

RESEARCH ARTICLE

Molecular characterization of beta-thalassemia reveals the presence of common mutations in the population of Himalayan region: Garhwal (Uttarakhand), India

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Abstract

Thalassemia is a dreadful heritable hemolytic disease, characterized by a genetic mutation in the hemoglobin subunit beta (HBB) gene. Mutation in HBB gene completely halts the production of the beta-globin protein, which leads to the defective production of functional hemoglobin. The prevalence of this disease is reported only in some specific geographical regions of India. Hence, the aim of this study was to screen the population of Garhwal for beta-thalassemia (β -thalassemia) and thus find out the prevalence in the inhabitants through molecular characterization. For this study, 4,081 individuals were considered, out of which only the ones with elevated HbA₂ levels (64) were subjected to molecular characterization. Mutational studies were carried out for the five most common mutations prevalent in the Indian subcontinent, that is, IVS 1-5 G-C, IVS 1-1 G-T, Codon 41/42 (-TCTT), Codon 8/9, and 619 bp deletion. The present study reports a frequency of 0.5% for β -thalassemia mutations among the subjects we have studied. The analysis of mutation spectrum revealed highest prevalence for IVS-1-5 (G-C) (18.75%) followed by Codon 8/9 (12.5%) and IVS-1-1 (G-T) with 6.25%. Codon 41/42 (-TCTT) and 619 bp deletion were found to be absent in our study population.

Keywords: Beta-thalassemia; Molecular characterization; Mutation; Beta-globin gene; India

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1. Introduction

Thalassemia is inherited, meaning that at least one of the parents must be a carrier of the disorder (Bajwa & Basit, 2022). It is caused by either a genetic mutation or a deletion of certain key gene fragments. Thalassemia patients have fewer than normal hemoglobin molecules. Red blood cells can carry oxygen because of hemoglobin. Anemia and fatigue are major symptoms of thalassemia, so patients of thalassemia in its severe variants may necessitate frequent blood transfusions. However, unlike thalassemia, in anemic condition, the body does not have enough normal and healthy red blood cells.

Thalassemia was originally thought to be the characteristic of tropics and subtropics but due to migration, it is now becoming a substantial global concern (De Sanctis

et al., 2017). Thalassemia is a group of inherited blood disorder transferred from parents to their offspring in an autosomal recessive manner. It is characterized by the abnormal production of hemoglobin. There are two forms of thalassemia: Alpha and beta. The beta-thalassemia (β -thalassemia) is characterized by the mutations in the beta-globin gene, which leads to reduced synthesis or absence of beta-globin chain and is responsible for causing β -thalassemia disorder in individuals from different ethnic groups. The DNA polymorphism in the β -globin gene shows a considerable amount of variability and form a series of patterns (i.e., haplotypes), which occur at varying frequencies among Asian Indians (Kazazian *et al.*, 1984). For the 1st time, the common seven mutations and their haplotypes in the Indians – Frameshift β 8-9 (+G), Nonsense Codon 15 (TGG-TAG), Frameshift β 41-42 (-TCTT), Frameshift β 16 (-C), IVS-1-5 (G-C), 619 bp deletion, and 13 and 25 nucleotide deletion, at 3' end of the gene were reported by Kazazian *et al.* team in the year 1984. According to them, deletion of 619 bp was found to be a common mutation in the Asian Indians. Recent studies reveal that each year between 300,000 and 400,000 babies is born with serious hemoglobin disorder in low- or middle-income countries, out of which 23,000 account alone for β -thalassemia major with about 90% of these births (De Sanctis *et al.*, 2017).

The carrier frequencies of β -thalassemia vary in different parts of the world from 1 to 20% or they may be even higher in some cases (Black *et al.*, 2010). In India alone, the numbers of β -thalassemia patients are approximately 30 million. About 10% of the total world thalassaemic individuals are born in India every year (Bashyam *et al.*, 2004). Certain communities in India, such as Sindhis (Colah *et al.*, 2010), Gujaratis (Bhukhanvala *et al.*, 2013), Punjabis (Grow *et al.*, 2014), and Bengalis (De *et al.*, 1997), are commonly affected with β -thalassemia and the incidence varies from 1% to 17% (Gupta *et al.*, 2003). As far as in studies conducted in different parts of the world, more than 150 – 200 mutations causing β -thalassemia have been reported (Cao & Galanello, 2010). While studies conducted in India have identified about 28 mutations in Indian populations (Old *et al.*, 2001), out of these 28 mutations, five to six mutations are found to be common. These include IVS 1-5 (G-C), 619 bp deletion, IVS 1-1 (G-T), Frameshift 8/9, 41/42, and Codon 15 (Bandyopadhyay *et al.*, 2004). The type of mutation varies across different ethnic groups (Bashyam *et al.*, 2004). The frequency of mutations in carriers varies from region to region with the predominant mutation being IVS 1-5 (G-C) (Agarwal *et al.*, 2000), which reflects the ethnic and genetic diversity of populations. The heterogeneous populations belonging to the Indian

subcontinent have been widely studied (Grow *et al.*, 2014), by various researchers in Pakistan (Usman *et al.*, 2009), Sindh (Jawahirani *et al.*, 2007), Punjab (Garewal & Das, 2003), Gujarat (Bhukhanvala *et al.*, 2013), Tamil Nadu (Colah *et al.*, 2009), Maharashtra (Ambekar *et al.*, 2001), and Kerala (Edison *et al.*, 2008). However, baseline data on thalassemia were completely unavailable in the populations of Garhwal region. Since the Garhwal Himalayan region is still unexplored due to the unpredictable climatic and weather conditions, the remoteness and location of the place make the research unfeasible in such an area. Individuals are limited to their daily needs and there is a lack of knowledge and education among the public in the Himalayan region. Therefore, considering the severity and the importance of the disorder in affecting people's health and well-being, there is a great need to carry out such studies in the population of this area.

2. Materials and methods

The present research was carried out on 4,081 (2,956 females and 1,125 males) unrelated individuals, from June 2016 until August 2019. The participants constituting the study were 0 – 60 years of age and had no chronic illness or history of infection. The ethical clearance for the present research was obtained by the Institutional Ethical Committee (I.E.C.) of H.N.B. Garhwal University before the commencement of the study.

Demographic information such as age, sex, marital status, occupation, prior history of infection, or chronic illness or surgery were obtained directly through face-to-face interview. Only after a written consent was obtained from the individuals, further study was carried out on the willingly participating ones.

Blood samples (whole blood) for the present study was collected from the district hospitals, public health centers, villages, schools, and local people residing in the five major districts of Garhwal region, including Chamoli, Pauri, Rudraprayag, Tehri, and Uttarkashi. A total of 5 ml of blood were collected from each sampled individual. The blood samples were collected in Vacutainer containing ethylenediaminetetraacetic acid as anticoagulant. Twenty microliters of anticoagulated blood were utilized for hemoglobin analysis. NESTROFT test (Naked Eye Single Tube Red Cell Osmotic Fragility Test) was done using 10 μ l of blood, following the protocol described by Thomas *et al.* (1996). Approximately 2 ml blood was utilized for HbA₂ analysis using high-performance liquid chromatography – HPLC (Variant BioRad 2).

Out of total 4,081 blood samples analyzed for hemoglobin and NESTROFT, only 648 individuals were found NESTROFT positive. Further screening for HbA₂

was done for all 648 individuals. Molecular investigation was done only for 64 individuals out of the total 648 screened for HbA₂, as their HbA₂ levels were more than 3.5%. The cutoff level of more than 3.5% of HbA₂ is considered as the gold standard for β -thalassemia trait (Ou *et al.*, 2011). All these 64 individuals were, thus, subjected to the detection of mutation through the amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR), which is a simple method for detecting any mutation involving single base changes or small deletions. ARMS is based on the use of sequence-specific PCR primers that allow an amplification of test DNA only when the target allele is contained within the sample (Little, 2001). Molecular analysis for the blood samples was carried out at the Central Molecular Research Laboratory of SGRRIHMHS, Dehradun, India. Genomic DNA from the blood samples was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA).

The amplification of the targeted sequences of the extracted DNA was carried out through ARMS-PCR technique. The five most common mutations prevalent in India, that is, IVS 1-5 G-C, IVS 1-1 G-T, Codon 41/42 (-TCTT), Codon 8/9, and 619 bp deletion were considered for detecting mutations. The PCR reaction was carried out with initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, 1 min annealing at 60°C, extension at 72°C for further 1 min, and final extension for 5 min at 72°C. After PCR reaction, the products of PCR are obtained in the form of amplicons. To analyze the PCR products, 10 μ l of amplicon was loaded on 1.5% agarose gel along with 1 μ L of 6X DNA loading dye (ML015). In a separate well 3 μ L of 50 bp DNA ladder (MBT084) was loaded as marker. The gel was then allowed to run for about 15 – 20 min at 125 volts and the results were recorded in Gel Documentation System.

3. Results

The distribution of the five mutations, that is, IVS 1-5 G-C, IVS 1-1 G-T, Codon 41/42 (-TCTT), Codon 8/9, and 619 bp deletion, among the 64 individuals was studied using ARMS-PCR. The most frequent mutation was IVS 1-5 (G-C), which was present in 12 study subjects, accounting for 18.75% of the study subjects. While Codon 8/9 was found in 12.5% of individuals, IVS 1-1 (G-T) was found to be present in 6.25% of the study population. On the other hand, 619 bp deletion and Codon 41/42 (-TCTT) mutations were absent in our studied population. The allelic frequency for all the three mutations, IVS 1-5 (G-C), IVS 1-1 (G-T), and Codon 8/9, was found to be same at 0.99. The distribution frequency of the five investigated β -thalassemia mutations is shown in Tables 1-4 according to the four major zones of India in

comparison with the previous reports on β -thalassemia in different regions of India.

4. Discussion

Numerous studies on thalassemia have been carried out throughout the globe. India, being a part of the thalassaemic belt, faces various problems, such as unawareness, lack of finance, resources, and social stigma for pre-marital screening (Mohanty *et al.*, 2013; Verma *et al.*, 1997). Despite the enormous amount of research and rapid developments seen during the past decade, it still continues to be a national concern. According to the World Health Organization (WHO), the total thalassaemic reported in India alone accounts for 80 – 90% and is a common hemoglobinopathy (Haritha *et al.*, 2012). β -thalassemia is considered to be the cause of morbidity and mortality along with the source of economic burden to the community (Piplani *et al.*, 2013). As per the WHO (WHO, 2001) records, β -thalassemia is a common hemoglobinopathy in India, and therefore, numerous studies have been carried out for Indian population for the distribution of thalassemia mutation. Since India represents an extremely heterogeneous population with numerous tribal pockets, diverse racial origin, and high inbred diseases frequency among certain communities, the prevalence rate of thalassemia is very high for some particular communities. There are diverse ethnicities residing in different parts of the Himalayan belt. A study carried out in western part of India, conveyed 22.7% thalassemia carrier women diagnosed with anemia (Mulchandani *et al.*, 2008).

Molecular confirmation was done to ascertain authentic reporting. In different local communities of India, the reported range for the five most common β -thalassemia mutations was between 0.3% and 17% (Agarwal & Mehta, 1982; Weatherall & Clegg, 2001a; 2001b; WHO, 2008). The present research reported a frequency of 0.5% for β -thalassemia mutations, which is within the range described for the Indian population. The analysis of mutation spectrum revealed the highest prevalence for IVS1-5 (G-C) (18.75%) followed by Codon 8/9 (12.5%) and IVS 1-1 (G-T) (6.25%). The frequency of mutation was compared with the other reported mutations of Indian population (Tables 1-4). Colah *et al.* (2009) discovered IVS 1-5 (G-C) as a pre-dominant mutation throughout India, with 44.8% prevalence in the north and 71.4% in the east. This pre-dominancy is in unison with our study where the frequency of mutation was found to be 18.75% for IVS 1-5 (G-C).

In contrast to our study, a higher frequency (88.6%) of IVS I-5 (G-C) was reported in Orissa, 78.8% in Andhra Pradesh, and 77.2% in West Bengal (Colah

Table 1. Distribution of five most common mutations for beta-thalassemia among the eastern zone of Indian population

State	Sample size	619 bp deletion	Co 8/9 (+G)	Co 41/42 (-CTT)	IVS-1nt 5 (G-C)	IVS-1nt 1 (G-T)	References
West Bengal	2,456	1.0	0	4.0	77.2	4.0	Colah <i>et al.</i> (2009)
	11	45.450	0	0	0	0	Bashyam <i>et al.</i> (2004)
East India	80	0	0	17.50	28.75	0	Kukreti <i>et al.</i> (2003)
Bihar	2,456	0	0	15.0	70.0	0	Colah <i>et al.</i> (2009)
Jharkhand	2,456	0	0	0	75.0	0	Colah <i>et al.</i> (2009)
Orissa	2,456	1.2	0	2.6	88.6	0	Colah <i>et al.</i> (2009)
	276	0	0.38	0	4.59	0.38	Sahoo <i>et al.</i> (2014)
Bihar, Chhattisgarh, and Jharkhand	1,642	0	3.6	5.4	51.8	0	Nagar <i>et al.</i> (2014)

Table 2. Distribution of five most common mutations for beta-thalassemia among the western zone of Indian population

State	Sample size	619 bp deletion	Co 8/9 (+G)	Co 41/42 (-CTT)	IVS-1nt 5 (G-C)	IVS-1nt 1 (G-T)	References
Maharashtra	2,456	1.7	1.7	2.0	66.5	0.7	Colah <i>et al.</i> (2009)
	126	2.38	6.34	3.96	65.07	9.52	Satpute <i>et al.</i> (2012)
Gujarat	2,456	6.5	12.6	6.9	48.6	5.0	Colah <i>et al.</i> (2009)
Gujarat (Sindhi)	1,233	49.2	0	0	0	25.5	Vaz <i>et al.</i> (2000)
Gujarat (Lohanas)	1,233	0	0	0	0	31.2	Vaz <i>et al.</i> (2000)
Gujarat (Punjabis)	1,233	0	0	0	0	34.7	Vaz <i>et al.</i> (2000)
Goa	2,456	0	0	0	15.4	0	Colah <i>et al.</i> (2009)
Rajasthan	2,456	1.1	2.3	9.9	72.4	3.3	Colah <i>et al.</i> (2009)
Madhya Pradesh	2,456	10.6	19.9	5.7	36.9	11.3	Colah <i>et al.</i> (2009)
Chhattisgarh	2,456	23.5	23.5	0	35.3	5.9	Colah <i>et al.</i> (2009)
East-west population							
Rajasthan, Maharashtra, Assam, and West Bengal	75	13.23	0	0	0	0	Shah <i>et al.</i> (2017)

Table 3. Distribution of five most common mutations for beta-thalassemia among the southern zone of Indian population

State	Sample size	619 bp deletion	Co 8/9 (+G)	Co 41/42 (-CTT)	IVS-1nt 5 (G-C)	IVS-1nt 1 (G-T)	References
Andhra Pradesh	2,456	0	0	0	78.8	0	Colah <i>et al.</i> (2009)
	39	0	0	0	76.92	0	Bashyam <i>et al.</i> (2004)
Karnataka	2,456	0	0.8	0.8	67.2	1.6	Colah <i>et al.</i> (2009)
	28	0	0	0	64.28	3.57	Bashyam <i>et al.</i> (2004)
Kerala and Tamil Nadu	2,456	0	0	6.31	56.3	0	Colah <i>et al.</i> (2009)

et al., 2009). The previous studies showed that Codon 8/9 had a higher prevalence of 37.2% in immigrants from Pakistan followed by 23.5% in Chhattisgarh population (Colah *et al.*, 2009), whereas in our study, it was found only 12.5%. Codon 41/42 (-TCTT) was found to be higher (17.50%) in East Indian population in the previous studies (Kukreti *et al.*, 2002) and was also reported in Punjab and

Haryana (13.5%) (Colah *et al.*, 2009), but for our study, the frequency of this mutation was nil. A study carried out in Himachal Pradesh/Jammu Kashmir region by Colah *et al.* (2009), reported 37.5% IVS 1-1 (G-T) mutation. The very same mutation is found to be the second highest among the Punjabi Hindu population of Gujarat (34.7%) followed by Lohanas of Gujarat (31.2%) (Vaz *et al.*, 2022). The previous

Table 4. Distribution of five most common mutations for beta-thalassemia among the northern zone of Indian population as well as Garhwal population

State	Sample size	619 bp deletion	Co 8/9 (+G)	Co 41/42 (-CTT)	IVS-Int 5 (G-C)	IVS-Int 1 (G-T)	References
Punjab, Haryana, and Uttar Pradesh	1,050	21	12.1	8.7	34.1	15.8	Verma <i>et al.</i> (1997)
Delhi	46	34.8	13	9.8	22.8	19.6	Madan <i>et al.</i> (1998)
Uttar Pradesh	376	2.5	0	0	64.3	0	Agarwal S <i>et al.</i> , 2000
	98	12.5	5.20	6.25	54.0	3.12	Agarwal S <i>et al.</i> , 2003
	48	0	21	4	46	12	Christopher AF <i>et al.</i> , 2013
Uttar Pradesh	2,456	0.8	1.6	9.6	64.8	5.6	Colah <i>et al.</i> (2009)
Jammu, Kashmir, and Himachal Pradesh	2,456	0	0	0	50.0	37.5	Colah <i>et al.</i> (2009)
Punjab	780	18.7	14.8	15.3	31.8	12.8	Garewal & Das., 2003
	997	22.47	16.35	10.83	24.97	14.44	Black <i>et al.</i> (2010)
Punjab and Haryana	2,089	9.6	7.7	13.5	28.8	17.3	Panigrahi & Marwaha (2007)
Chandigarh (Sindhi)	87/1233	0	0	0	25.5	0	Panigrahi & Marwaha (2007)
Chandigarh (Punjabi)	87/1233	0	0	0	34.7	0	Panigrahi & Marwaha (2007)
Chandigarh (Lohanas)	87/1233	0	0	0	31.2	0	Panigrahi & Marwaha (2007)
Other North Indian States	62	94.1	0	0	0	0	Chakrabarti <i>et al.</i> (2005)
Uttarakhand							
Garhwal (Uttarakhand)	4,081	0	12.5	0	18.75	6.25	Present study

studies (Baig *et al.*, 2006) reported a high frequency of 37.3% for IVS 1-1 (G-T) mutation in Punjabi population residing in Pakistan. On the contrary, our study exhibited only 6.25% of the screened population having IVS 1-1 (G-T) mutation. In our study, Co 41/42 (-CTT) and 619 bp deletion were completely absent.

Of the total 64 individuals who were screened for mutational spectrum, mutations were found only in 24 individuals and the remaining 40 individuals did not reveal the presence of any of these five common mutations. The remaining 40 individuals may possess a novel mutation or can only be carrier. The confirmation of novel mutation can be done through DNA sequencing. However, DNA sequencing for the study presented could not be carried out due to economic constraint and limitation of resources. Furthermore, only 64 individuals out of the total 648 screened for HbA₂. The small sample size may suffer from some biases in our findings and thus the cautions are needed in interpreting our findings.

Despite some limitations, our study could have important implications. There are various programs implemented by the state governments and non-governmental organizations (NGOs) around the world, including India, for the awareness of thalassemia and also about its fatal effects. However, nothing has been done to make the population of Garhwal aware of this disorder. In the developing country like India, thalassemia is not

rare, and our nation is not an exception. In Pakistan, a bill is passed by the government where it is mandatory to carry out carrier testing for relatives of thalassemia patients. A similar system of bill is also placed in Dubai, Saudi Arabia, and Abu Dhabi where the frequency of beta-thalassemia is 19% (Cao & Kan, 2013). All these together with our findings signal the importance of initiatives and implementations of some targeted intervention programs in our study areas.

Furthermore, some caveats are noteworthy. Consanguineous marriage causes clustering of the mutations among some of the communities and, therefore, should be avoided. Clinical management, genetic counseling, and prenatal diagnostic techniques should be made available to the individual level. Awareness programs regarding anemia and thalassemia must be implemented for the general public through media and other modes, to make the public aware about this deadly disorder. As, the burden of thalassemia can be reduced at community and country level only through awareness, screening, and prevention strategies in conjunction with each other (Kumar *et al.*, 2015).

5. Conclusions

It is the first study to report and provide baseline data on the prevalence of β -thalassemia among the studied population of Garhwal, India. This research exhibited prevalence rate

of 1.5% for beta-thalassemia trait (BTT) on the basis of elevated level of HbA₂ of ≥3.5%. The present study reported a frequency of 0.5% for β-thalassemia mutations. The analysis of mutation spectrum revealed highest prevalence for IVS 1-5 (G-C) (18.75%) followed by Codon 8/9 (12.5%) and IVS 1-1 (G-T) with 6.25%, whereas Codon 41/42 (-TCTT) and 619 bp deletion were found to be absent.

The present research is a preliminary attempt to record the prevalence rates of thalassemia, prior for Himalayan region of Uttarakhand. The study suggests that emphasis should be laid down on the implementation of population screening programs to reveal the exact number of individuals carrying the gene for BTT and having anemia. The information provided in this research can be used for planning population-based mutation screening strategy along with pre-marital screening.

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Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

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Ethics approval and consent to participate

The ethical clearance for the present research was obtained by the Institutional Ethical Committee (I.E.C.) of H.N.B. Garhwal University (2016/01). Written consents were obtained from all the participants.

Consent for publication

Not applicable.

Availability of data

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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