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## Research Article

### STUDY OF SR. IRON, TIBC LEVELS AND HAMP GENE MUTATIONS IN SICKLE CELL DISEASE

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Sickle cell disease, Serum Iron, Iron Binding Capacity, HAMP gene mutation

#### ABSTRACT

**Background:** Sickle cell disease is common hereditary hemoglobinopathy that occurs primarily in the African Americans, Arabians and Indians. It is a multi - system disease, associated with episodes of acute illness and progressive organ damage.<sup>[1]</sup> It is a genetic disorder, characterized by the presence of the hemoglobin S (HbS), where valine is replaced by glutamic acid ( $\beta^s 6 \text{Glu} \rightarrow \text{Val}$ ) at the beta globin chain, that has a single point mutation (GAG  $\rightarrow$  GTG) at the sixth codon of the  $\beta$ -globin (*HBB*) gene. The sr. iron is regulated by HAMP gene. So the aim of our study was to find out the Sr. Iron, TIBC levels and HAMP gene mutation in sickle cell disease.

**Aim:** To study the Sr. Iron, TIBC levels and HAMP gene mutation in sickle cell disease.

**Material and Methods:** This study was being conducted in the Department of Biochemistry, People's College of Medical Sciences and Research Centre (PCMS & RC) and Centre for Scientific Research and Development (CSRD), People's University Bhopal. The study protocol had been approved by Institutional Ethics committee (IEC) of our institute. This was the hospital based case control study, included 111 SCD patients and 111 healthy controls after applying inclusion and exclusion criteria. The biochemical markers like Sr. Iron and Total Iron Binding Capacity TIBC by Kit. Ferrozine / MgCO<sub>3</sub> method and HAMP Gene Polymorphism: Genotyping of G71D of *HAMP* variants was performed by polymerase chain reaction-single nucleotide polymorphism.

**Results:** The Sr. Iron was found significantly decreased in cases ( $57.22 \pm 25.42$ ;  $106.33 \pm 26.62$ ) compared to controls. Serum Total iron binding capacity (TIBC) was found increase significantly very high ( $57.22 \pm 25.42$ ;  $289.85 \pm 33.40$ ) in cases of SCD. The results of Genotyping of HAMP-G71D variant in SCD patients revealed that, 111 out of 111 (100%) patients showed wild type genetic profile, 0/111 (0%) had variation in HAMP-G71D. So, no any single mutation found in SCD patients. The healthy control studies, (100%) were carried a wild type genetic profile in HAMP-G71D gene. So, no statistically significant difference found in gene frequencies of G71D mutation was observed in between SCD patients and controls.

**Conclusions:** The study concluded that, as the serum iron level decreases the total iron binding capacity (TIBC) increases in Sickle cell disease patients, this could be biochemically important diagnostic tests for the sickle cell disease. The HAMP gene is not showing mutation in the molecular level so we need to find out another parameter in the diagnosis of SCD at molecular level.

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#### INTRODUCTION

Sickle cell disease is common hereditary hemoglobinopathy that occurs primarily in the African Americans, Arabians and Indians. It is a multi - system disease, associated with episodes of acute illness and progressive organ damage.<sup>[1]</sup> It is a red cell disorder with multiple acute and chronic complications, the complications of sickle cell disease (SCD) is due to multiple factors such as intrahepatic sinusoidal sickling, bilirubin,

transfusion - related hepatitis infections or excess iron deposition and different in patho-physiological outcomes, remains a healthcare challenges, especially in sub-Saharan Africa.<sup>[2, 3, 4, 5]</sup>

It is a genetic disorder, characterized by the presence of the hemoglobin S (HbS), where valine is replaced by glutamic acid ( $\beta^s 6 \text{Glu} \rightarrow \text{Val}$ ) at the beta globin chain, that has a single point mutation (GAG  $\rightarrow$  GTG) at the sixth codon of the  $\beta$ -globin

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(HBB) gene. This point mutation is responsible for the alteration in the properties of the hemoglobin tetramer, with a tendency to polymerize in the deoxygenated state altering normal, flexible, biconcave shaped red blood cells (RBCs) are changes in to stiff, rigid, sickle cell RBCs. If the mutation affects only one  $\beta$ -globin chain and the other is normal, the patient is said to have the sickle cell trait, which is relatively benign carrier state and does not have the classic phenotypic features of sickle cell disease (SCD). When both  $\beta$ - chains carry HbS mutation, the patient exhibits phenotypic features of SCD which may include recurrent painful crisis, anemia, infections, stroke, organ failure and premature death due to various complications and end organ damage. Sickle cell disease is a group of disorders associated with a mutation in the  $\beta$  globin gene, associated with multi organ damage, with various diseases like; hemoglobinopathy, causes sickling of red blood cells, resulting in vessel blockage, stroke, anemia, inflammation, and extreme pain.<sup>[6]</sup> The sr. iron is regulated by HAMP gene, so the aim of our study was to find out the Sr. Iron, TIBC levels and HAMP gene mutation in sickle cell disease.

## MATERIAL AND METHOD

This study was being conducted in the Department of Biochemistry, People's College of Medical Science and Research (PCMS & RC) and Centre for Scientific Research and Development (CSR D), People's University Bhopal. The study protocol was approved by Institutional Ethics committee (IEC) of our institute.

### Study Design

This hospital based case control study included 111 subjects, after applying inclusion and exclusion criteria. All the socio-demographic data of the participants was entered in a self designed quaternary. All the participants will be will be the steady state, characterized by absence of blood transfusion in a period of four months prior to blood draw. In addition, patients included in this study did not show any infection, hospitalization or vaso-occlusive event, and were not under antibiotics, corticosteroids or hydroxyurea (HU) treatment but all patients were under treatment with folic acid. The control group 111 healthy normal subjects were characterized by absence of hematological disorders or inflammatory conditions. All procedures followed will be in accordance and approved by the Institutional Ethics Committee of the People's College of Medical Sciences & Research Center, Bhopal, and also with the Helsinki Declaration of 1975 and its revisions. Informed consent forms will be obtained from all patients.

### Inclusion criteria for normal healthy subjects

Normal healthy person are comprised of staff, medical students posted for internship or the relatives who were healthy and accompany their IPD ward or OPD.

**Inclusion criteria:** A) Normal healthy individuals, more than 5years age and less than 80 years of age were selected and recruited for the control group.

### Exclusion criteria

- a) Patients less than 5years and more than 80 years.

- b) Individuals with any other hemoglobinopathy, and the patients having any history of blood transfusion within 3 months.
- c) SCD patients under the treatment of chemotherapy will be excluded from the study.

### Screening tests for the sickle cell disease subjects

The following screening tests were carried out with the blood sample of SCD patients for the confirmation of sickle cell RBCs.

1. **Sickling test with 2% meta-bisulphite:** It was the principle of sickling test, was based on microscopical observation of sickling of red blood cells when exposed to a low oxygen tension.
2. **Solubility test with 0.02% sodium dithionate:** It was the principle of solubility method, based on turbidity created when Hb S is mixed with sodium dithionate.
3. **Peripheral blood film method:** Thin blood films, stained with giemsa stain were examined by light microscopy ( $\times 100$ ).
4. **Hb electrophoresis:** The cellulose acetate membrane Hb electrophoresis method will be used to determine the presence of Hb-S in the sample.
5. **Patient's history and blood cell counts such as;** RBC, WBC, and HCT, MCH, MCV, MCHC, were carried out.

To confirm any diagnosis, a sample of blood were examined under a microscope to check for large number of sickle cells, patient's history and blood cell counts i.e. RBC, WBC, and HCT, MCH, MCV, MCHC, was carried out.

### Sample collection

The 10 ml overnight fasting venous blood was collected from patients and controls under aseptic conditions. 6 ml blood will be collected in plain vacutainer, and remaining 4ml blood will be poured in EDTA anticoagulated vacutainer. The Sample was centrifuged at 3000 rpm for 10 minutes; serum were separated and immediately stored in deep freezer at  $-20^{\circ}\text{C}$  until further analysis. The biochemical markers like Sr. Iron and Total Iron Binding Capacity TIBC by Kit. Ferrozine /  $\text{MgCO}_3$  method.<sup>[8]</sup> and HAMP Gene Polymorphism: <sup>[9]</sup> Genotyping of G71D of HAMP variants was performed by polymerase chain reaction-single nucleotide polymorphism.

All PCR reactions were performed in a total volume of 25 $\mu\text{l}$  containing 12.5  $\mu\text{l}$  Master Mix (Qiagen kit), 1  $\mu\text{l}$  forward primer (25 pmol), 1  $\mu\text{l}$  reverse primer (25 pmol), 7.5  $\mu\text{l}$  nuclease- free water, and 3  $\mu\text{l}$  genomic extracted DNA. For G71D genotyping, the following primers were used, Forward primer: 5'- TGCTCACATTCCCTTCCTTC -3' and Reverse prime: 3'- CAAGACCTATGTTCTGGGGC -5' The thermocycler program applied was initial heating at  $94^{\circ}\text{C}$  for 5 min, followed by 30 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $60^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 60 s. A final extension step was carried out at  $72^{\circ}\text{C}$  for 10 min. The digested products were then run on a 2.5% agarose gel for 1 hour and photographed under UV-light. A DNA molecular weight marker was also run to identify the site of bands and then amplification; purification with PEG-NaCl, sequencing PCR and purification, COI sequencing, Trace file, NABI database and then analysis is carried out to see the mutation.

**Polymerase Chain Reaction (PCR)**

SrNo	Gene	Primer Name	Sequence (5'-3')	Bases	Ann. Temp.	Amplicon size (bp)
1	HAMP	HAMP3F	TGCTCACATTCCCTTCCTTC	20	55°C	200 bp
		HAMP3R	CAAGACCTATGTTCTGGGGC	20		

[10, 11]

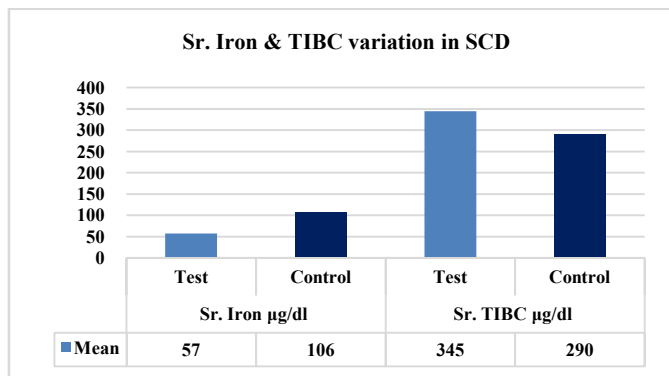
**RESULTS**

The observations and inferences obtained from this study are summarized in following tables:

**Table 1** Sr. Iron and Sr. TIBC level variations in Sickle Cell Disease

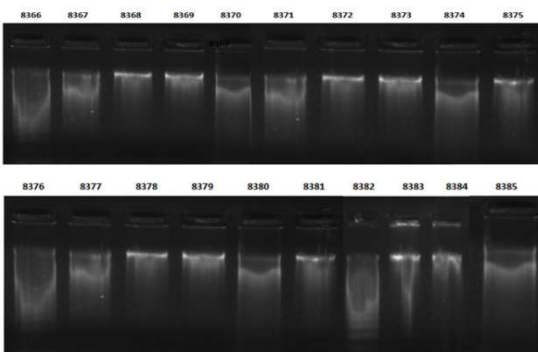
Variable	Group	Mean ± SD	t-stat	p-value	Significant level
Sr. Iron µg/dl	Test	57.22 ± 25.42	14.05	0.0000	Highly Significant
	Control	106.33 ± 26.62			
Sr. TIBC µg/dl	Test	344.57 ± 45.03	10.29	0.0000	Highly Significant
	Control	289.85 ± 33.40			

The Sr. Iron was found significantly decreased in cases (57.22 ± 25.42; 106.33 ± 26.62) compared to healthy controls. Serum Total iron binding capacity was found increase significantly high (57.22 ± 25.42; 289.85 ± 33.40) in cases of SCD.

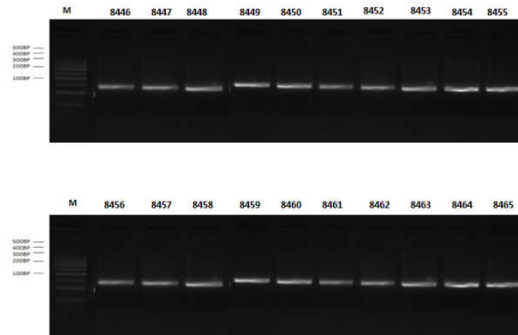


**Graph 1** Sr. Iron and Sr. TIBC level variations in Sickle Cell Disease

And in the graphical presentation, The Sr. Iron is declined in the cases and serum TIBC is increased above the normal level (highly significant) in cases as compared to the controls.



**HAMP gene: Figure. 1.** gDNA image 1% (w/v) Agarose Gel Electrophoresis:



**Fig 2** HAMP: Agarose Gel Electrophoresis Gel Images: Image: 2% (W/V) Lane M: 100 bp DNA marker with Sr. sample product

**Table 2** Frequency of HAMP gene in sickle cell disease

Genotypes	Mutations	No. of mutations	Frequency in %	Interpretation
HAMP gene	G71D	0.0	0.0	Normal activity of HAMP
	G71G	0.0	0.0	Normal activity of HAMP

**DNA Sequencing**

**HAMP GENE**

>Ref seq  
 GGCCCCCTGCCATCCTCTGCACCCCCCTTCTGCTTTTAC  
 AGACGGGACAACCTGACAGAGCTGCAACCCAGG  
 ACAGAGCTGGAGCCAGGGCCAGCTGGATGGTGAGCG  
 CAACAGTGATGCCTTTCCTAGCCCCCTGCTCCCTC  
 CCCATGCTAAGGCCGTTCCCTGCTCACATTCCCTTCC  
 TTCCCACAGCCCATGTTCCAGAGGCCGAAGGAGG  
 CGAGACACCCACTTCCCCATCTGCATTTTCTGCTGCGR  
 CTGCTGTCATCGATCAAAGTGTGGGATGTGCTG  
 CAAGACGTAGAACCCTACCTGCCCTGCCCCCGTCCCCT  
 CCCTTCCTTATTTATTCCTGCTGCCCCAGAACAT  
 AGGTCTTGAATAAAAATGGCTGGTTCTTTTGTTTTCCA  
 AACCAGAGTGTCTGTTGTCCTTTCTCTCTGCCG  
 AGTGTCTGTGCTAAGAGCTTGTCTGACCCTGCCTTGC  
 AAGCACCAGTGCTTGGTGGGTCATGTGGGGCTG  
 GTGT

sample sequences:

>C28(HMP3F)  
 GTCTCCAAGGCCGAGCAGCGAGAACCCACTTCCCCAT  
 CTGCATTTTCTGCTGCGGCTGCTGTCATCGATCA  
 AAGTGTGGGATGTGCTGCAAGACGTAGAACCCTACCTG  
 CCCTGCCCCCGTCCCCTCCCTTCCTTATTTATTC  
 CTGCTGCCCCAGAACATAGGTCTTGAA  
 >C29-8447(HAMP3F)  
 CATCCCAAAAGCGGAAGAGCGAGAACCCACTTCCCC  
 ATCTGCATTTTCTGCTGCGGCTGCTGTCATCGATC  
 AAAGTGTGGGATGTGCTGCAAGACGTAGAACCCTACCT  
 GCCCTGCCCCCGTCCCCTCCCTTCCTTATTTATT  
 CCTGCTGCCCCAGAACATAGGTCTTG  
 >C30-8448(HAMP3F)  
 ATCCCAAAGGGGGAAGGAGCGAGAACCCACTTCCCC  
 ATCTGCATTTTCTGCTGCGGCTGCTGTCATCGATC  
 AAAGTGTGGGATGTGCTGCAAGACGTAGAACCCTACCT  
 GCCCTGCCCCCGTCCCCTCCCTTCCTTATTTATT

CCTGCTGCCCCAGAACATAGGTCTTGA  
 >C31-8449(HAMP3F)  
 TTTCAGAGGGCGGGGGAAGGCGAGAACCCACTTCC  
 CCATCTGCATTTTCTGCTGCGGCTGCTGTCATCGA  
 TCAAAGTGTGGGATGTGCTGCAAGACGTAGAACCTAC  
 CTGCCCTGCCCCGTCCCCTCCCTTCCCTTATTTA  
 TTCTGCTGCCCCAGAACATAGGTCTTGA  
 >C32-8450(HAMP3F)  
 CTTCAAAGGGCGAAGGAAGCGAGAACCCACTTCCCC  
 ATCTGCATTTTCTGCTGCGGCTGCTGTCATCGATC  
 AAAGTGTGGGATGTGCTGCAAGACGTAGAACCTACCT  
 GCCCTGCCCCGTCCCCTCCCTTCCCTTATTTATT  
 CCTGCTGCCCCAGAACATAGGTCTTAAA

HAMP: Results of Genotyping of HAMP-G71D variant in SCD patients revealed that, 111 out of 111 (100%) patients showed wild type genetic profile, HAMP-G71D gene in Sickle Cell Disease state. 0/111 (0%) had variation in HAMP-G71D. So, no any single mutation found in SCD patients. The healthy control studies, (100%) were carried a wild type genetic profile in HAMP-G71D gene. So, no statistically significant difference found in gene frequencies of G71D mutation was observed between SCD studied patients and controls.

## DISCUSSION

Hepcidin is regulated by a number of mechanisms, including genes, signals released from the erythroid bone marrow, iron stores, circulating iron, oxygen homeostasis, and inflammatory cytokines. <sup>[12]</sup> Merryweather-Clarke AT *et al.*, stated that, the G71D mutation in the *HAMP* gene was detected in the general northern European population at an allele frequency of 0.3% <sup>[13]</sup> and Jacolot S, *et al.*, Biasiotto G, *et al.*, and Merryweather-Clarke AT *et al.*, all been also found the same mutations in the France population. <sup>[14]</sup> Italy <sup>[15]</sup> and UK. <sup>[13]</sup>

In the patients with sickle cell anaemia, chronic haemolysis results in increased availability of iron directly from the broken red cells and also from increased absorption of iron from the gastrointestinal tract. <sup>[16]</sup> Additionally, the high load of iron provided by multiple blood transfusions. <sup>[17, 18]</sup> However, reduced frequency of transfusion implies a reduction in sources of iron and, therefore, increased vulnerability to iron deficiency anemia. This assertion is buttressed by a study in the USA which suggested that iron deficiency was commoner than expected in untransfused patients with sickle cell anemia, explained by Vichinsky E., *et al.* <sup>[19]</sup> Jeyakumar *et al.* stated that, the Serum iron concentration is a balance between intakes on the one hand and excretion as well as increased utilization on the other. Since there is no empirical reason to believe that HbSS subjects had lower dietary intake of iron, attention will have to be focused on excretion. <sup>[20]</sup> Koduri *et al.* reported that the sickle cell anemia patients lose excessive amounts of iron through excretion through the body. <sup>[21]</sup>

Our present study is also supporting with above all with same hypothesis, because the Sr. Iron was found significantly decreased below the healthy controls in the SCD cases ( $57.22 \pm 25.42$ ;  $106.33 \pm 26.62$ ); But the Sr. Total Iron Binding Capacity (TIBC) were found significantly increased as compared to the healthy controls. ( $344.57 \pm 45.03$ ;  $289.85 \pm 33.40$ ). Here again the Vichinsky E *et al*, Rao KRP *et al.*, and Haddy TB *et al.*, described in their studies that, the low serum iron and elevated

total iron binding capacity commonly occurs in patients with increased in iron deficiency in sickle cell disease. <sup>[19, 22, 23]</sup>

Hussain MA, *et al.*, studied that, there were some correlations between blood transfusion (BT) and serum ferritin. High iron status was found only in children who needed frequent BT but, according to study, none of our patients had serum iron more than 1000 ng/ml. He also observed that 6% of SCD had ferritin level greater than 1000 ng/ml. <sup>[24]</sup> Vichinsky *et al.*, (2005), ZA Jenkins (2007), described that, 43 adult patients with SCD who were previously transfused for a mean of 6 years, resulting in elevated mean ferritin levels at 2916 ng/ml. <sup>[25, 26]</sup>

Results of Genotyping of HAMP-G71D variant in SCD patients revealed that, 111/111 (100%) patients showed wild type genetic profile, HAMP-G71D gene in homozygous and heterozygous state. 0/111 (0%) had variation in HAMP-G71D. The healthy control and SCD patients studied was carried (100%) a wild type genetic profile in HAMP-G71D gene. So, no statistically significant difference in gene frequencies of G71D mutation was observed between studied SCD patients and in the controls.

Lyon E, *et al.*, In their study, the G71D mutation of the *HAMP* gene revealed higher frequency among SCD patients than the normal controls, 8.5% vs. 6.7% respectively, although statistically non-significant. The H63D mutation has a prevalence of approximately 16% in the European population. <sup>[27]</sup> Viana-Baracioli LM, *et al.*, in (2011) reported that, the H63D allele frequency had been found in brazilian population that the 10.7% in sickle cell disease patients in Brazil. <sup>[28]</sup> El-Rashidi FH *et al* in (2008) and Madani HA *et al.*, in (2008) stated that, the allele frequency of H63D mutation ranged from 13 to 30% in thalassemia patients and between 10 and 11% in controls; in Egypt population, <sup>[29,30]</sup> In this study, the frequency of H63G heterozygous mutation was significantly higher in SCD patients than in the controls, 27.7% vs. 8.9%, respectively,  $p=0.02$ . The overall allele frequency of H63D in SCD patients and normal controls in our study were 13.8 % and 4.4%, respectively. <sup>[29, 30]</sup>

Hala A. Abdel Rahman *et al.*, (2014) noted in his study that, the SCD patients carrying either *HAMP*-G71D or *HFE*-H63D variants did not show statistically significant difference in iron overload parameters in relation to the patients with wild genotype. <sup>[31]</sup> Previous studies reported contradictory results regarding the impact of G71D mutation of *HAMP* gene as a possible modifier in iron overload diseases. <sup>[13, 14, 15, 32]</sup>

## CONCLUSION

The study concluded that, as the serum iron level decreases, the total iron binding capacity (TIBC) increases in Sickle cell disease patients, this could be biochemically important diagnostic tests for the sickle cell disease. The *HAMP* gene is not showing mutation in the molecular level so we need to find out another parameter in the diagnosis of SCD at molecular level.

### Conflict of Interest:

None declared.

### Funding Sources:

Nil

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