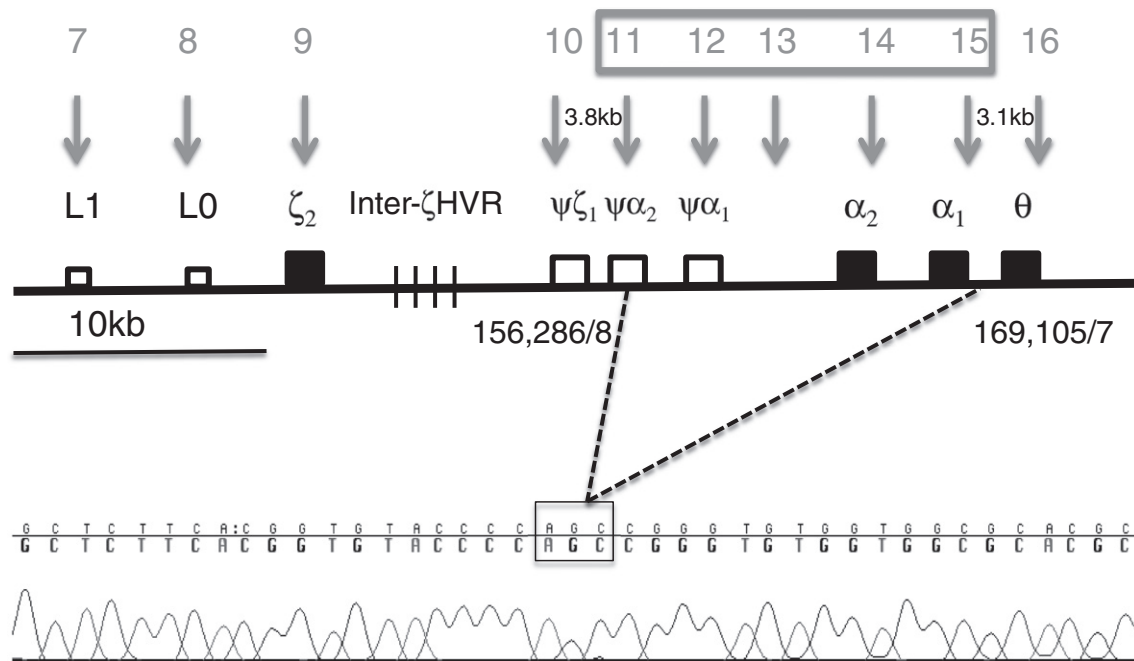
Fig. 2. The extent of the $--^{SL}$ deletion. The arrangement of the α gene complex on chromosome 16 is shown. Numbers 7–16 with arrows refer to the MLPA probes. A sequence chromatogram spanning the deletion is shown with the sequence co-ordinates using NCBI36/hg18 as a reference. The broken lines summarise the size of the deletion.

for this non-deletion form of α thalassaemia; the other α globin genes were normal.

The propositus in pedigree 7 was a 30-year-old woman who presented with symptoms of anaemia and was found to have a haemoglobin level of 6.7 g/dl associated with the typical picture of HbH disease. Sequence analysis revealed that she was homozygous for a point mutation in the $\alpha2$ globin gene, AATAAA to AATAAG, in the polydenylation signal sequence. The propositus in pedigree 8 was a 40-year-old male who presented with symptoms of anaemia and chronic leg ulcers and who also had a haematological picture typical of HbH disease. He was also found to be homozygous for this mutation. Homozygosity for this mutation has been previously reported to be associated with HbH disease in Middle Eastern and Mediterranean populations [14].

The propositus in pedigree 9 was a 41-year-old woman who presented with a haemoglobin level of 8.6 g/dl in pregnancy. Her blood picture showed marked hypochromia and microcytosis, while her serum ferritin level was in the normal range. Southern blot analysis of her α-globin genes revealed that she was heterozygous for the common $-\alpha^{3.7}$ deletion and sequence analysis revealed an A-C mutation in the termination codon of $\alpha1$, a mutation that has previously been reported in Indian populations and called Hb Koya Dora [15].

α Thalassaemia and HbE β thalassaemia

The α-globin genes in 167 patients with HbE β thalassaemia were examined by Southern blotting. The phenotypic severity based on up to fifteen years of observation was classified as previously described [8]. In short, class 1 describes patients who have grown and developed well without transfusion while class 2 patients are those in whom it was possible to stop transfusion without any deleterious effects. Classes 3 and 4 are much smaller groups where there are still phenotypic uncertainties, while class 5 is made up of patients who require regular transfusion. Overall, 15 patients (8%) were found to have the deletional form of α^+ thalassaemia, 13 with the $-\alpha^{3.7}$ deletion and 2 with the $-\alpha^{4.2}$ deletion. Thirteen of these patients were

found among those with the mild phenotypes of class 1 and 2; two were in the uncertain phenotypes and none were found in the severe, class 5 phenotypes who required regular transfusion. From previous studies of the frequency of α^+ thalassaemia in normal individuals from the same region 14% of the patients with HbE β thalassaemia would have been expected to have this mutation. The finding that only 8% were affected indicates a significant reduction over the expected figure ($\chi^2 = 4.43$, $p = 0.035$).

As well as their extremely mild phenotypes those patients who had co-inherited α thalassaemia with HbE β thalassaemia also showed remarkably low levels of HbF. In those without α thalassaemia the mean level of HbF was 26.9% (median 28.6%, range 2.6–55%, IQR 18.3–34.6%). In those with α thalassaemia the mean level of HbF was 11.6% (median 9.5%, range 2.7-25.2%, IQR 6.5–15.9%). $p < 0.001$.

To determine whether there were other forms of α thalassaemia in the patients with HbE β thalassaemia the α-globin genes were sequenced in 28 cases without an α gene deletion who showed mild phenotypes with HbF values of 20% or less. One patient was found to be heterozygous for the non-deletion form of α thalassaemia associated with Hb Evanston, as described earlier. This 52-year-old female had a remarkably mild form of HbE β thalassaemia, having never required transfusion; her HbF value was 12.5%.

Extended α-chain numbers

By Southern blotting analysis seven of the patients with HbE β thalassaemia were found to be heterozygous for chromosomes with extended α gene numbers, five with $\alpha\alpha\alpha$ arrangements, one with $\alpha\alpha\alpha\alpha$ and one with $\alpha\alpha\alpha\alpha\alpha$. Two of the patients with duplicated α genes were found in the group with mild phenotypes while the remainder were in the transfusion-dependent category. The patient with five α genes on one chromosome presented with severe anaemia during the first year of life at which time his spleen was enlarged to 4 cm below the costal margin. Despite adequate transfusion his spleen continued to enlarge and had reached 8 cm by the age of 3 years. His brother also had HbE β thalassaemia which was

diagnosed while he was being investigated for jaundice aged 2 years. For several years he did not require transfusion but there was gradual enlargement of the spleen and a fall in haemoglobin and he started on regular transfusion at the age of 6 years. His α-globin chain gene arrangement was normal.

Discussion

While it is now established that there is a relatively high frequency of the deletion forms of α^+ thalassaemia and that α^0 thalassaemia and non-deletion forms of the condition occur sporadically in parts of India less is known about the occurrence of these conditions in Sri Lanka. We have previously reported that approximately 14% of the Sri Lankan population are heterozygous for the deletion forms of α^+ thalassaemia, mainly the $-\alpha^{3.7}$ form [7,16]. However, apart from two reports of HbH disease [17,18] nothing is known about the occurrence of the α^0 or non-deletional forms of α thalassaemias in the island population.

In the present study we have identified two varieties of α^0 thalassaemia in Sri Lanka, one novel mutation that we have called $--^{SL}$ and the other which was previously described in Thailand. Although, as judged by the absence of these mutations in the large number of patients with HbE β thalassaemia who were studied, they are probably rare, the fact that the families in which the Sri Lankan form were discovered were all members of the Muslim population, and because of the relatively high frequency of consanguineous marriages on the island, the finding of this condition is of importance with respect to the risk of the production of babies with the Hb Bart's hydrops syndrome in these families. Indeed it seems very likely that a baby with this condition was born in pedigree 2.

The finding of five different non-deletion forms of α thalassaemia in what is a relatively small island population was surprising. Most of these conditions have been found sporadically in other populations, although Hb Koya Dora occurs quite commonly among southern Indian populations [15]. Although further work is required, it seems likely that these non-deletion forms of α thalassaemia are uncommon in the Sri Lankan population; in our search for them among the patients with HbE β thalassaemia we only found one example, Hb Evanston, and in an earlier study in which the α globin genes of 190 newborns were sequenced, none were found.

The extreme mildness of the phenotype in patients with HbE β thalassaemia who have inherited a form of α thalassaemia confirms our previous findings and those of others in different populations [8,19,20]. In this context the observation that only 8% of the patients with HbE β thalassaemia inherited the deletional form of α^+ thalassaemia, while this mutation occurs in about 14% of the Sri Lankan population [7], is of particular interest. We have previously noted a discrepancy between the frequency of β thalassaemia major and HbE β thalassaemia as estimated by the Hardy–Weinberg formula in the hospital population in Kurunegala where these studies were carried out. While the frequency of β thalassaemia major was as expected from the local heterozygote rate there was a paucity of HbE β thalassaemia based on the same population data. This suggested that fewer patients with this condition than expected were attending the hospital. The extreme mildness of the phenotypes of patients with HbE β thalassaemia who co-inherited α thalassaemia in the present study, together with the relative paucity of those with this genotype, suggests that this genotype results in a phenotype that is so mild that many patients with it do not present for treatment. The finding of significantly reduced levels of HbF in association with this genotype was surprising and, apart from its diagnostic value, suggests that the co-inheritance of α thalassaemia reduces the degree of globin chain imbalance such that there is less selective pressure on survival of red cells with relatively higher levels of fetal haemoglobin.

The low frequency of the different types of extended α gene numbers in the patients with HbE β thalassaemia, similar to that of the normal population in Sri Lanka, suggests that this has little selective effect on the phenotype. The finding of a chromosome with five α genes has only been reported once before, in a 33-year-old Sudanese male with a normal haemoglobin level and mild microcytosis and hypochromia of the red cells [21]. From the findings in the patient in the present study it appears as though it had a deleterious effect on the genotype of HbE β thalassaemia when compared to that of his brother with the same disease who had normal α-globin gene numbers. This observation is supported by the distribution of the patients with extended α gene numbers between the mild and severe phenotype groups in this study.

In summary, the novel form of α^0 thalassaemia described here has so far been restricted to Muslim families which comprise only 7% of the population of Sri Lanka. The relatively high risk of HbH disease and fetal loss due to Hb Bart's hydrops, particularly in consanguineous marriages, underlies the importance of micro-mapping gene frequencies for the thalassaemias and not excluding ethnic sub-groups. The significant paucity of α thalassaemia genes in the patients with HbE β thalassaemia, and the quite remarkably mild phenotypes in those who have co-inherited α thalassaemia, provides further evidence regarding the ameliorating effect of α thalassaemia in this condition. This effect is much greater than the co-inheritance of α thalassaemia in other forms of β thalassaemia [6], probably because of the less severe imbalance of globin chain synthesis in HbE β thalassaemia. This observation points to the considerable therapeutic benefit that would follow the ability to selectively suppress α-chain synthesis, even to a small degree, in patients with HbE β thalassaemia.

Author contributions

SS identified the families with α thalassaemia; CF and HA carried out the molecular analysis of the α thalassaemias; NO, A Prem, A Per and DB assessed the patients; and CF and DW wrote the paper.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgments

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