

Frequency of Alpha Thalassaemia in homozygous Beta Thalassaemia paediatric patients and its clinical impact at a blood disease centre in Karachi, Pakistan

Jawad Hassan, Muhammad Nadeem, Saqib Hussain Ansari, Shariq Ahmed, Sadia Parveen, Tahir Shamsi

Abstract

Objective: To find frequency of alpha Thalassaemia in homozygous beta Thalassaemia patients, and to see any difference in frequency and age of first transfusion and mean haemoglobin concentration.

Method: The single-centred, descriptive cross-sectional study was conducted at the National Institute of Blood Disease and Bone Marrow Transplantation, Karachi, from June 1, 2012, to May 31, 2013. Patients of homozygous beta thalassaemia, diagnosed by polymerase chain reaction, were tested for coinheritance of alpha Thalassaemia and foetal haemoglobin XMN1 polymorphism using polymerase chain reaction. SPSS 17 was used for data analysis.

Results: Of the 286 patients, 119(41.6%) were males, and 99(34.6%) showed coinheritance of alpha thalassaemia. In the coinheritance group, 50(50%) and 11(11%) patients received 1-20 and 21-40 times transfusions per year respectively, while in the non-coinheritance group, the corresponding numbers were 125(67%) and 27(14.4%). Overall, 73(25.5%) patients had never been transfused, including 38(13.3%) patients in the alpha Thalassaemia group. XMN1 polymorphism was found in 86(41%) of the 208 patients who were tested and analysed on this count.

Conclusions: Alpha thalassemia was present in more than one-third homozygous beta thalassemia patients.

Keywords: Alpha thalassaemia, DNA mutation analysis, β -thalassaemia major, Co-inheritance. (JPMA 69: 959; 2019)

Introduction

Thalassaemia is inherited autosomal recessive disorder of haemoglobin (Hb) synthesis. It has two main types i.e. alpha (α) and beta (β) thalassaemia. Alpha Thalassaemia (AT) is caused by deletions or mutations of α -globin gene complex. Deletion of all four α -globin genes (homozygous α^0 thalassaemia $(--/--)$) is not compatible with life and results in a fatal condition called Haemoglobin Bart's Hydrops Foetalis. Deletions of one to three normal α -globin genes causes an increasing reduction in α -globin chain synthesis in carriers of α^+ Thalassaemia ($-\alpha/\alpha\alpha$, $--/\alpha\alpha$ or $-\alpha/-\alpha$), α^0 thalassaemia ($-\alpha/\alpha\alpha$ or $-\alpha/-\alpha$), α^0 thalassaemia ($--/\alpha\alpha$) and haemoglobin H disease ($--/-\alpha$).¹⁻³ Commonest deletional ATs found in Pakistani population are $-\alpha$ 3.7 with a frequency of 8.3% and the rare forms include $-\alpha$ 4.2 (0.2%) and $\alpha\alpha\alpha$ anti-3.7(0.9%).⁴ Beta Thalassaemia (BT) results in reduced synthesis (β^+) or complete suppression (β^0) of β -globin chain synthesis. BT carriers are generally

asymptomatic; while BT major (BTM) is characterised by severe anaemia requiring frequent blood transfusions and iron chelation.^{1,2}

Thalassaemia -or β -forms, but in Asia it is not unusual to find AT and BT co-inherited in the same individual as Thalassaemia is highly prevalent in this region³ AT, when co-inherited with homozygous BT, can ameliorate the clinical condition known as Thalassaemia intermedia (BTI), a form of BT that does not require regular blood transfusions for survival. On the other hand, interaction with triplicated α -globin gene expression with heterozygous BT (carrier) is also able to produce BTI. These findings seem attributable to excess α chain in the development of clinical phenotype.³

The standard diagnostic marker for BT trait (BTT) is elevation of Hb alpha 2 (HbA2) level ($>3.5\%$). Low mean corpuscular volume (MCV) and mean cell Hb (MCH) with a raised HbA2 indicate a Thalassaemia carrier in the absence of iron deficiency anaemia. Staining for HbH inclusion bodies is also carried out as part of the screening for AT.

National Institute of Blood Disease, Karachi.

Correspondence: Jawad Hassan. e-mail: jawadkazmi2003@gmail.com

In addition, haematological tests used for screening show no significant difference between double heterozygosity for AT and BT, and heterozygous BT. Both disorders show a similar haematological picture, except for a slightly higher MCV value in double heterozygous individuals.⁵ Therefore, deoxyribonucleic acid (DNA) analysis is necessary to find out the co-inheritance. XMNI polymorphism is frequently associated with a higher baseline Hb at diagnosis. This is due to increased synthesis of γ -globin (G) chains, thereby reducing imbalance between alpha and non-alpha globin chains and resulting in decreased disease severity.⁶⁻⁸ The current study was planned to find out the frequency of AT in homozygous BT patients in our population as majority of the studies regarding the topic are international while local studies are limited.

Patients and Methods

The single-centre, descriptive cross-sectional study, conducted at the National Institute of Blood Disease and Bone Marrow Transplantation, Karachi from June 01, 2012 to May 31, 2013. Subjects of homozygous BT were selected on the basis of consecutive sampling technique. Patients of either gender who presented with transfusion dependent anaemia and had started to receive transfusions at more than 6 months of age, or homozygous BT patients diagnosed on the basis of DNA analysis were included in the study from the Thalassaemia Clinic. Compound heterozygote cases of HbD, HbE, HbS or HbC trait with BTT either diagnosed on Hb electrophoresis or DNA analysis were excluded.

The study was approved by institutional review board and informed consent was taken from all the participating subjects.

Bias was controlled by inclusion and exclusion criteria as well as confounding factors like age, weight and number of transfusions which were controlled by stratification techniques at the time of analysis. Complete physical examination including general and systemic, and investigations complete blood count (CBD), reticulocyte count and Hb electrophoresis.

AT was diagnosed by employing amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) to see the presence or absence of $-\alpha$ -3.7, $-\alpha$ -4.2 and $\alpha\alpha\alpha$ anti-3.7 were performed at molecular department of laboratory. All informations were recorded on a pre-designed proforma.

DNA was extracted (QIAGEN DNA extraction Kit, Germany) from 200- μ L of blood sample collected in ethylene diamine tetra acetic acid (K3-EDTA), and $-\alpha$ 3.7 kb deletion was detected by amplifying the α -globin genes using forward and reverse primers (Integrated DNA Technologies, USA) in separate reactions. PCR was performed and its products were electrophoresed in an ethidium bromide stained 1% agarose gel with λ -Hind III digest as a marker 1.

The $-\alpha$ 4.2 kb deletion was detected by a gap PCR procedure using the same PCR conditions as for the $-\alpha$ 3.7 kb deletion. A forward primer 1 (Integrated DNA Technologies, USA) and a reverse primer 2 (Integrated DNA Technologies, USA) were used. The two primers were 5519bp apart, thus, in the PCR conditions described normal gene was not amplified. Only when α 4.2 kb α 2 gene deletion was present, a fragment of 1.319 kb was obtained. As a control for the PCR reaction, a normal region of DNA was also amplified by using forward primer 3 and reverse primer 4, in the same reaction.

For XMNI polymorphism, 10 ml of blood was collected in EDTA tubes, and the DNA was extracted. A 641 bp fragment of DNA flanking C-T polymorphism at-158 to the G γ gene was amplified with primers (Invitrogen, Carlsbad, CA). The amplified fragments were digested overnight at 37°C with 10 units of XMNI I restriction enzyme (Fermentas Life Sciences, Vilnius, Lithuania). Results were recorded after electrophoresis on 2% agarose gel and staining with ethidium bromide.

Data was analysed using SPSS17. Frequencies and percentages were computed for gender and AT. Mean and standard deviation (SD) were computed for age, weight and height. Effect modifiers, like age, weight, number of transfusion and gender, were controlled by stratification analysis to observe the effect on outcome.

Results

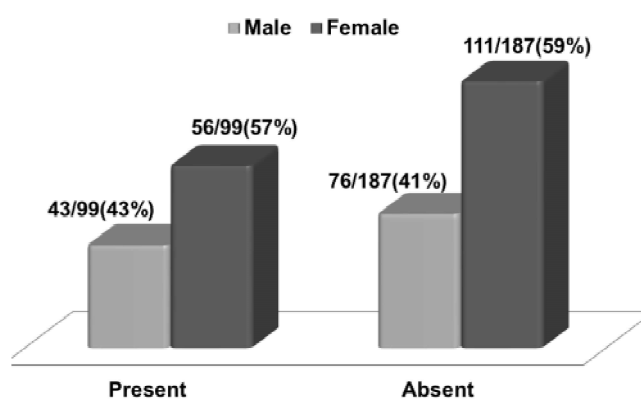
Of the 286 patients, 119(41.6%) were males. Among these, 99 (34.6%) patients showed AT coinheritance (ATC) (Table 1). The male to female ratio was 0.75:1 (Figure-1). In the ATC group, median age was 4.0 years. Mean Hb at presentation in ATC group was 7.1 ± 1.8 g/dl and 6.9 ± 1.7 g/dl in the AT non-coinheritance (ATNC) group (p-value >0.05). In the ATC group, 73 patients (73.7%) had $-\alpha$ 3.7 single gene deletion, 19(19.2%) had $-\alpha$ 3.7 double gene deletion, while $-\alpha$ 4.2 single gene deletion was found in 6 (6.1%) and $\alpha\alpha\alpha$ anti-3.7 was found in only one patient

Table-1: Patients Characteristics (n=286).

Gender n (%) Male	119 (42)
Female	167 (58)
Mean age of patients (years)	6.5±6.4
ATNC, n (%)	187(65)
ATC, n (%)	99 (35)

ATNC: Alpha Thalassemia non co-inheritance, ATC: Alpha Thalassemia co-inheritance

(Figure 2). In the ATC group, 50(50.5%) and 11(11.1%) patients received 1-20 and 21-40 times transfusions per year respectively, while the corresponding numbers in ATNC group were 125(67%) and 27(14.4%). Overall, 73(25.5%) patients had never been transfused, including 38 (13.3%) patients in the ATC group (Table 2). Mean age at first transfusion in the ATC group was 1.62±2.3 years whereas in ATNC group it was 1.43±2.2 years (Table 3). The commonest beta mutation was IVS1-5 in both groups (Table 4). XMN1 polymorphism was performed in 208(73%) patients and among them, 86(41%) were positive. Effect on transfusion frequency was assessed in these patients. In ATNC, XMN1 presence was causing a significant reduction in transfusion frequency (*p*-value 0.004) (Table 5).

**Figure-1:** Frequency of Alpha Thalassemia in Homozygous Beta Thalassemia stratified by Gender (n=286) n(%)**Table-2:** Frequency of Packed Red Cell Transfusions per year (n=286).

Number of transfusions/year	α-Thalassaemia n (%)			p-value	
	Present (n=99)		Absent (n=187)		
No history of Transfusion, n(%) (n=73) n (%)	38 (38.3)			35 (18.7)	0.001*
	Single gene deletion	Double gene deletion	Anti 3.7		
	29 (76)	9 (24)	-		
1 - 20 n (%) (n=175) n (%)	50 (50.5)			125 (67)	
	Single gene deletion	Double gene deletion	Anti 3.7		
	42 (84%)	7 (14)	1 (2)		
21 - 40 n (%) (n=38) n (%)	11 (11.1)			27 (14.4)	
	Single gene deletion	Double gene deletion	Anti 3.7		
	8 (73)	3 (27)	-		

Chi square test was applied - *p* value ≤0.05 was considered as significant, * Significant at 0.001 level.

Table-3: Age at first transfusion in transfusion dependent groups (n=213).

α-Thalassaemia	Age (years)			p-value
	<1	1-5	>5	
Present, n (%) (n=61)	30 (49)	29 (47.5)	2 (3.3)	0.008
Absent, n (%) (n=152)	103 (68)	39 (25.6%)	10 (6.5%)	

Chi square test was applied - *p* value ≤0.05 was considered as significant.

*Significant at 0.008 level

Table-4: Homozygous Beta Thalassemia mutations, n (%) .

Mutations	ATC cohort 71/99(72)	ATNC cohort 118/187(63)
IVS1-5	45(63.4)	43 (36.4)
IVS1-1	7(10)	8 (7)
Fr 8-9	6(8)	26 (22)
Cd 30	6(8)	6 (5)
Cd 5	2(3)	7(6)
Del 619	2(3)	13(11)
Uncharacterized	2(3)	6(5)
Cd 15	-	5(4.2)
Fr 16	1(1.4)	-
Fr 41-42	-	3 (2.5)
Cap +1	-	1 (1)

IVS: Intervening sequence, Fr: Frame shift, Cd: Codon, Del: Deletion, Cap+1: Cap site change of amino acid at position +1. ATC: Alpha Thalassemia co-inheritance, ATNC: Alpha Thalassemia non-co inheritance

Table-5: Effect of XMN on transfusion frequency (n=208).

Alpha	XMN	n	Number of transfusions per year Mean ± SD	p-value
Present	Present	32	5.06±8.4	0.86
	Absent	49	8.27±7.9	
Absent	Present	54	7.07±8.4	0.004
	Absent	73	11.34±7.7	

XMN: XMN 1 polymorphism

Discussion

Thalassemia is a globin gene disorder that results in a diminished rate of synthesis of one or more globin chains and consequently a reduced rate of synthesis of Hb in which that chain constitutes apart.^{1,2}

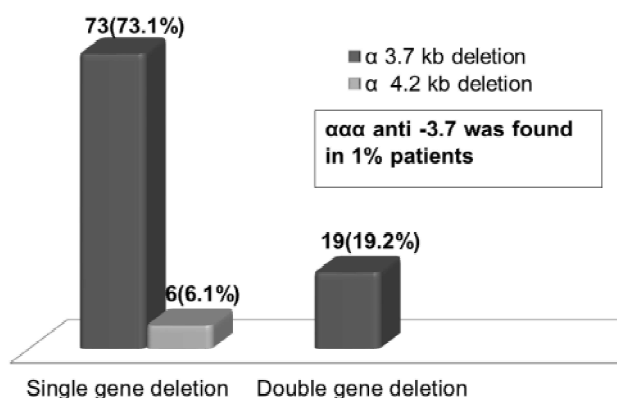


Figure-2: Types of Alpha Thalassemia Deletions (n=99).

The World Health Organisation (WHO) has identified control of haemoglobinopathies, particularly BT in the developing world, as a priority.⁵ Estimated 5000 to 9000 children with BT are born annually and the projected carrier rate is 5% to 7%, with 9.8 million carriers in the total population.⁹

There is a wide variation in the phenotypic presentation of BT ranging from severe transfusion-dependent β TM to less severe β TI.^{1,2} The mechanism underlying the pathophysiology of BT relates to imbalance in chain production with decreased or no beta chains and normal alpha chains.¹⁰ Genetic modifiers can impact the phenotypic severity of BT primarily by affecting the degree of globin chain imbalance and secondarily by moderating complications of the disease. Homozygotes or compound heterozygotes for BT who co-inherited AT have less redundant α -globin and therefore present with less severity. Patients who have co-inherited HbH ($--/\alpha$) with homozygous β are found to have TI.¹¹

The present study was planned to identify frequency of AT in homozygous BT patients in Karachi region. Previously, an Italian study¹¹ found that only a minority of the β TM patients had concomitant AT, but upto 19% of β TI patients with severe β chain mutations were having $-\alpha$ 3.7 mutation which is in comparison to but lower than the results of our study (34.6%). Similarly a study in northern India concluded that out of 73 cases of β TI, α -deletions were found in 16 out of 50 patients (32%).¹² Chen et al found β^+ -thalassaemia mutation as a main contributor of genes for the β TI patients in southern China; he also found reduction in disease severity in homozygous BT patients with ATC [(14/59), (23.7%)]¹³ which is slightly lower compared to our study.

Out of 285 thalassaemia patients in a local study, 24.6% patients had one or two α genes deleted which shows a clear difference from our study (99 of 286).

The α -thal-2 (α^+) deletions are the most common form of AT defects, with two major forms, $-\alpha$ 3.7 and $-\alpha$ 4.2 prevalent throughout the world. The 3.7 kb deletion is most prevalent in Mediterranean and African ethnic groups, while the 4.2 kb deletion is most common in Southeast Asians. These alleles have also been reported in Asian Indians.³

We have identified $-\alpha$ 3.7, $-\alpha$ 4.2, and $\alpha\alpha$ anti-3.7 alleles in the Pakistani population. The most prevalent alpha deletion in our cohort was $-\alpha$ 3.7 which is found in 92 patients (32.7%) with single gene in 73 (25.5%), whereas double gene in 19 (6.6%) respectively. The $-\alpha$ 4.2 and $\alpha\alpha$ anti-3.7 were the other two deletions observed in our cohort.

Mean age at first transfusion in the coinheritor group was 1.62 years whereas it was 1.43 ± 2.2 years without the coinheritor group which shows that presence of AT leads to a slight decrease in disease severity causing comparatively delayed presentation of symptoms.

The percentage of un-transfused patients in ATC group was significantly higher 38% compared to the ATNC group 18.7%. Transfusion-dependent group didn't show a significant difference between the two groups as 50.5% patients received 1-20 times and 11.1% received 20-40 times PRC transfusions per year in ATC group 125 (67%) and 27 (14.4%) respectively in the ATNC group.

In our study, single gene deletions were predominantly found and seems attributable to the findings in previous studies which mentioned that a single α gene deletion does not produce a significant ameliorating effect in β^0 -thalassaemia. This form was mostly prevalent in our cohort. However, among patients with β^+ thalassaemia, even a single α gene deletion is an important contributing factor in shifting the clinical spectrum of the disease.¹² The commonest beta mutations found in our cohort was IVS1-5, both in homozygous and heterozygous forms and in both ATC and ATNC cohorts.

XMN1 polymorphism was found in 86 of 208 patients. Effect on transfusion frequency was assessed in these patients. In the AT present group, XMN was not causing a significant effect on transfusion frequency ($p=0.86$). However, in ATNC group, XMN1 presence was causing a significant reduction in transfusion frequency ($p=0.004$).

This shows an independent effect of XMN1 polymorphism on transfusion frequency.

Increased expression of γ globin gene was seen in BT children who had XMN1 polymorphism at position-158. Variable expression was seen in heterozygous and homozygous states. This increased expression of γ globin gene and production of γ globin chain ameliorate the clinical phenotype of homozygous BT. Therefore, β T children with XMN1 polymorphism showed better response to HbF augmentation agents^{8,14} which also led to reduced or complete cessation of packed red cell transfusion.^{15,16}

A significant difference among un-transfused group and minor difference among transfusion-dependent groups is pointing towards the fact that there are other genetic modifiers which play an important role in disease pathogenesis and clinical amelioration of symptoms which need to be identified.

Detection of AT patients in homozygous BT may have therapeutic implications. An association between certain β gene mutations and a better response to hydroxyurea has been found in a previous study.¹⁷ AT, being one of the genetic modifiers of BT may produce much better outcome in ATC patients than in those with ATNC.

The main limitation noted in our study was that TI patients were also included and genetic modifiers, such as B-cell lymphoma/leukaemia 11A (BCL11) gene mutation etc, were not assessed. Larger cross-sectional studies are required to validate our results and identify other genetic modifiers.

These results points to the need for an extensive disease awareness programme, as well as molecular diagnosis before initiating treatment of Thalassaemia patients, and also to identify TI in various ethnic groups in Pakistan.

Conclusion

AT was present in more than one-third of homozygous BT patients. A significant proportion of co-inheritance patients were transfusion-independent, possibly because TI patients were also included in the sample, and the presence of IVS1-5 and XMN1 polymorphism might have caused amelioration of disease severity. Mean Hb as well as mean age at first transfusion were not significantly different between the groups. This could be due to the presence of single alpha gene deletion in majority of patients.

Disclaimer: The study was presented as a poster at the 8th International Hematologic Malignancies Conference ' Bridging the Gap 2017 (BTG2017) 'Bangkok, Thailand, on January 12-14, 2017, and its abstract was published in the BTG 2017 Abstract Book of the Conference (poster#1, page #1).

Conflict of Interest: None.

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