

Frequency of Cap+1 Mutation in Beta Thalassemia and its Associated Haematological Features

Muhammad Ijaz Iqbal, Helen Mary Robert, Nabeela Khan, Asad Mahmood, Ali Haider

Armed Forces Institute of Pathology/National University of Medical Sciences (NUMS) Rawalpindi Pakistan

ABSTRACT

Objective: To study the frequency of Cap+1 mutation and associated hematological parameters in suspected beta thalassemia patients.

Study Design: Analytical cross-sectional study.

Place and Duration of Study: Department of Hematology, Armed Forces Institute of Pathology (AFIP) Rawalpindi from Aug 2017 to Aug 2018.

Methodology: 960 patients suspected to have beta thalassemia were inducted into the study. After detailed history and examination basic hematological parameters (Hemoglobin, Mean Corpuscular Volume, Mean Corpuscular Hemoglobin) were analyzed using automated analyzer (Sysmex XE-5000). Molecular genetic analysis by conventional PCR was carried out for CAP+1 mutation. Genomic DNA purification kit (Gentra system USA) was used for extracting DNA from whole blood in order to study the molecular genetics for Cap+1 mutation. Primers were designed for detection and analysis of normal and mutant DNA.

Results: The frequency of Cap+1 mutation was observed in $3.2 \pm 1.7\%$ (31/960) in all suspected cases of beta thalassemia with a normal range of Hemoglobin (12.4 ± 1.1 g/dl), Mean Corpuscular Volume (86.4 ± 2.1 fl/red cell) and Mean Corpuscular Hemoglobin (29 ± 1.7 pg/cell).

Conclusion: Cap+1 mutation is a silent mutation and its diagnosis remains a challenge because of its normal clinical presentation and normal to mildly deranged basic hematological parameters. Detection of CAP+1 at molecular level has revolutionized the thalassemia prevention program in Pakistan.

Keywords: Beta thalassemia Cap+1 mutation, Hemoglobin, Polymerase chain reaction silent mutation.

How to Cite This Article: Iqbal I M, Robert M H, Khan N, Mahmood A, Haider A, Khan A. Frequency of Cap+1 Mutation in Beta Thalassemia and its Associated Haematological Features. *Pak Armed Forces Med J* 2022; 72(Suppl-2): S245-249. DOI: <https://10.51253/pafmj.v72iSUPPL-2.3080>

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INTRODUCTION

Thalassemia are a diverse group of inherited hematological disorders in which one or more of globin chains are either absent or reduced.¹ Prevalence of thalassemia is high in Northwest Africa, through the Middle East, Indian subcontinent to South East Asia. The frequency ranges from 2-30% amongst different populations.² Studies in Pakistan have revealed that the thalassemia has a carrier rate of 5%, Based on this carrier rate it is estimated that each year approximately 5000 new individuals are born with thalassemia major in Pakistan.³

Thalassemia is divided into three subgroups on the basis of the type of globin chain synthesis affected alpha thalassemia, beta thalassemia and delta thalassemia. Beta thalassemia is characterized by impaired synthesis of beta globin chains due to a mutation of gene located on chromosome 11.⁴ Worldwide occurrence of beta thalassemia varies from 1.7-9%. Pakistani

population has a carrier rate of approximately 5% for Beta Thalassemia trait, which ranges from 1-7%. Annual Incidence beta thalassemia in children of Pakistan is 6%.⁵ This high incidence is attributed to increasing trend of consanguineous marriages.⁶

Individuals with thalassemia major are diagnosed at the age of 6 months to 2 years. They require frequent blood transfusions and progressively become pale and gives a jaundiced look. Iron overload due to red cell breakdown is the most frequent complication encountered in such patients, along with hypersplenism, hepatitis etc. Prognosis of beta thalassemia is quite poor with a mean survival age of 5-35 years. Majority of the patients do not live longer than 5 years of age and only 50-65% individuals reach upto 35 years of age.

There are over 200 mutations causing beta thalassemia.⁷ Mutations associated with beta thalassemia include Fr 8-9, IVSI, Fr41-42, CD5, CD15, CD30(G-C) and CAP+1 (β^+). CAP+1 mutation is the most prevalent in Pakistani and South Asian populations.⁸ The purpose of this study was to study the relationship between CAP+1 mutation in individuals with beta

Correspondence: Dr Muhammad Ijaz Iqbal, Department of Hematology, AFIP Rawalpindi-Pakistan.

Received: 03 Jul 2019; revision received: 20 Feb 2020; accepted: 28 Feb 2020

thalassemia thus establishing its importance in the prevention of thalassemia in Pakistan.

METHODOLOGY

The cross sectional study was conducted on suspected beta thalassemia patients, after taking approval from ethics review committee AFIP (FC-HEM 17-29/READ-IRB/18/947) over a duration of 1 year at the department of Haematology/Molecular Pathology, Armed Forces Institute of Pathology, Rawalpindi. AFIP is a tertiary care referral centre receiving patients from different parts of the country. A sample size of 960 was calculated using WHO sample size calculator considering Level of significance 5%, Power of test 80% and confidence interval of 95%.

Inclusion Criteria: Patient selection was done on non-probability consecutive sampling technique after which they were stratified into 3 groups, Group 1 included Patients whose mutational analysis was done for suspicion of β thalassemia with positive family history, Group 2 comprised of patients whose mutational analysis was done due to Borderline Hemoglobin A2 levels, which was taken 3-4% for the purpose of study and group 3 patients whose mutational analysis was done as a part of antenatal screening.

Exclusion Criteria: All diagnosed cases of β thalassemia Major, Intermedia and Minor as well as Patients with normal Haemoglobin A2 levels were excluded from the study.

After taking detailed history and examination, Venous blood samples of 960 patients were collected in EDTA tube. Basic haematological parameters were determined by automated analysers Sysmex XE- 5000. Capillary electrophoresis for all patients was performed on SEBIA and Hb A-2 levels were estimated. DNA extraction was done from whole blood by using Sol Gent Genomic DNA prep kit. Amplification refractory mutation system (ARMS) primers were designed for detection of DNA (normal and mutant). Amplified PCR products were observed using PAGE (polyacrylamide Gel electrophoresis). Using Cap 1 specific primers, mutation was detected along with positive n negative control (Figure-1 & 2).

Data was analysed using SPSS version 25.0. Mean and SD was calculated for numerical variables such as age, HB, MCV and MCH. Percentage and frequency was calculated for categorical variables like gender, type of mutation. Data was divided into groups based on the presence or absence of CAP+1 mutation. Association between these groups and haematological

parameters was calculated using independent samples t-test, considering *p*-value of ≤ 0.05 to be significant.

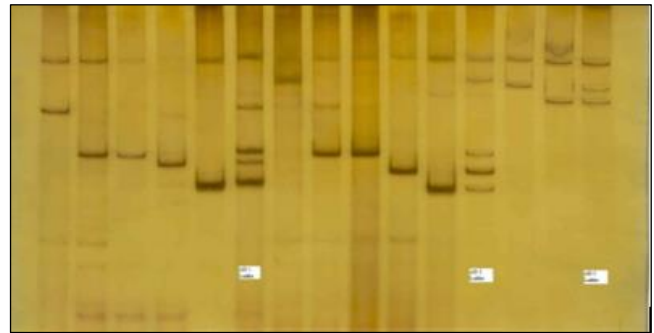


Figure-1: Multiplex PCR showing various thalassemia mutations.

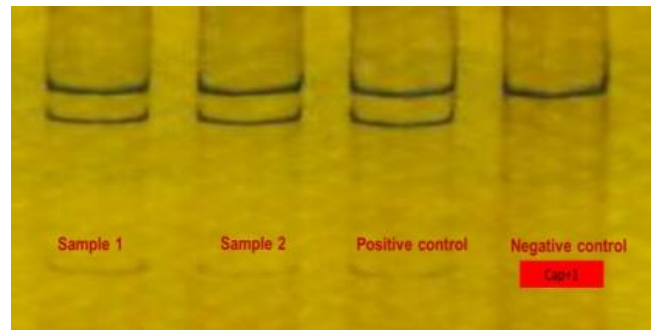


Figure-2: Detection of CAP+1 mutation on PAGE.

RESULTS

A total of 960 patients were recruited into our study. Out of these 510 (53.1%) were females and 450 (46.8%) were males with an age range of 21-43 years, with a mean age of 31 ± 5.3 years. Cap +1 mutation was positive in 31 (3.22% ± 1.7) patients. Patients screened positive for Cap+1 mutation were 10 (2.6%) patients out of total 380 patients in Group 1, 17 (4.3%) out of 390 patients in Group II and 4 (2.1%) patients out of total 190 in Group III. In total Cap+1 positive patient (31), there were 19 females (61.2%) and 12 males (38.8%), Figure-3 & 4.

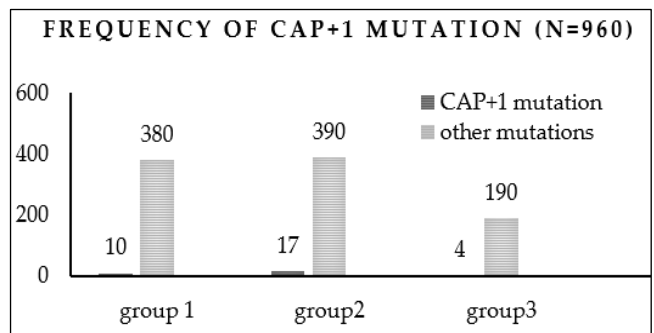


Figure-3: Frequency of Cap+1 mutation.

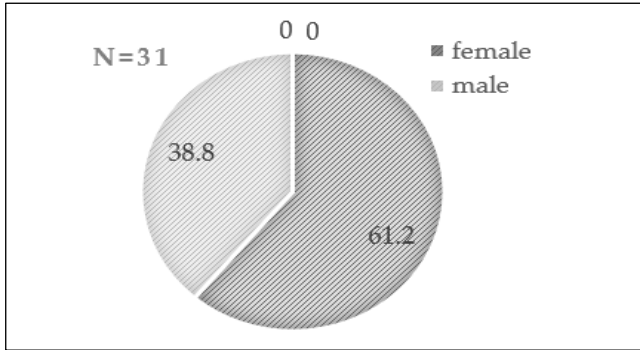


Figure-4: Gender distribution in patients with Cap+1 mutation.

We also analysed the haematological parameters with CAP+1 patients and compared them with patients having mutations other than CAP+1. Mean haemoglobin in Cap +1 patients was 12.4 ± 1.1 mg/dl where as in other mutations it was 7.8 ± 1.2 mg/dl. Mean MCV of 78.4 ± 2.1 fL/red cell was observed in Cap+1 positive patient however in other mutations MCV of 68.1 ± 1.8 fL/red cell was recorded. 25.6 ± 1.7 pg/cell MCH was recorded in Cap+1 mutation patients, as mentioned in Table.

Table:Various haematological parameters in CAP+1 and other mutations.

Parameter	CAP+1 mutation (n=31)	Other mutations (n=929)	p-value
Hb (g/dl)	12.4 ± 1.1	7.8 ± 1.2	0.04
MCV (fL/red cell)	78.4 ± 2.1	68.1 ± 1.8	0.02
MCH (pg/cell)	25.6 ± 1.7	19.4 ± 1.1	0.01

DISCUSSION

Beta thalassemia are categorized into $\beta 0$ or $\beta +$, based on beta globin chain synthesis. In thalassemia where the gene is incapable to translate for any viable translator mRNA and therefore, no beta chain is synthesized is termed as $\beta 0$. However, in $\beta +$ thalassemia's, fewer amount of beta chains is still formed.¹⁰ This leads to development of severe anaemia which is highly reliant on blood transfusions for its improvement. Repeated transfusion of blood products is required on monthly basis or more. Major side effect of repeated blood transfusion is iron deposition in the body which is highly toxic and cause debilitating side effects to major organs like liver, heart and pancreas. Iron chelation therapy is required in such cases for which the drugs required are quite expensive. Affordability of such treatment is a major dilemma for the thalassemia patient due to which a wide population among thalassaemic children die before they reach 5 years of age. Thalassemia patients suffer from various

transfusion dependent complications. These include iron overload, infections including HIV, HCV, HBV, hemolytic and non-hemolytic reactions.¹¹

The first step in diagnosing such cases is the Assessment of Red cell parameters.¹² Typical $\beta 0$ or severe $\beta +$ thalassemia, demonstrate lower Hemoglobin, Mean Corpuscular Volume and Mean Corpuscular Hemoglobin levels. However, some carriers have very mild form of β thalassemia that it is phenotypically silent with normal haematological parameters and no clinical sign and symptom of anemia. Accurate diagnosis prenatally can be done via molecular and genetic studies. Disease conditions which mimics homozygous beta thalassemia include ALA synthase deficiency, juvenile chronic myeloid leukemia, aplastic anemia etc. The ideal time for genetic risk assessment, carrier rate detection and counselling of the patient is before conceiving. Patients who are carriers and are at a greater risk should be offered genetic counselling, with options of prenatal diagnosis and various effects on the offspring.

CAP+1 is among one of the mild beta thalassemia mutation which has minimal influence on production of beta globin chain leading to less clinical severity and normal ranges of basic haematological parameters, thus is known as a silent mutation.¹³ Cap +1 mutation has a high prevalence in our population ($3.22\% \pm 1.7$) as shown in our study. Our study showed a female predominance with 62.1% than males (38.8%). This poses serious screening and diagnostic difficulties because of contradictory clinical and haematological picture. As shown in our study in which individuals with CAP+1 positive mutation showed a normal range of Hb (12.4 ± 1.1), MCV (78.4 ± 2.1) and MCH (25.6 ± 1.7). SAK khattak *et al*,¹⁴ showed similar results in his study, when the patients detected with Fr 8-9 mutation were analysed for these parameters, they had the lowest mean MCV of 63.7fl and MCH of 19.1pg, of all the other mutations analysed. Whereas, patients with CAP+1 mutation had mean Total Red Blood Cell count of $5.5 \times 10^{12}/L$, mean Hb of 13.5g/dl, MCV of 78.0fl MCH of 24.7pg and RDW 41.9fl respectively. Showing that basic haematological parameters were near normal range.

Karim *et al*, reported a 5% frequency of CAP+1 mutation in targeted beta thalassaemic families and 2% in overall thalassaemic population in Pakistan.¹⁵ Similar results were shown by Garewal *et al*, in north Indians the frequency of CAP+1 allele was 3.2%¹⁶

Detection of carriers, counselling of the families who are at risk and prenatal diagnosis using amniocentesis or chorionic villus sampling are 3 main highly important domains for prevention of this disease.¹⁷ As shown in our study patients in group 3 whose mutational analysis was done as a part of antenatal screening depicted a 2.1% positivity for CAP+1 mutation.

Beta thalassemia is inherited in an autosomal recessive pattern. In Couples who are at a risk, if one of the parent is an identified β thalassemia carrier and other individual is clinically normal and symptomless i.e. he/she is a silent carrier, joint inheritance of an allele of β thalassemia and CAP+1 allele in a developing foetus may lead to thalassemia intermedia after birth. As silent carriers (CAP+1 mutation) remain a diagnostic challenge for the clinician due to normal routine haematological variables (Blood Complete Picture, HPLC and Hb electrophoresis) they may give birth to individuals with beta thalassemia intermedia if they get married to individuals with beta thalassemia minor.

Diagnosis of beta thalassemia starts from the proper medical history and analysing routine basic haematological parameters (Hb, MCV, MCH). When microcytic, hypochromic anemia with target cells are identified further classification of thalassemia can be done by measuring HbA2 levels. Accurate diagnosis can be carried out by molecular and genetic studies. Since the basis of diagnosis is basic haematological parameters and they appear to be in normal range in patients with CAP+1 mutation, so its detection plays a crucial role in premarital screening of patients. As shown in our study that patients in group whose mutational analysis was done for suspicion of β thalassemia with positive family history showed 2.63% individuals screened positive for CAP+1.

Due to high mortality rate associated with thalassemia in our population.¹⁸ Prevention of births of thalassemia children should be promoted as definitive treatment like haematopoietic stem cell transplantation and gene therapy are quite expensive treatment options and are still under trial. Mutational analysis for CAP+1 should be made a mandatory part of suspected families. Continued genetic education and public awareness programs are fundamental for premarital screening and genetic counselling to gain optimal acceptability. After establishment of such enlightening programs, a premarital screening program will have a

better chance in successful reduction of the high prevalence of this disease in our country.

CONCLUSION

One of the diagnostic challenge in thalassemia patients is to identify the presence of a silent CAP+1 (A-C) mutation which has very minimal effects on synthesis of beta hemoglobin chains. Cap+1 (A-C) mutation is an unanticipated cause of beta thalassemia transmission in Pakistani population and presents itself with normal red cell values. Combined inheritance of an allele of β thalassemia (major or minor) along with CAP+1 allele in a developing embryo may in turn lead to an individual with thalassemia intermedia after birth. Therefore, in individuals with high clinical suspicion proper prenatal screening and diagnosis plays a pivotal role in detection of carriers and presence of CAP+1 mutation by DNA analysis.

Conflict of Interest: None.

Author's Contribution

MII: Direct contribution to conception, design, analysis, interpretation, HMR: Data analysis, NK: Intellectual contribution to analysis and interpretation, AM: Manuscript preparation, AH: Data collection.

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