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ORIGINAL ARTICLE

Association of alpha hemoglobin–stabilizing protein (AHSP) gene mutation and disease severity among HbE—beta thalassemia patients

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Abstract

In this study, we aimed to investigate the pattern and association of genetic mutations occurring within the alpha hemoglobin–stabilizing protein (AHSP) gene among HbE beta thalassemia patients with varying phenotypic expressions. Fifty-four diagnosed cases of HbE beta thalassemia (transfusion dependent and independent) were included in the study. Among them, 38 patients with similar genotypes (IVS 1–5, alpha gene deletion and triplication, Xmn polymorphism) were selected for further analysis. AHSP gene sequencing was done for these 38 samples to study associated mutations in AHSP gene. HbE beta thalassemia patients with similar genotypes but different phenotypic expressions were found to have mutations in the AHSP gene. There were five mutations found most prevalent among the samples analyzed for AHSP gene sequencing. Among these, two mutations were from intron 1 region of AHSP and three mutations were found in exon 3. The most prevalent mutation was found at the Oct binding site at intron 1 of AHSP. The mutations in exon 3 were more prevalent among the TDT groups. A mutation in exon 3 changing the amino acid (33rd) from serine to phenylalanine was found to be associated with only TDT group. This study documents that among the HbE beta thalassemia patients with varying severity, an exon mutation in AHSP is significantly prevalent only among the TDT group. Further understanding of the mechanism will shed light upon the impact of AHSP in modifying the disease severity in thalassemia.

Keywords Thalassemia · Globin gene · Transfusion · Phenotype · HbE beta thalassemia

Abbreviations

NTDT	Non-transfusion-dependent thalassemia
TDT	Transfusion-dependent thalassemia
HPFH	Hereditary persistence of fetal hemoglobin
AHSP	Alpha hemoglobin–stabilizing protein
RBC	Red blood cells
Hb	Hemoglobin
HbF	Fetal hemoglobin

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Introduction

Thalassemia and other hemoglobinopathies are the most common monogenic disorders in the world. Thalassemia is known to be associated with a diverse range of clinical expression [1]. Hemoglobin E-beta thalassemia (HbE/β-thalassemia) is the genotype responsible for one of the most common forms of thalassemia worldwide. The highest frequencies of HbE/β-thalassemia are observed in India, Bangladesh, and throughout Southeast Asia. The phenotypic presentation of HbE β-thalassemia is highly variable ranging from those who never require a blood transfusion to those dependent on it for survival. In the last decade, various genetic factors like the type of beta gene mutation, alpha gene mutations, various polymorphisms, presence of HPFH, and environmental factors like malarial infection have been identified as a modifier of the severity of this disease but still, the reasons for such a varied clinical presentation are not completely understood. Considering pathophysiology of thalassemia, the level of

Table 1 Phenotypic assessment of patients

		Sample number	Mean	Minimum	Maximum	*p* value
Age	TDT	33	18.48	5	58	
	NTDT	21	30.19	19	61	0.002
	Total	54	23.04	5	61	
Age of diagnosis (year)	TDT	33	5.26	2	42.0	
	NTDT	21	20.57	3.0	55.0	< 0.001
	Total	54	11.21	2	55.0	
Ferritin (ng/ml)	TDT	33	1737.60	479.40	3500.00	
	NTDT	21	685.16	145.00	3000.00	< 0.001
	Total	54	1328.31	145.00	3500.00	
Height (cm)	TDT	33	131.621	90.0	172.0	
	NTDT	21	154.214	140.0	167.0	< 0.001
	Total	54	140.407	90.0	172.0	
Weight (kg)	TDT	33	32.79	18	60	< 0.001
	NTDT	21	45.19	36	58	
	Total	54	37.61	18	60	
Baseline hemoglobin (g/dl)	TDT	33	7.46	3.80	10.40	
	NTDT	21	6.50	5.10	8.90	0.021
	Total	54	7.09	3.80	10.40	
Hb F(percentage)	TDT	33	8.98	.6000	40.70	
	NTDT	21	20.03	3.2000	42.50	< 0.001
	Total	54	13.28	.6000	42.50	

excess alpha globin chain causes RBC damage which is the major factor that impacts upon the clinical expression in thalassemia. The amount of excess alpha globin chain can be influenced by three major factors: (i.) amount of alpha globin chains produced; (ii.) amount of non-alpha globin chains available (beta, gamma chains); (iii.) the mechanism and efficiency of chaperone activity that stabilizes the insoluble free alpha globin chain. The amount of free alpha chain finally may be related to the efficiency of the proteolytic mechanism of the erythroid precursors and/or to the mechanism associated with stabilization of insoluble alpha globin chain [2]. Alpha hemoglobin–stabilizing protein (AHSP) is an abundant erythroid protein that specifically binds free alpha globin, stabilizes its structure, and limits its ability to participate in chemical reactions that generate reactive oxygen species [3–7]. The evidences suggest that AHSP acts as a molecular chaperone for free α-globin chain, and the altered expression or function of AHSP might account for some of the clinical variability observed in thalassemia patients [8]. Several studies have attempted to assess the relationship between AHSP and the severity of β-thalassemia [5, 7, 9–14]. Literature review shows that there are few studies on AHSP in thalassemia patients worldwide. In this study, we aimed to investigate the pattern and association of genetic mutations occurring within the AHSP gene in HbE beta thalassemia and its correlation with disease phenotypes.

Methodology

Fifty-four diagnosed cases of HbE beta thalassemia, both transfusion dependent (TDT) and non-transfusion dependent (NTDT), were recruited for this study. Diagnosis of HbE beta thalassemia was done by HPLC (Bio-Rad Variant II machine using beta thalassemia short program). All age groups and both sexes were included.

Clinical parameters

A detailed history of age at diagnosis, age of first transfusion, transfusion requirement/year, chelation therapy, etc. were taken from the patients/parents. Thorough clinical examination was done and clinical parameters—degree of pallor, spleen size, and growth were recorded.

Laboratory data/evaluation

(a) Hematological parameters

Complete hemogram was performed on Sysmex (KX-21) automated cell counter. Hemoglobin analysis was estimated by high-performance liquid chromatography (HPLC, from Bio-Rad Variant II), and Feritin level was measured by ELISA based method.

Table 2 Transfusion parameters among TDT and NTDT patients

	TDT	NTDT
Number	33	21
Transfusion requirement	≥ 8 BT/year	< 8 BT/year
Average of total transfusions received	162.93 BT	24 BT

DNA isolation and mutation analysis

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) by proteinase-K digestion and a standard high salt-extraction method. Beta globin gene mutations [CD26 (G→A) for HbE and other common beta mutation] analysis were carried out by ARMS PCR. XMN 1 polymorphism was identified following primer sets and protocol by Sanjana et al., 2012 [15]. Alpha deletions (3.7 and 4.2) were detected by using the primer set and protocol by GAP PCR [16] with necessary modifications with the concentrations of primer and high GC content stabilizers (DMSO, Betaine) to amplify the respective amplicons using Qiagen Hot Taq polymerase. Alpha gene triplication (3.7 and 4.2) was detected by GAP PCR [17].

DNA sequencing analysis

The DNA sequencing analysis was done by Applied Biosystems® 3500 Series Genetic Analyzers following the manufacturer protocol. To sequence the AHSP gene, an amplicon of 895 base pair was amplified spanning from intron 1 to exon 3 of AHSP gene. The PCR and primer designing was done following Viprakasit et al. [7] and dos Santos et al. [10]. The amplicons then were sequenced using manufacturer protocol. Analysis of the sequences and SNP detection were done using Globin Gene Server, NCBI database, and SecScape (Applied Biosystems®), SnapGeneViewer, ClustalW software. The reference sequence for analysis was used from NCBI database (NG_046852 for AHSP gene).

Table 3 Genotype analysis

Type of mutation	Mutation obtained ($n = 54$)	Percentage (%)
IVS 1–5 heterozygous	43	79.0
Alpha 3.7 deletion	4	7.4
Alpha 4.2 deletion	1	1.9
Alpha triplication 3.7	0	0
Alpha triplication 4.2	0	0
XMN heterozygous	38	70.4

Table 4 Phenotypes of the selected 38 patients having similar mutation profile

	Patients with similar mutation profile (after excluding alpha mutation, XMN homozygous, beta mutations other than IVS 1-V)	
	TDT	NTDT
Number	23	15
Average age (year)	18.34	30.33
Average age of diagnosis (year)	4.92	19.35
Mean ferritin value (ng/ml)	1543.26	546.17
Mean baseline Hb (gm/dl)	7.24	6.7
Mean Hb F level (percentage)	8.84	20.43
Average total transfusion	155.52 BT	9.73BT

Statistical analysis

Categorical variables are expressed as the number of patients and percentage of patients and compared across the groups using Pearson's chi square test for independence of attributes. Continuous variables are expressed as mean standard deviation and compared across the 2 groups using unpaired *t* test. The statistical software SPSS version 20 has been used for the analysis. An alpha level of 5% has been taken, i.e., if any *p* value is less than 0.05, then it has been considered as significant.

Result

Phenotypical parameter Fifty-four HbE beta thalassemia patients were initially included in the study. The phenotypic parameters (age, age of diagnosis, height, weight, baseline

Table 5 Description and prevalance of mutations in AHSP gene

Type of mutation	Mutation found		*p* value	Position at AHSP (Ng_046852)
	TDT ($n = 23$)	NTDT ($n = 15$)		
OCT binding	18 (78%)	12 (80%)	0.898	5258 (G-C) intron 1
-1 to start codon	8 (35%)	7 (47%)	0.464	5288 (G-C) intron 1
Leu > Leu 31st AA	14 (61%)	0 (0%)	< 0.001**	5624 (C-T) exon 3
Phe > Phe 47th AA	15 (65%)	0 (0%)	< 0.001**	5672 (C-T) exon 3
Ser > Phe 33rd A.A	15 (65%)	0 (0%)	< 0.001**	5629 (C-T) exon 3

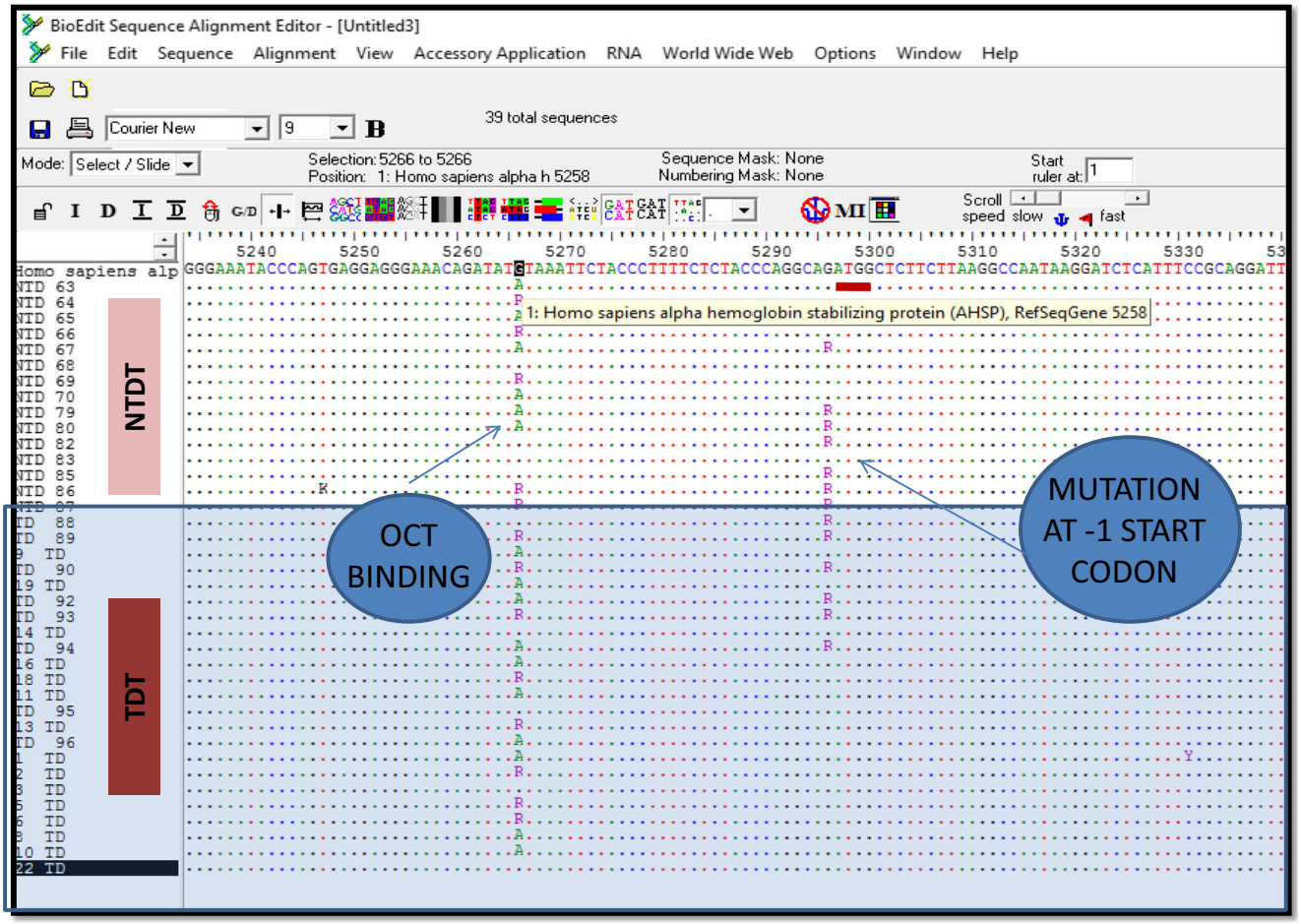

Fig. 1 AHSP mutation identification by ClastalW homology analysis. (AHSP gene mutation analysis shows the presence of mutation at the Oct binding site and -1 to start codon in intron 1 by ClastalW homology analysis among NTDT and TDT groups)

Hb, HbF, transfusion requirement, total transfusion received) were compared among transfusion dependent (TDT) and non-dependent (NTDT) arms (Tables 1 and 2). Age at presentation/age at diagnosis was significantly lower in TDT arm (p value < 0.001) which is understandable as TDT patients with higher severity show symptoms at an earlier age than NTDT HbE beta thalassemia patients. HbF percent was higher in NTDT arm in comparison with TDT (p value < 0.001). Ferritin level was lower (p value < 0.001) in NTDT (685.16) in comparison with the TDT group (1737.60).

Genetic mutations The prevalence of various (Table 3) genetic mutations analyzed showed that forty-three out of 54 samples (79%) had IVS 1–5 mutation in the beta globin gene. None of the 54 samples were detected to have alpha triplication (3.7 and 4.2). Four samples were found to have alpha 3.7 alpha deletion and one had alpha 4.2 deletion. Xmn polymomorphism (heterozygous) was found to be present in 70% of the samples (38 out of 54). To have a homogenous genetic background, samples carrying IVS 1–5 mutation, normal alpha globin genotype (i.e., absence of alpha 3.7/ 4.2 deletion and triplication), and Xmn polymorphisms were selected for further analysis. Thus out of 54 samples, 38 HbE beta patients having similar genotype with varying phenotypic severity were subjected to AHSP gene mutation analysis by DNA sequencing. Among these 38 patients, 23 were transfusion dependent (TDT) and 15 were non-transfusion dependent (NTDT). The phenotypic parameters (age, age of diagnosis, height, weight, baseline Hb, HbF, transfusion requirement) were compared (Table 4) among the selected 38 patients (TDT and NTDT) having homogenous genetic background with IVS 1–5 mutation, normal alpha globin genotype (i.e., absence of Alpha 3.7/ 4.2 deletion and triplication), and Xmn (heterozygous) polymorphism. The values were consistent with the patterns found among the TDT and NTDT groups in the total sample populations (Tables 1 and 2).

AHSP sequence analysis Thirty-eight samples were subjected to AHSP mutation identification by DNA sequencing analysis. For AHSP gene sequencing, an amplicon of 895 base pair spanning from intron 1 to exon 3 of AHSP gene was amplified and

Fig. 2 Position of the intron mutations in AHSP gene. [Identification and analysis of position of AHSP mutations at the Oct binding site (indicated by triangle) and -1 to start codon mutation (indicated in circle) in intron 1 of AHSP by SnapGene viewer analysis]

purified. Five mutations (Table 5) in AHSP gene were found to be most prevalent among the samples studied. Two mutations were found in the intron 1 (Figs. 1 and 2) region of AHSP and three mutations in exon 3 (Fig. 3). The mutations at OCT binding site at intron 1 of AHSP were the most prevalent (more than 75%) in the studied population. The distribution of this mutation was almost equal in both TDT and NTDT arms. A mutation in the exon 3 causing a change in the 33rd amino acid from **serine** to **phenyl alanine** (C-T at 5629 nucleotide position human AHSP gene NG_046852) was present only among the TDT group (p value < 0.001) (Table 5). Among the TDT patients, 65% had the mutation causing a change in the 33rd amino acid from **serine** to **phenyl alanine**. The phenotypic parameters (age, age of diagnosis, height, weight, baseline Hb, HbF, transfusion requirement) were compared (Table 6) among the patients carrying the AHSP changing 33rd A.A against the patients not carrying the same mutation. The average of total transfusions was found to be higher (170 BT) among the patients carrying the above-said mutation. Two other prevalent mutations (Table 5) in exon 3 were silent mutations coding for the same amino acid at the 31st amino acid (CT***C*** to CT***T*** > Leu) and 47th amino acid (TT***C*** to TT***T*** > Phe) position of AHSP.

Discussion

It has already been well understood that it is not only the mutation in beta globin gene but also the ratio of beta globin:alpha globin level determining the level of free alpha globin chain that leads to phenotype expression in thalassemia. The excess alpha globin induces ROS generation and promotes RBC destruction. Alpha hemoglobin–stabilizing protein (AHSP, also known as erythroid differentiation-related factor EDRF, and erythroid-associated factor, ERAF) is a chaperone protein that binds free α-globin and stabilizes it [2]. Interaction with AHSP inhibits reactive oxygen species production by α-globin and prevents the precipitation of highly unstable, cytotoxic free α-globin chains, which aggregate in erythroid precursors, damaging the membrane and triggering cell death [3].

It was shown that when AHSP-deficient mice were bred to β-thalassemia mice, loss of AHSP increased the severity of the thalassemia [4]. AHSP mutations in thalassemia patients are not well studied and, to date, the association between AHSP mutation and β-thalassemia variability has not been clearly established; however, there exist evidences showing the association between β-thalassemia severity and AHSP

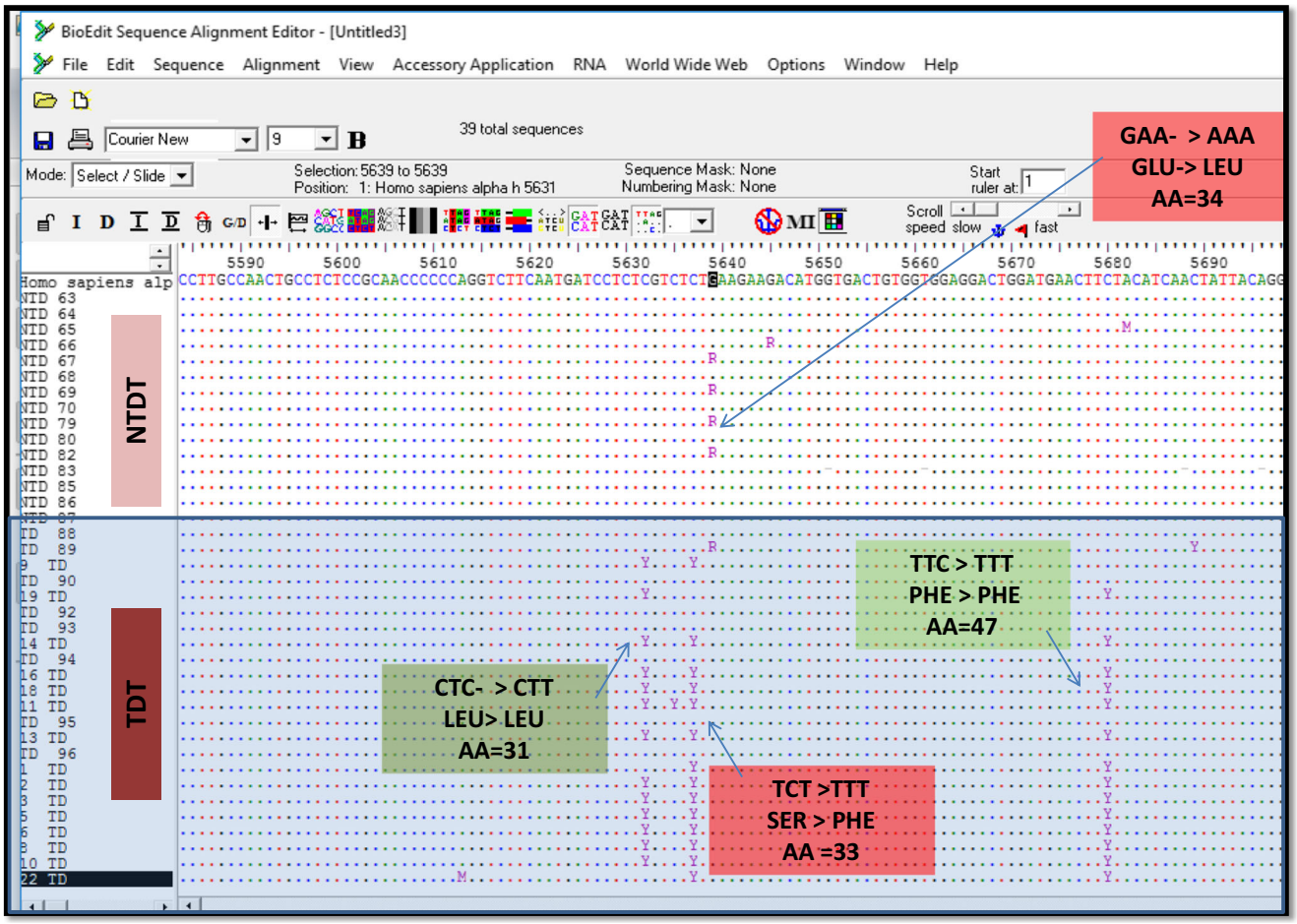

Fig. 3 Mutation identification by ClastalW homology analysis [AHSP gene mutation analysis shows the presence of mutations (as indicated by arrows) in the exon 3 by ClastalW homology analysis among NTDT and TDT groups]

genotype [7, 8, 12]. Dos Santos (2008) [10] conducted a study of AHSP genetic mutation in population and observed the presence of missense mutation (N751) altering the protein function of AHSP beside the prevalence of a commonly found mutation at intron 1 of AHSP in the binding site of Oct transcription factor which regulates AHSP expression. Other than the mutation in the Oct transcription factor binding site, mutations at the GATA binding site have also been observed to be associated with altered function of AHSP [11].

In this study, we did not find any mutation at GATA binding site; the mutation at Oct binding site in intron 1 was the most prevalent among the five mutations found in the study. It had a similar prevalence in both the TDT and NTDT groups. The impact of this mutation at expression level and functionally is not known as this mutation has not been reported earlier. There were two silent mutations observed in exon 3 (Leu > Leu) at the 31st amino acid and Phe > Phe at the 47th amino acid of AHSP) present only among the TDT groups. However,

Table 6 Phenotypes of the patients with and without the AHSP (33rd A.A) mutation

	TDT with AHSP mutation (33rd amino acid)	TDT without AHSP mutation (33rd amino acid)
Number	15	8
Average age (year)	21.6	12.25
Average age of diagnosis (year)	5.34	4.15
Mean ferritin value (ng/ml)	1486.43	1649.82
Mean baseline Hb (gm/dl)	6.83	8.01
Mean Hb F level (percentage)	8.87	8.86
Average total transfusion	170 BT	128.37 BT

these two mutations at exon 3 were found in high prevalence (> 60%) among the TDT group and were statistically significant, but they should not have any impact on the phenotypes as these do not produce any change in amino acid sequences. There was another mutation changing the 33rd amino acid from Ser to Phe in the exon 3 present only among the TDT group.

The exon mutation in AHSP that is prevalent only among the TDT patients causes the substitution of the hydrophilic and water soluble amino acid moiety (Serine) to a hydrophobic amino acid (Phe) which is likely to affect the structural formation of AHSP and thus its function. AHSP (PDB 3ovu) which is a 102 residue protein adopts a three helical chains (helices 1, 2, and 3). Helices 1 and 2 form a loop which is key segment to the recognition and interaction with α-Hb [3, 6]. A proline residue (Pro-30) resides within the loop segment (PDB 3ovu) that directs the cis and transform of AHSP [6]. The Pro 30 residue in AHSP has been found to play an important role in regulating AHSP-mediated stabilization of α-Hb [18]. The mutation found at the 33rd amino acid (Ser-Phe) in this study is adjacent to Pro 30 and also resides within the loop (Fig. 4) between helix 1 and helix 2; thus, a change in the Ser-33 is likely to affect the tertiary structure of the protein. Beside this, serine side chains are hydrogen bonded and this amino acid is known to be commonly present in the active site whereas the phenylalanine amino acid, that is replacing serine, is a hydrophobic amino acid and does not have a free OH group in the side chain. The bioinformatics structural simulation analysis by Jasmol and Pymol Program (Fig. 4) postulates that the OH group at the side chain of Ser33 is likely to form a hydrogen bond with the side chain Asp 36 in the tertiary structure formation of AHSP. Replacing Ser 33 with Phe will disrupt the hydrogen bond between the free OH group of Ser side chain with the adjacent amino acid which is likely to be crucial for the structural integrity of helix 1 and the loop (Fig. 4). Thus, the substitution of Ser with Phe is likely to affect the structural formation of AHSP impacting on its function.

Conclusion

This study documents that among the HbE beta thalassemia patients with varying severity, an exon mutation in AHSP is significantly prevalent only among the TDT group. Further

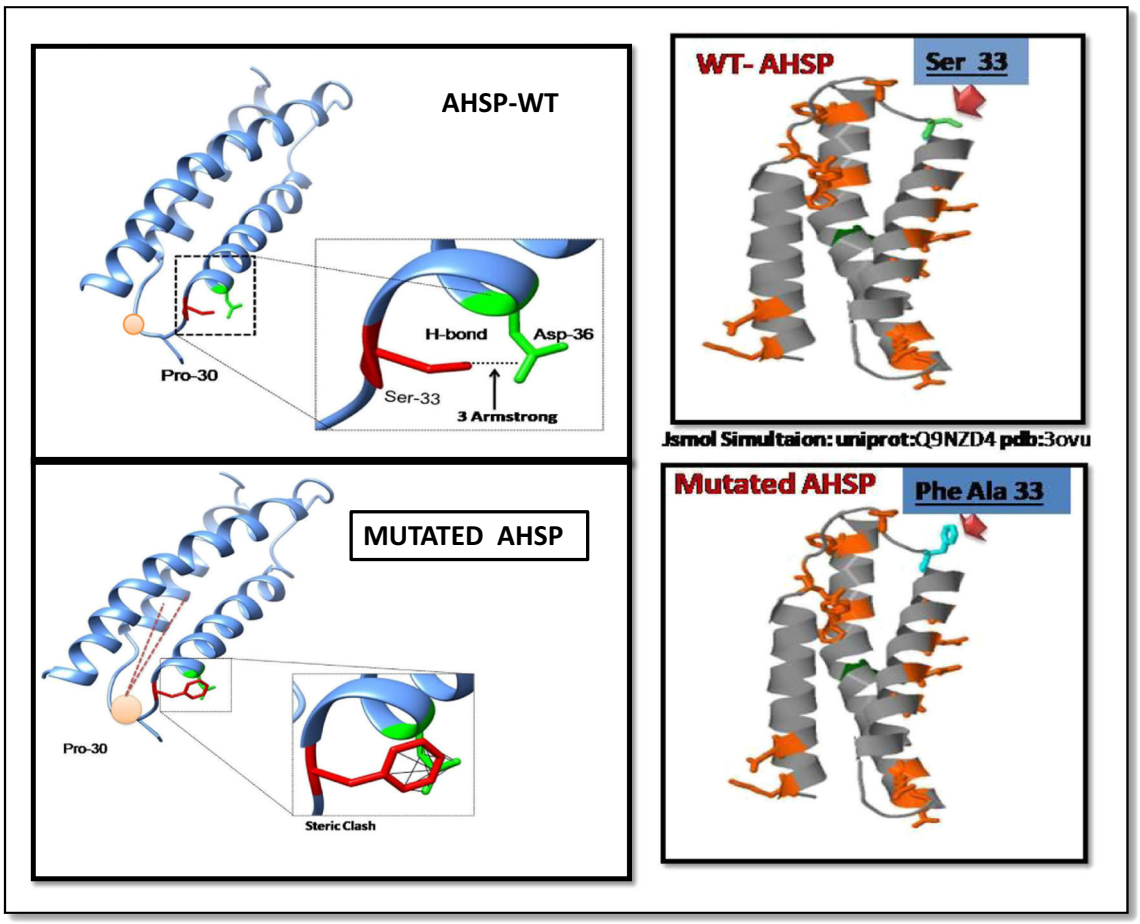

Fig. 4 Structure of AHSP and impact of mutation (Ser 33) in AHSP (replacing Ser33, adjacent to the loop between two helices and the Pro 30 residue, with phenylalanine which lacks free hydrogen group at its aromatic side chain is likely to alter the intramolecular bonding)

understanding of the mechanism will shed light upon the impact of AHSP in modifying the disease severity in thalassemia.

Compliance with ethical standards

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

Informed consent was obtained from all patients for being included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

Financial relationship There is no financial relationship involved with the organization sponsored the research.

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