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Molecular, Biochemical and Hematological Investigations of β- Thalassemia Children in Bannu KPK Pakistan

Sabir khan[1], Irum Saba[2], Abeed Ullah[2] Xiao Junhua[1]*
[1.] Department of Chemistry Chemical Engineering and Biotechnology Donghua University Shanghai PR China
[2.] University of Sciences & Technology Bannu Kpk Pakistan

Abstract:-Hereditary anemia's like thalassemia are particularly prevalent in the Mediterranean, equatorial, or close to equatorial regions of Africa and Asia. They are divided into four categories: thalassemia,,,, and, depending on which specific globin chain are synthesized in a lower level. More than 300 people in the Bannu Strip have been identified as having : α, β, δβ, δ, and γ,δ thalassemia major they are currently receiving transfusions and receiving treatment in nearby facilities.

I. INTRODUCTION

Thalassemia, a hereditary anemia, is caused by mutations that interfere with the synthesis of globin, the protein component of hemoglobin. In many regions of the world, thalassemia causes serious public health problems(1). The majority of those who have them reside in equatorial or close equatorial parts of Africa and Asia, particularly in the Mediterranean region. They are the most common hereditary ailments that mankind is aware of, and they have essentially been seen in every ethnic group and region of the globe (2). Thalassemia is characterized based on which specific globin chain are generated in a lower proportion. This may result in uneven globin chain synthesis, ineffective erythropoiesis, hemolysis, and various degrees of anemia. They are the main thalassemia subtypes. The most prevalent classes are and thalassemia and thalassemia is the most significant and pervasive kind that results in severe anemia in homozygous and compound heterozygous conditions(3).

Clinically, thalassemia is divided into three categories based on the severity of the condition: thalassemia major, which necessitates ongoing blood transfusions; thalassemia intermediate, which causes anemia but is not severe enough to require ongoing blood transfusions; and thalassemia minor or trait, which is the asymptomatic carrier state(4) (5). The type of mutation in the â gene determines the severity of the clinical condition associated with â-thalassemia. The globin gene has more than 400 known mutations, all of which have been linked to the emergence of thalassemia (6, 7). Although uncommon cases can entail significant deletion mutations, point mutations account for the bulk of thalassemia types (3, 8, 9). There are well-known relationships between the type of the - thalassemia mutation and the hematological-clinical phenotype (10). Finding the patient's mutation is therefore very important for a better care regimen (11, 12). Blood transfusions are being introduced by medical professionals to reduce thalassemic symptoms. However, because humans have a relatively limited ability to remove iron, frequent blood transfusions invariably end in iron overload. Organs with apparent iron deposition include the liver, heart, pancreas, thyroid, parathyroid, adrenal, renal medulla, bone marrow, and spleen.

This parenchymal iron burden is the primary cause of illness and mortality in severe -thalassemia. A variety of clinical conditions, including diabetes, hyperparathyroidism, adrenal insufficiency, and liver failure, will arise if the typical growth spurt of adolescence does not occur, including hepatic, endocrine, and cardiac problems. Either secondary sexual development is slowed down or not present (13). For the treatment of severe β-thalassemia illnesses, three cornerstones are necessary: routine blood transfusions, the removal of excess iron using chelating medications like deferoxamine and Exhale, and splenectomy when the frequency of transfusions is increasing (13).

II. MATERIALS AND METHODS

A. *Study design*

This work was performed according to the cross-sectional descriptive study design.

B. *Target population Inclusion criteria*

All β-thalassemia unrelated children aged 5-12 years old at public hospitals in Bannu city who are currently being transfused and managed for the clinical symptoms and manifestations of the disease were considered as a target for the present study. Only one child was included for each couple.

C. *Exclusion criteria*

All other β-thalassemia related children were excluded from the study. Children less than 5 or older than 12 years were also excluded. Any unconfirmed blood transfusion dependent children were also excluded.

D. *Sample size*

Sample size was 54 transfusion dependent β-thalassemia children (28 boys and 26 girls) and 54 apparently healthy children (28 males and 26 females) served as a control group. The cases and controls were age and sex matched.

E. Data collection

The data of the study was collected via questionnaire and also from laboratory investigation of blood sampled for the type of causative mutation, biochemical and hematological parameters of the of β-thalassemia children.

F. Venous blood withdrawal

Each participant (cases and controls) in the current investigation provided five ml of venous blood, which was divided almost evenly (2.5 ml) into 12x56mm K3-EDTA polypropylene tubes (Meus, Piove Di Sacco, Italy) and serum tubes (2.5 ml). Patients had blood drawn immediately before the scheduled blood transfusion for the children with â-thalassema. Using a Cell Dyne 1700 electronic counter, a complete blood count (CBC) was performed on the blood in the K3-EDITA tubes. This test measures the number of white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), and (Sequoia-Turner corporation, California, USA).

A total of 400 units of K3-EDTA blood were used for spotting, DNA extraction, and purification in order to perform molecular diagnosis and locate the mutated gene that causes the disease using PCR-based methods. All of the study's - thalassemic youngsters had their HbF levels measured using K3-EDTA blood. Three blood films from the children with -thalassemic disease were made. In the meantime, the serum tube containing the blood was centrifuged to separate the serum, which was then kept in fresh plastic screw tip tubes and used to calculate the following biochemical values using commercial test kits:

- *Liver function enzymes:*
  Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) serum bilirubin.

- *Kidney function tests:*
  Urea, creatinine, uric acid.

- *Blood chemistry*
  Total protein, albumin, globulins, serum ferritin, serum calcium, serum phosphorus.

G. DNA extraction and purification

Using BL and BLM lysis buffers (Agowa, GmbH, Berlin, Germany) and a magnetic bead-based DNA extraction and purification process, DNA was extracted and purified from two dried blood spots (about 200 L). The super paramagnetic particles used in the beads-based kit are used to isolate DNA from whole blood.

H. HBB mutations screening

At the molecular medicine facilities of the Bernhard Nocht Institute (BNI), Germany, the screening for potential HBB gene mutations was carried out using the Dynamic Allele-Specific Hybridization (DASH) method with the goal of identifying the most prevalent single nucleotide polymorphisms (SNPs) in HBB reported in the literature for the Arabic and/or Mediterranean populations: IVS-I-1 (G - -> A), IVS-I-6 (T --> C), IVS-I-110 (G --> A), codon 37 (G --> A), and codon 39 (C --> T) Using the DASH approach, three PCR-based hybridization tests were developed a type for these 5 mutations that had been established. There were discovered specific annealing temperatures for the hybridization of the probes for the wild types and for each of the mutations (Table 2.1) This made it possible to identify the 5 mutations' homozygous, heterozygous, and compound heterozygous genotypes.

Table 2.1 Characteristics of hybridization assays used for genotyping

PCR	Screened Mutation	Amplicon	Primers 5í- 3í	Probes 5í- 3í Mutation position underlined	Annealing Temperature
Assay 1	IVS-I-1 IVS-I-6 Wildtype	186 bp	Forw TGAGGAGAAGTCTGCCGTTA Rev CCAATAGGCAGAGAGAGTCA	Anchor Cy5- ACAAGACAGGTTTAAGGAGACCAATA GAAACTGG- Phosphate Sensor for IVS-I-1 G>A and IVS-I-6 T>C GCAGGTTGGCATCAAGGT-Fluorescein	58 ^{o}C
Assay 2	CD37 CD39 Wildtype	208 bp	Forw AAGGTTACAAGACAGGTTTA AG Rev TTAGGGTTGCCCATAACAGC	Anchor Cy5- CTTTGAGTCCTTTGGGGATCTGTCCAC- Phosphate Sensor for CD37 G>A and CD39 C>T CCCTTGAACCCAGAGGT-Fluorescein	55 ^{o}C
Assay 3	IVS-I-110 Wildtype	208 bp	Forw AAGGTTACAAGACAGGTTTA AG Rev TTAGGGTTGCCCATAACAGC	Anchor Cy5-TCCCACCCTTAGGCTGCTGGT- Phosphate Sensor for IVS-I-110 A>G CTCTCTCTGCCTATTAGTCTATT- Fluorescein	58 ^{o}C

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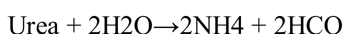
I. Biochemical analysis

Establishing serum urea levels According to Thomas's approach serum urea was measured using the "Urease-GLDH" enzymatic UV test with DiaSys reagent kits.


Urea + 2H2O→2NH4 + 2HCO

2-Oxoglutarate + NH4 + NADH→ L-Glutamate + NAD + + H2O

➢ *Reagents*

Concentrations are those in the final test mixture.

Reagent	Concentration
R1: TRIS	121 mmol/l
2- Oxoglutarate	8 mmol/l
ADP	0.7 mmol/l
Urease	≥ 0.6 ku/l
GLDH	≥ 1 ku/l
R2: NADH	0.25 mmol/l
Standard	50.5 mg/dl

➢ *Assay Technique*

By combining four parts of R1 and one component of R2, the working solution was created.
340 nanometers
1 cm optical path Climate: 37 IC
Against distilled water for measurement.
● 1 ml of the working reagent was combined thoroughly with 10 l of the standard (sample or control).
● After 30 seconds of incubation, absorbance (A1) was measured.
●Exactly 60 more seconds later, the absorbance (A2) was measured.

➢ *Calculation*

ΔA = (A1 ñ A2) sample or stander

$$\text{Urea (mg/dl)} = \frac{\Delta A \text{ sample X concentration standard}}{\Delta A \text{ standard}}$$

Child	5 - 30 mg/dl
Adult	13 - 43 mg/dl

Reference value (Pakistan Clinical Laboratory Tests Guide)

J. Hematological analysis

➢ *Complete blood count (CBC)*

Children's complete blood counts (CBCs) were measured using Cell-Dyn 1700, a full set of control and calibrator reagents, at the Central Blood Laboratory-Thalassemia Center in Bannu

➢ *Determination of serum ferritin.*

Microparticle Enzyme Immunoassay (MEIA) technology was used in the current investigation to measure serum ferritin levels. The full automated Axsym immunoassay analyzer ferritin test system from Abbott Laboratories in the United States was employed for this purpose.

➢ *Fetal hemoglobin (HbF) quantitation*

HbF is usually quantitated based on it.s resistance to denaturation at alkaline pH **.** In,modified the original method so that it can accurately measures HbF when present in relatively small amounts However slightly modified the method so that it gives highly reproducible results over the range of HbF 0.5- 50% **.** In the present study we followed the method.

➢ *Principle of the method*

When exposed to an extremely alkaline solution, the majority of human hemoglobin denatures. Ammonium sulphate which precipitates the denatured hemoglobin is added to cease denaturation. Fetal hemoglobin, on the other hand, is not denatured and is still soluble, allowing it to be filtered and quantified using spectrophotometry.

➢ *Reagents*

1- 1.2N sodium hydroxide. To make 1000 cc, dissolve 48g of NaOH in distilled water. Keep in a polyethylene bottle at 5 oC.
2. Ammonium sulfate that is saturated. For a 1000 ml solution, dissolve 500g of (NH4)2SO4 in distilled water.
3. Drabkin's remedy. Make a 1000 ml solution by dissolving 0.05g of potassium cyanide (KCN) and 0.2g of potassium ferricyanide (K3FeCN6) in distilled water. Keep in opaque bottles.

➢ *Procedure*

1- To make haemolysate, combine 200 ml of whole blood and 800 ml of distilled water completely. Remix after 5 minutes of standing.
2- To 10 ml of Drabkin's solution, 0.6 ml of haemolysate is added. Both test and reference measurements will be made with this diluted haemolysate.
3- To 2.8 ml of the diluted haemolysate, about 0.2 ml of 1.2N NaOH is added. The mixture is gently stirred, and then it is let to stand for two minutes.
4- After vigorously shaking the mixture for exactly two minutes, 2 ml of saturated ammonium sulphate at room temperature is added. The mixture is then let to stand for at least five minutes.
6- While filtering, make 1.4 ml of the diluted haemolysate, 1.6 ml of distilled water, and 2 ml of saturated ammonium sulphate to make a standard or total haemoglobin solution. Mix 0.5 ml of the standard with 4.5 ml of Drabkin's solution in a separate tube to make a 1:10 dilution with the standard.
7- Compare the absorbance of the standard (As) and the fetal hemoglobin solution (AF) at 415 nm to a blank made up of 1.4 ml of Drabkin's solution, 0.2 ml of 1.2N NaOH, and 2 ml of saturated ammonium sulphate solution.
8- The formula used to determine HbF % is as follows.

➢ *Blood film*

Giemsa staining agent is used (Lobachemie. Laboratory reagents and fine chemical- India).
1. Fix the slide for 2–5 minutes with 96% methanol.
2. Use distilled water to clean the slide.

3. Spread stain all over the slide and wait 20 minutes.
4. Use tap water to clean the slide.
5. Use an oil immersion lens to examine the slide under a microscope to see the morphology of the red blood cells.

K. Statistical analysis

Using IBM SPSS Statistics, the data from the questionnaire, CBC, and biochemical tests were collated, encoded, and statistically evaluated (version 17, IBM Corporation, Somers, NY). The following statistical analyses were conducted with the goal of describing, identifying important relationships, correlating, and comparing the study items, variables, and parameters. Chi square test and Z test are these tests. The one-way analysis of variance and the independent-samples t test (ANOVA).

III. RESULTS

54 children with β-thalassemia major who are currently receiving blood transfusions and receiving treatment at the Woman and Children hospital for the disease's clinical symptoms and manifestations were included in the current study. 54 seemingly healthy kids were also randomly chosen from primary healthcare clinics to form the study's control group. The ratio of cases to controls was 1:1, with the controls being age and sex matched to the cases. To illustrate the number and frequency of the subjects according to the various factors and characteristics, the research results are presented as cross tabulation tables in this section. The t-test analysis was used to compare the mean values of the hematological and biochemical parameters of the various groups and subgroups.

A. General and some clinical characteristics of the patients

The general and clinical features of the patients are given in table 3.1 which revealed that the majority (67.0%) of the patients are receiving blood transfusion each 2- 3 week and 33.1 % each 4 -5 weeks. Only one (1.8%) of the patients required transfusions at intervals greater than five weeks. Only 7.5% of the instances involved thalassemic individuals with splenoctomies. Nearly half of the patients undergoing iron chelation remove the excess iron using subcutaneous pumps (desferal pumps), 43.5% using intramuscular infusion, and 8.4% using intravenous infusion. 3.9% of patients utilize oral iron chelation (X-Jade). It is evident that there are additional thalassemic patients in the same family (brothers or sisters), and 67.0% of the current patients have an additional thalassemic sibling brother and /or sisters which were excluded for biasness issues.

Table 3.1 Represent General and some clinical characteristics of the patients

	Number	Percentage
Blood transfusion		
2-3 weeks	35	67.0
4-5 weeks	17	33.1
More than 5 weeks	2	1.8
Iron chelation		
Desferal pump	24	45.3
Intramuscular infusion	22	43.5
Intravenous infusion	5	8.4
Oral (x-Jade)	3	3.9
Splenoctomy		
Yes	5	6.5
No	49	93.5
Thalassemic brothers/sisters		
0	18	33.6
1	22	40.9
2	12	23.2
4	2	1.8

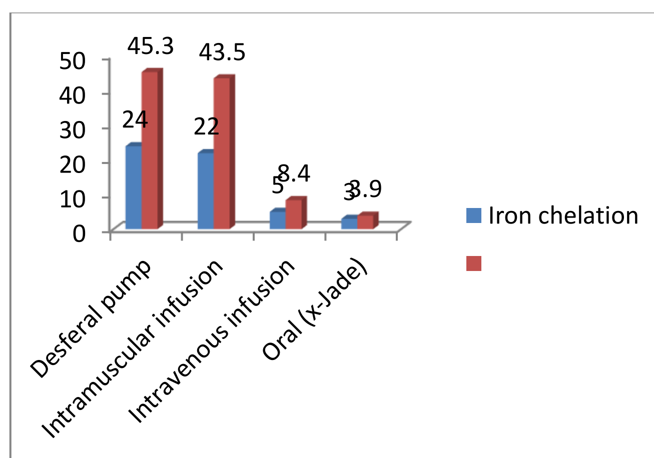


Fig 1 Represent General and some clinical characteristics of the patients

B. Hematological characteristics of the study groups

The hematological features of the study groups are displayed in Table 3.2. With the exception of MCH, all CBC values and indices assessed in the current study showed significant differences between patients and controls. In contrast to the controls, the patients displayed severe anemic symptoms. When compared to controls, patients' hemoglobin levels are roughly 30% lower at 9.0 1.0 vs. 10.4 0.8 g/dL, respectively. With MCV and MCH values of 74.97.3 fl and 25.12.3 pg in patients compared to 78.54.4 fl and 25.7 2.2 pg in controls, respectively, and p-values of 0.003 and 0.149, it is evident that patients have microcytosis without hypochromia. Additionally, patients exhibited considerably notable secondary thrombocytosis (thrombocythemia) compared to controls, With a p-value of 0.001, PLT counts were 371.7152.9 and 283.865.2x108/L, respectively. Additionally, patients had a substantial leukocytosis (10.5 12.0 vs. 7.1 2.1 X 109/L vs. controls, respectively; p-value = 0.009).

Table 3.2 Represent Hematological Characteristics of the Study groups

Parameters	Patients n=54	Controls n=54	*P*-value
	Mean ± SD	Mean ± SD	
WBC X 10^9/L	11.5 ± 12.0	7.1±2.1	0.009
RBC X 10^{12}/L	3.3±0.5	4.6±0.4	<0.001
Hb (g /dL)	8.0±1.0	11.4±0.8	<0.001
HCT (%)	25.1±3.2	36.5±2.7	<0.001
MCV (fl)	75.9±7.3	79.5±4.4	0.003
MCH (pg)	24.1±2.3	24.7 ± 2.2	0.148
MCHC (g /dL)	31.8±0.7	31.1±1.2	0.001
PLT 10^9/L	370.7±152.9	284.8±65.2	<0.001
RDW %	21.6±10.9	15.3±2.7	<0.001

WBCs: white blood cells, **RBCs:** red blood cells, **Hb:** hemoglobin, **HCT:** heamtocrit, **MCV:** mean corpuscular volume, **MCH:** mean corpuscular hemoglobin, **MCHC**: mean corpuscular hemoglobin concentration, **PLT:** platelets count.

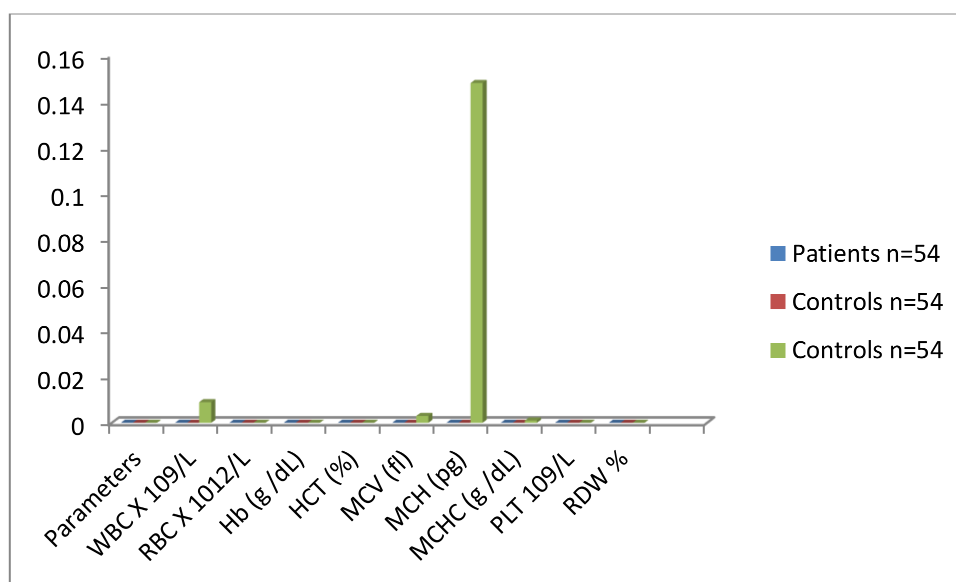


Fig 2 Represent Hematological Characteristics of the Study groups

C. Biochemical characteristics of the study groups

Table 3.4 displays the biochemical traits of the patients in comparison to the controls. When compared to controls, patients' liver and kidney function tests both considerably worsened, with the exception of urea. Patients' protein contents—including total protein, albumins, and globulins—are significantly lower than those of the control group. Additionally, there is a considerable rise in iron overload in patients compared to healthy controls, with serum ferritin reaching 3231.01560.5 ng/l vs 46.823.1 ng/l, with a p-value of 0.0001 for both comparisons. Despite the fact that patients' serum calcium levels were substantially lower (9.20.6) than those of controls (9.70.7), there were no discernible variations in phosphorus levels (4.80.8 vs 4.70.6, respectively; p-value = 0.396).

Table 3.3 Represent Biochemical Characteristics of the study groups

Parameters	Patients n=54	Controls n=54	*P*-value
	Mean ± SD	Mean ± SD	
ALT	54.3±50.0	17.4±15.2	< 0.001
AST	61.1±36.8	27.9±6.1	< 0.001
Urea	23.5±6.2	23.7±5.8	0.871
Creatinine	0.45±0.08	0.54±0.10	< 0.001
Uric acid	4.2±1.0	3.6±0.8	0.001
Total Bilirubin	1.2±0.8	0.7±0.1	< 0.001
Direct Bilirubin	0.44±0.40	0.26±0.09	0.001
Total protein	6.4±0.6	7.0±0.4	< 0.001
Albumin	4.2±0.3	4.7±0.3	< 0.001

Globulins	2.1±0.5	2.3±0.3	0.061
Ferritin	3231.0±1560.5	46.8±23.1	< 0.0001
Calcium	9.2±0.6	9.7±0.7	< 0.001
Phosphorous	4.8±0.8	4.7±0.6	0.396

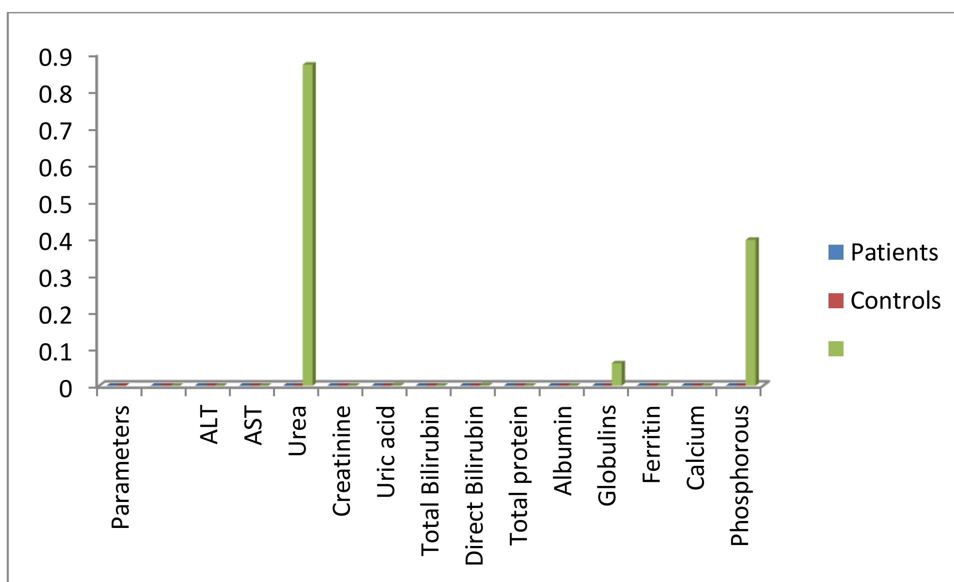


Fig 3 Represent Biochemical Characteristics of the study groups

D. Mutation spectrum of β-thalassemia major patients

The five mutations screened in the present work were found in 74/106 chromosomes with a relative allelic frequency of 0.7, according to the molecular characterization (Table 3.4) of the HBB gene in the Palestinian children with major -thalassemia. The remaining 32/106 chromosomes carry a different mutation of the HBB gene that was outside the scope of our study. Following the ß+ mutation IVS-I-110, which was discovered in nearly a quarter (24/106, relative allele frequency of 0.25), were the o mutations IVS-I-1 and CD39, which were discovered in 18 and 17 patient chromosomes, respectively, with relative allele frequencies of 0.18 and 0.15. IVS-I-6 and CD37 were discovered as less prevalent alterations, with relative allele frequencies of 0.09 and 0.07, respectively.

Table 3.4 Relative allelic Frequencies of HHB variants in Bannu region patients

HBB variants	SNP number	Classification	Number of alleles	Relative frequency
IVS-I-110	rs35004221	β^+	24	0.25
IVS-I-1	rs33971441	β^0	18	0.18
CD39	rs63750242	β^0	17	0.15
IVS-I-6	rs35724774	β^+	8	0.09
CD37	rs33974935	β^0	7	0.07
Subtotal			**74**	***0.70***
Others			**32**	***0.30***

E. Genotype of patients according to the identified variants

More than half of the patients (54.7%) had a homozygous genotype, as is shown in Table 4.5 based on the discovered variations. While homozygotes for the CD39 mutation and the IVS-I-1 mutation respectively constituted 13.2% and 9.3% of the patients, homozygotes for the IVS-I-110 mutant represented 20.7% of patients. 15.0% of the patients are double heterozygotes for two distinct mutations. Samples from the remaining patients (16/54) are either unidentified (11/54) or semi-identified (5/54).

Table 3.5 Genotype of patients according to the identified variants

Genotype	Number	Frequency
Homozygous IVS-I-110	11	20.7
Homozygous CD39	7	13.3
Homozygous IVS-I-1	5	9.3
Homozygous IVS-I-6	3	5.8

Homozygous CD37	3	5.8
Subtotal	*29*	*54.9*
Double heterozygous	8	15.0
One variant identified	6	10.1
Unidentified	11	20.6
Total	**54**	**100**

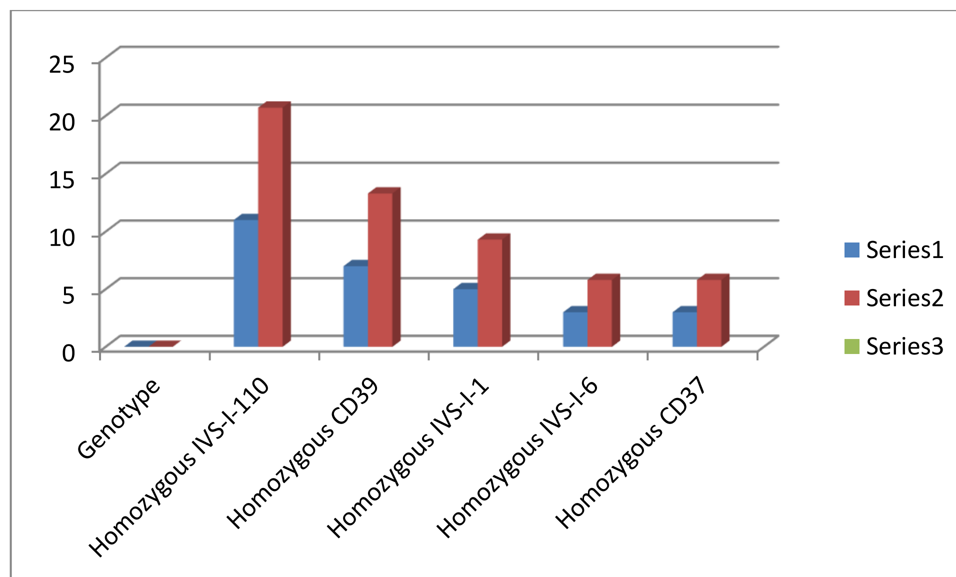


Fig 4 Genotype of patients according to the identified variants

IV. CONCLUSIONS

- Compared to the control group, where the percentage is less than 2.0%, the parents of -thalassemia major children are first-degree cousins to about 71% of them.
- All hematological parameters assessed in the current study, with the exception of MCH, showed substantial differences between patients and controls.
- When compared to the controls, the patients displayed a severe anemic appearance. Patients' hemoglobin levels fall to roughly 30% of what is observed for controls.
- Patients were found to have significantly more secondary thrombocytosis (thrombocythemia) and leukocytosis than controls.
- Except for urea, liver and kidney function tests in patients considerably declined as compared to controls.
- Patients' protein contents—including total protein, albumins, and globulins—are significantly lower than those of the control group.
- Patients have more iron than controls do, with serum ferritin levels reaching 3231.01560.5 ng/l vs. 46.823.1 ng/l (P 0.0001).
- Patients had significantly lower serum calcium levels than controls, but there was no discernible change in serum phosphorus levels.
- A poikilocytosis score of 4-5 was present in more than 58% of the individuals.
- The ß+ mutation IVS-I-110, the ßo mutation IVS-I-1, and the ßo CD39 were all discovered in approximately a quarter of the patient's chromosomes.
- Although at low relative allele frequencies, the mutations IVS-I-6 and CD37 were shown to be less common.
- Patients who were homozygous for the IVS-I-6 mutation displayed hematological traits that were significantly different from those of patients with other genotypes.

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