Effect of deletions in the α-globin gene on the phenotype severity of β-thalassemia

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Abstract

Thalassemia is the most common inherited hemoglobinopathy worldwide. Variation of clinical symptoms in this hemoglobinopathy entails differences in disease-onset and transfusion requirements. The aim of this study was to investigate the role of α-globin gene deletions in modulating the clinical heterogeneity of β-thalassemia (β-thal) syndromes. A total number 270 β-thal subjects were enrolled. Hematological parameters were recorded. β-Globin mutations were determined by amplified refractory mutation system-polymerase chain reaction (ARMS-PCR), gap-PCR and Sanger sequencing. α-Globin gene deletions were determined by multiplex PCR. Out of 270 β-thal subjects, 19 carried β⁺/β⁺, 74 had β⁰/β⁺ and 177 had the β⁰/β⁺ genotype. When we determined the severity of the different β-thal subjects in coinherited with the α gene deletion, it was revealed that, 84.2% β⁺/β⁺ subjects carried a non severe phenotype and did not have an α gene deletion. Of the β⁰/β⁺ individuals, 95.9% presented a severe phenotype, irrespective of α-globin gene deletions. In cases with the β⁰/β⁺ genotype, 19.2% subjects also carried a deletion on the α gene. Of these, 61.8% presented a non severe phenotype and 38.2% were severely affected. Only in the β⁺/β⁺ category did α gene deletions make a significant contribution (p < 0.001) toward alleviation of clinical severity. Therefore, it can be stated that α-globin gene deletions play a role in ameliorating the phenotype in patients with a β⁺/β⁺ genotype.

Introduction

Thalassemia syndromes are inherited monogenic disorders, primarily associated with anemia. Patients suffering from thalassemia display a wide variation in clinical presentations such as the age of presentation, transfusion requirements, hepatosplenomegaly and others. This ailment is caused by inherited mutations primarily in the β-globin gene (HBB) that limit the synthesis of hemoglobin (Hb) [1]. In the HBB gene, about 400 different aberrant loci have been identified that are responsible for β hemoglobinopathies or β-thalassemia (β-thal) [2]. Based on their position and nature, they result in β-thal major (β-TM) or β-thal intermedia (β-TI) types of β hemoglobinopathies. Mutations on the HBB gene include defective splicing or early frameshifts, resulting in complete disruption of mRNA synthesis, thus categorized as β⁰ mutations. On the other hand, certain substitution mutations may lead to alternative amino acids being placed or mutations in the promoter or locus control region (LCR), which may lead to retarded synthesis of the β chain, categorized as β⁺ mutations [3]. However, it has been observed in different populations, that the phenotypic heterogeneity and variations in clinical presentation and manifestations are not always explainable by the mutations solely on the HBB gene [4,5]. This leads to a diagnostic and therapeutic dilemma.

Several reports are available urging the need to delineate mutations/deletions on the α-globin genes for clinical management. The most common variations on the α-globin genes are large deletions of variable lengths. It has been shown that in most cases, despite there being mutations on the α-globin gene, the subjects remain clinically asymptomatic as there are two copies of the duplicated α gene in our genome. The role of α mutations as secondary modifiers, in lowering the disease burden when coinherited with mutations on the β-globin gene, has been under scrutiny. Several studies from Thailand, Sri Lanka, Bahrain, Central Africa, North America and as well as from India, have shown the...
the role of \(\alpha\)-globin gene deletions in the prediction of disease severity in subjects suffering from \(\beta\)-thal [6–13]. It has also been observed that in several cases of \(\beta\)-thal, the presence of \(\alpha\) mutations may not have distinct clinical effects [14]. Similarly, it has been noted, that patients with similar types of \(\beta\)-globin gene defects have clinically different phenotypes [4]. It is also noteworthy, that to date, about 400 such mutations have been described, but only about 20 of them are responsible for 80.0% of \(\beta\)-thalassemias [15]. Various authors have tried to explain this mild phenotype with \(\alpha\)-globin gene mutations, but this model failed to predict the phenotype in its entirety [16,17]. However, the association between \(\alpha\) and \(\beta\)-globin mutations remains to be elucidated with a proper molecular framework. Thus, the present study aimed to investigate the type of \(\beta\) hemoglobinopathy whose disease severity maybe influenced by deletions of the \(\alpha\)-globin gene, which may help in building the proper diagnostic protocol for better thalassemia management.

Fetal Hb (Hb F) level by itself as a compensatory mechanism and polymorphism \(-158\) (C>T) on the \(G\gamma\) gene, plays a significant role in the amelioration of clinical symptoms [18]. Taking all these variables into account and negating any bias, we tried to investigate the role of deletions on the \(\alpha\)-globin gene in phenotype severity.

Materials and methods

Subject information

Subjects of both sexes, with primary symptoms of hemolytic anemia, were screened. Those found positive for a \(\beta\) hemoglobinopathy (homozygous/compound heterozygous states) by high performance liquid chromatography (HPLC); the VARIANT II \(TM\) system (Bio-Rad Laboratories, Hercules, CA, USA) was used. Accordingly, 270 thalassemia subjects were recruited for the present study. A total of 89 normal subjects, diagnosed as normal by the HPLC method, were included in this study as controls. All the subjects were from West Bengal and the surrounding states of Eastern India. The subjects were further classified based on their phenotype status. Approval for this study was given by the clinical ethics committee, The University of Burdwan, Burdwan, West Bengal and the surrounding states of Eastern India. Written informed consent was obtained from each subject before the enrollment in this study.

Hematological data analysis

Clinical and hematological data were collected from the individuals’ previous medical records for the thalassemia subjects. Hematological parameters were determined for the control subjects according to standard laboratory procedures using an automated hematolgy analyzer, Abacus, 380 (Diatron, Budapest, Hungary).

Clinical severity or phenotype status

The clinical condition in terms of severity was determined based on the Burdwan University Thalassemia Severity (BUTS) scoring system [19]. In the present study, of the 270 thalassemia subjects, 200 carried severe phenotypes, and 70 were non-severe phenotypes (Table 1).

Sample collection and DNA extraction

Peripheral venous blood samples or buccal swab samples were collected for DNA extraction. DNA was extracted following the phenol chloroform extraction method. The quality and quantity of the extracted DNA were checked spectrophotometrically.

HBB mutation detection

For the detection of \(HBB\) mutations, we followed a stepwise mutation detection guide based on our earlier study [20]. As the frequencies of IVS-I-5 (G>C) (HBB: c.92+5G>C) and codon 26 (G>A) or Hb E (HBB: c.79G>A) were higher, ARMS-PCR was performed in the first step to detect the presence of these mutations as described previously [21]. In case the result turned out to be negative, in the second step, Sanger sequencing was performed, comprising part of the 5’ untranslated region (5’UTR), exon 1, intron 1, exon 2 and part of intron 2 of the \(HBB\) gene [20]. For the samples that showed no detectable mutation through the first Sanger sequencing, were further sequenced, comprising part of intron 2 and exon 3 of the \(HBB\) gene. If the above steps failed to detect the \(HBB\) mutations, in step four, gap-PCR was performed to detect the Asian Indian 619 bp (NG_000007.3: g.71609_72227del619) deletion on the \(HBB\) gene as described elsewhere [22]. All the PCR reactions were performed in a dual block thermal cycler, Prima Duo (HiMedia Laboratories, Thane West, Maharashtra, India).

HBB mutation category

The identified \(HBB\) mutations were clustered into \(\beta^+\) and \(\beta^0\) categories based on their functional and/or \(\beta\) chain production capacity, after comparing with the information laid down in the HbVar database [2]. The \(HBB\) mutations that result in low synthesis and/or altered function of the \(\beta\) chain were clustered under \(\beta^+\). Those \(HBB\) mutations that result in null or extremely reduced synthesis of \(\beta\) chain, were clustered under \(\beta^0\). Accordingly, the subjects were categorized into \(\beta^+/\beta^+\), \(\beta^0/\beta^+\) and \(\beta^0/\beta^0\) based on their \(HBB\) genotypes.

Detection of \(\alpha\) mutations

Eight common deleitional forms of \(\alpha\)-globin gene mutation \(-\alpha^{3.7}\) (rightward), \(-\alpha^{4.2}\) (leftward), \(-\alpha\)20;5, \(-\alpha\)SEA (Southeast Asian), \(-\alpha\)SA (South African), \(-\alpha\)MED (Mediterranean), \(-\alpha\)THAI (Thailand), \(-\alpha\)FIL (Filipino) were screened by the single tube multiplex gap-PCR method, as described earlier [23]. The \(-158\) (C>T) \(G\gamma\), XmnI polymorphism was identified by the PCR-restriction fragment length polymorphism (RFLP) described elsewhere [24].
Sixteen different mutations on the HBB gene were identified (Supplementary Figures 1 and 2). Based on the HbVar database, out of 16 mutations, 10 were categorized as β^0 type and six were categorized as β^+ type (Supplementary Table 1).

**α-Globin gene deletions**

Of the 270 subjects, 68 (25.2%) carried one or more deletions on the α-globin gene, while 202 subjects were negative for α gene deletions. Out of the eight types of deletions screened, only –α^3.7, –α^4.2 and –α^0/SEA were detected in this cohort. The remainder [–(α)^20.5, –α^0/SEA, –α^0/MED, –α^0/THAI, –α^0/FIL] were not detected in any of the studied subjects. Out of the 68 subjects, the –α^3.7 deletion was detected in 50 individuals (73.5%), of whom 46 were heterozygotes and four were homozygotes. The –α^4.2 deletion was found in 13 (19.1%) subjects, of whom 10 were heterozygotes and three were homozygotes. Two subjects (2.9%) were positive for the heterozygous –α^0/SEA deletion. Other than these, three subjects (4.4%) were positive for compound heterozygosity of both –α^3.7 and –α^4.2 deletions (Supplementary Figures 3 and 4). In the 89 control subjects, 37 (41.6%) had the dele- tional α gene mutations, of these, 14 were heterozygous for –α^0/THAI deletion and two were homozygous. Deletion of –α^4.2 was found in 16 subjects, of whom 11 were heterozygous and five were homozygous. Five subjects were positive for the compound heterozygosity of both –α^3.7 and –α^4.2 deletions.

**Comparisons of hematological data**

The mean Hb level, mean corpuscular volume (MCV), mean corpuscular Hb (MCH), red cell distribution width (RDW), and red blood cell (RBC) count of different α genotype subjects of β-thal and control group, are shown in Table 2. The change in the MCV value was not significant between the control subjects and thalassemia subjects with α gene deletion (p > 0.05). The changes in the parameters, such as MCH, RDW, RBC count, Hb level were significant between thalassemia subjects and normal controls in both α genotype groups, with α gene deletion(s) and normal α gene (p < 0.05) (Table 2).

**Effects of α-globin gene deletions on the clinical severity of HBB mutant genotype individuals**

Out of 19 individuals with a β^+ /β^+ genotype, 16 had no α gene deletions (αα/αα), and 12 of these were not clinically severe (Table 3). On the other hand, out of 74 β^0 /β^0 genotype individuals, 43 did not have α gene deletions, while 31 carried α gene deletions of any of the types reported in the previous section. In the 31 subjects, 29 presented as clinically severe. Finally, out of the 177 β^0 /β^+ individuals, 143 were without α gene deletion, while 34 carried α gene deletions. It was observed that, only in the β^0/β^+ category, α gene deletions made a significant contribution (p < 0.001) to the modulation of clinical severity (Table 3).

**Effects of Hb F**

The distribution of the mean level of Hb F was 28.5% in not severe and 35.2% in the severe phenotype group (Supplementary Table 3). The result shows a statistically significant correlation.

**Discussion**

In the present study, 270 subjects with confirmed diagnosis of β-thal with variations in clinical presentation were investigated. Of these, 70 were classified into the non-severe...
phenotype category and 200 into the severe phenotype category. To explain the phenotypic heterogeneity, they were also categorized by HBB genotypes into $\beta^+ / \beta^+$, $\beta^0 / \beta^+$ and $\beta^0 / \beta^0$ types, for revealing the genotypic variation of the primary modifier. To establish the role of the $\alpha$ gene deletion in the clinical heterogeneity, they were examined for the presence of the eight common types of $\alpha$ gene deletions.

The clinical severity of thalassemia patients varies from individual to individual based on the cumulative effects of different pathophysiological conditions, such as the age of presentation, baseline Hb value, transfusion interval, spleen size, and growth velocity [25]. It is well known that the pathophysiological effects depend on the position of the mutation on the HBB gene, resulting in a quantitatively and/or qualitatively altered protein. The modification affects its normal physical and/or biochemical characteristics [26].

Table 3. Distribution of subjects with $\alpha$-globin deletion mutations in the not severe and severe group in different $\beta$-thalassemia categories.

| HBB genotype | HBA genotype | Total n | Non severe | Severe | p Value |
|---------------|--------------|---------|------------|--------|---------|
| $\beta^0 / \beta^+$ (n = 19) | $\alpha^0 / \alpha^0$ (WT) | 16 | 12 | 4 | >0.99 |
| $\beta^0 / \beta^+$ (n = 177) | $\alpha^0 / \alpha^0$ (WT) | 143 | 31 | 112 | <0.0001 |
| $\beta^0 / \beta^+$ (n = 74) | $\alpha^0 / \alpha^0$ (WT) | 43 | 1 | 42 | 0.568 |

WT: Wild type.

mutation, i.e., $\beta^+ / \beta^+$ genotype, should have a less severe effect than the individual with two copies of $\beta^0$ mutation, i.e., $\beta^0 / \beta^0$ genotype. Accordingly, in the current study, it was observed that 15 (78.9%) subjects with a $\beta^+ / \beta^+$ genotype were non severe, while 71 (95.9%) subjects with a $\beta^0 / \beta^0$ genotype carried a severe genotype (Table 3). Thus, the clinical severity of these two extreme HBB genotypes can be explained by the HBB mutation itself. However, in the subjects with a $\beta^+ / \beta^0$ genotype, as there is a chance of synthesis of $\beta$ chain with or without altered function, the clinical condition may not be reflected through the HBB genotype only [27]. Therefore, in this study, in 177 subjects, 52 belonged to the non severe category and 125 to the severe category.

To understand both types of clinical conditions with the same type of HBB genotype, the role of the $\alpha$ gene deletions was hypothesized for further investigation.

Previous studies from Congo, USA, India and Bahrain, have shown the role of $\alpha$-globin gene number in disease severity of Hb S (HBB: c.20A>T)/$\beta$-thal [10–13]. Similarly, coinheritance of $\alpha$ gene deletions was found to be a disease modulating factor of Hb E/$\beta$-thal in Thailand, India and Malaysia, occurring in around 18.0, and 12.9 and 6.7% patients, respectively [4,8,28,29]. However, when we analyzed the extent of deletion on the $\alpha$ gene, based on heterozygosity ($-\alpha / \alpha\alpha$) and homozygosity/compound heterozygosity ($-\alpha / -\alpha\alpha$ or $-\alpha / -\alpha\alpha$), coherited with different types of HBB genotype, it was observed that in the $\beta^+ / \beta^+$ genotype, 63.2% (12/19) subjects were non-severe and without an $\alpha$ gene deletion. On the other hand, in the $\beta^0 / \beta^0$ genotype, 95.9% (71/74) subjects were of the severe category, with and without an $\alpha$ gene deletion. Accordingly, it was deduced that there is no significant role of $\alpha$ gene deletion in the modulation of clinical severity in the $\beta^+ / \beta^+$ or $\beta^0 / \beta^0$ patients ($p > 0.99$ and $p = 0.568$, respectively) (Table 3). In contrast, it appeared that $\alpha$ gene deletion significantly ($p < 0.001$) modulated clinical severity in the $\beta^+ / \beta^0$ genotype, where 11.9% (21/177) subjects with an $\alpha$ gene deletion were of the non severe category (Table 3). Similar observation was also reported from Sri Lanka and Thailand, where 9/50 (18.0%) and 9/58 (15.5%) $\beta$-thal subjects ($\beta^+ / \beta^0$) with an inherited $\alpha$ gene deletion showed a
milder phenotype [9,30]. Thus, the observations of the present study support the earlier reports, that an α gene deletion can ameliorate the severity of β-thal, but only in the case of a β⁺/β⁰ genotype [9,30].

The sample analysis revealed that the mean level of Hb F was higher in the severe group and lower in the non severe group, the observation was statistically significant (Supplementary Table 3). The XmnI polymorphism was observed to be present in heterozygous and homozygous states in 71.8% of randomly selected samples from the defined population under study. There was no statistically significant difference between the presence and absence of the XmnI polymorphism in the clinically severe and non severe groups (Supplementary Table 2). Thus, the bias of Hb F level or one of its modifier gene loci, was negated before the role of α gene deletions were ascertained in this study.

Our attempt to explain the clinical heterogeneity based on α gene deletions yielded insignificant results in both β⁺/ β⁰ and β⁺/β⁺ genotype groups, possibly because amelioration is only feasible when a parameter can be synthesized enough to be modified. For example, in β⁺/β⁰ individuals, where β-globin was not synthesized, the presence of α-globin cannot modulate the Hb production. The impact of α gene deletions in lowering the clinical severity with the β⁺/β⁰ genotype, may be due the reduced synthesis of α-globin chain to maintain α- to β-globin ratio. In β⁺/β⁰ individuals with a normal α-globin gene, the relative excess of α-globin chain triggers the ineffective erythropoiesis [3]. Thus, α gene deletion in these individuals can maintain the α- to β-globin ratio and produce the non severe phenotype.

There are certain limitations of the present study. Firstly, it was reported that the α-globin gene may possess several mutations other than deletional mutations, and the present study was based only on the deletional mutations. Secondly, it could not explain the presence of a significant number of individuals with the β⁺/β⁰ genotype and a single individual with a β⁰/β⁰ genotype not carrying α deletions who was still clinically presenting as non severe. This may be due to the effect of other modifier genes.

In summary, subjects carrying the β⁺/β⁰ mutant allele have one nonfunctional β allele and one partially functional allele. The clinical severity in these subjects varies from mild to severe. Therefore, the α-globin gene deletion may play a role to compensate the α- to β-globin chain ratio and thus help in producing the mild phenotype in the β⁺/β⁰ genotype subjects. The results were derived from the patients of West Bengal and the surrounding states of India and can be valid for other populations. The findings of the present study may also be helpful in thalassemia management.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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