Journal of Bioresource Management

Volume 9 | Issue 2 Article 9

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Recommended Citation

Kanwal, A., Mahmood, M., & Irshad, S. (2022). Hepcidin Antimicrobial Peptide (HAMP) Screening for P.CYS70ARG Variant and Iron Overload in β -Thalassemia Major Patients, *Journal of Bioresource Management, 9* (2).
ISSN: 2309-3854 online
(Received: Aug 9, 2021; Accepted: Mar 27, 2022; Published: Jun 15, 2022)

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Cover Page Footnote

This work was performed in the School of Biochemistry and Biotechnology, University of the Punjab, Lahore.

HEPCIDIN ANTIMICROBIAL PEPTIDE (*HAMP*) SCREENING FOR P.CYS70ARG VARIANT AND IRON OVERLOAD IN β-THALASSEMIA MAJOR PATIENTS

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ABSTARCT

Hereditary Hemochromatosis is a rare genetic iron overload disorder characterized by iron accumulation in vital body organs such as the lungs, liver, and pancreas. *HAMP* mutations are reported as one of the principal sources for the disturbance of iron homeostasis. This study was designed to screen the involvement of p.Cys70Arg *HAMP* variant in iron overload in the β-thalassemia patients. For the purpose, bioinformatics tools were used for the structural and functional manifestation of mutated protein which revealed 1.93 kcal/mol energy differences between the wild-type and mutated proteins, causing the stability decline. Following that, clinical data was collected for 106 β-thalassemia major (β-TM) patients which showed a higher prevalence of splenectomy, hepatomegaly and ascites. The PCR-RFLPs were performed to screen the *HAMP* p.Cys70Arg in 27 controls and 106 β-TM patients. Sac II restriction enzyme was used to screen genetically affected and ethnically matched control samples but no control was found with *HAMP* p.Cys70Arg variant. Out of these 106 β-thalassemia patients, eight patients were HCV^+ with higher levels of ferritin in blood. *HAMP* exon 3 Sanger sequencing did not reveal any mutation in these patients conferring iatrogenic hemochromatosis. Future recommendations include sequencing of complete *HAMP* gene with its three exons in a large sample size.

Keywords: Hepcidin, variant, sangers sequencing, HCV^+, PCR-RFLPs.

INTRODUCTION

Transfusion dependent thalassemia (TDT) is a group of heterogeneous genetic disorders of which the most common is β-thalassemia major (β-TM) characterized by diminished β-globin chain production leading to globin chain discrepancy and stark anemia (Al-Khabori et al. 2014). Patients suffering from β-TM require regular blood transfusions on weekly or monthly basis. These blood transfusions lead to a number of complications like iron overload, hepatitis B, hepatitis C and HIV infection (Al-Khabori et al. 2014) along with pallor, ascites, oedema, cardiomyopathy and increased transferrin saturation. Serum ferritin level is used to find the extent of iron overload in β-TM patients.

In normal individuals, toxicity of iron is managed due to its storage in liver, pancreas or kidney which is mobilized only when needed. When the level of iron is beyond its storage capacity, it becomes toxic (Haq et al. 2020). Hemosiderosis is a state of iron overload in different body tissues and organs which can be genetics (hereditary hemochromatosis HH) or iatrogenic due to subsequent blood transfusions (Kawabata 2018, Sato et al. 2020).

Iron metabolism is regulated with a number of proteins, the most important one is HAMP protein (Camaschella 2013, Kroot et al. 2011). Mutations in *HFE*, transferrin receptor 2 (*TFR2*), *TFR1*, hemojuvelin (*HJV*) or ferroportin (*SLC40A1*) might also be causative for HH

(Pietrangelo 2015). HH type1 which is caused due to *HFE* p.Cys282Tyr in homozygous state is prevalent in Asia. HH type2 is further divided into type2A and type2B due to multiple variants in *HJV* and *HAMP*, respectively (Krause et al. 2000).

The *HAMP* is synthesized as a precursor molecule which consists of 84 amino acids. This precursor molecule cleaves into small peptides of 25, 22 and 20 amino acids. Hepcidin consisting of 25 amino acids, is the most active form. A striking feature of these peptides are cysteines, (Nicolas et al. 2002) which make up 32% of total amino acid content. This higher amino acid content gives this structure, rigid and tight conformation (Ahmad et al. 2002). Promoter analysis reveals consensus sequences for the transcription factor CCAAT/enhancer-binding protein-α (CEBP/α), which shows that it is hepatic in origin (Pigeon et al. 2001). The single *HAMP* gene is present in humans, sheep, horses (Badial et al. 2011) and dogs (Lou et al. 2004).

The *HAMP* mutations are identified as responsible for Hereditary Hemochromatosis (Roetto et al. 1999). A small number of patients are found with the variants in *HAMP* (McDonald et al. 2013). Only sixteen variants in *HAMP* promoter region or coding region have been identified causing HH (accessed Human Gene Mutation Database HGMD, June 2022), out of which only three have been reported in the Asian region, p.Arg42Serfs in Pakistan, p.Arg75* in Japan and p.Cys78Thr in the Israel population (McDonald et al. 2013). *HAMP* p.Cys70Arg has been reported in the Italian population affecting 1 out of 8 conserved cysteines that form disulfide bonds which are essential for the stability of polypeptide (Roetto et al. 2004). Uptil now, sixteen HAMP mutations (eight missense, five regulatory and three small deletions) have been reported causing juvenile hemochromatosis with iron overload in liver (accessed through HGMD June 2022).

To define the prevalence of p.Cys70Arg among β-TM patients and the role of *HAMP*, we screen the blood samples of 106 β-TM patients with iron overload complications along with 27 normal ethnically matched controls. The discovery of *HAMP* mutations and their ultimate consequences could lead to new therapies for hemochromatosis (Ganz 2003).

MATERIALS AND METHODS

Bioinformatic Analysis

Massenger sequence (mRNA) (NCBI reference sequence: NM_021175.3) (https://www.ncbi.nlm.nih.gov/) of *HAMP* gene exon 3 was aligned by Bioedit software (https://bioedit.software.informer.com/7.2/) and mutation was analyzed by RegRNA 2.0 (https://bio.tools/regrna). Expasy translate tool (https://web.expasy.org/translate/) was used to convert nucleotide sequence into the amino acid sequence. The 3D structure was retrieved from Protein Data Bank (https://www.rcsb.org/) (PDB: 1M4F) and the mutated protein (C70R) 3D structure was built using PyMoL program (https://pymol.org/2/). The mutated and the wild-type protein structures were analyzed for energy calculations through FoldX (http://foldxsuite.crg.eu/). PROVEAN (http://provean.jcvi.org/index.php) and Verify3D (https://bip.weizmann.ac.il/toolbox/structure/3d.htm) were used to evaluate the 3D model of the mutated protein.

Sample Collection

Ethical approval was taken from the School of Biochemistry and Biotechnology (SBB), University of the Punjab, Lahore to collect blood samples of 27 ethnically matched controls and 106

clinically diagnosed β-thalassemia major patients (β-TM). β-TM patient samples were taken from Sundas Foundation, Lahore with age range from one month to 27 years old. A complete history of these patients were taken on a proforma including their current age, the phenotype of blood, age of disease diagnosis, age of first blood transfusion, frequency of transfusions, ferritin level, presence of secondary complications including oedema, jaundice, pallor, ascites, splenomegaly, lymphadenopathy, hepatomegaly and HCV reactive antigens.

Clinical Analysis

During clinical analysis, clinical data of 106 β-TM patients were collected. For the estimation of patients' immune level, values of WBCs and platelets were taken.

DNA Isolation and PCR Amplification

The DNA of 27 controls and 106 β-TM patients was isolated using the salting out and sucrose lysis method (MWer et al. 1988). For qualitative analysis of genomic DNA, 0.8% agarose gel electrophoresis was performed and spectrophotometry was performed for quantitative analysis. *HAMP*, exon 3 was amplified from the blood of patients and controls. Primers were designed by using Primer3D (https://bioinfo.ut.ee/primer3-0.4.0/). 25µl volume mixture was prepared containing 100ng of genomic DNA, 10mM of dNTPs, 10mM of forward and reverse primers, 10X PCR buffer, 1 unit of Taq polymerase and 5µl of 25mM $MgCl_2$ for PCR amplification for primer set (Table 1).

Thermocycler profile for PCR amplification was set with an initial denaturation at 95^{o}C for 2 min, leading to 32 cycles of denaturation at 95^{o}C for 30sec, annealing at 57.1^{o}C for 30sec and extension at 72^{o}C for 30 sec with a final extension at 72^{o}C for 10 min. PCR product was observed on 1.5% agarose gel electrophoresis.

Mutational Analysis

For restriction, Cfr421 (Sac II) restriction enzyme with palindromic sequence 5′-CCGC↕GG-3′ and 3′-GG↕CGCC-5′ was used. All samples were run on 1.5% agarose gel and Gene Genius Bio Imaging System by Syngene was used to take photographs.

Sanger Sequencing

The preparative gel of PCR product of eight HCV^{+} β-TM patients with higher ferritin level in blood and 2 controls was prepared. Gel band was extracted using Invitrogen Quick Gel Extraction Kit (Cat no. FAGPK001-1, Lot no. BF127115123). Eluted samples were sequenced by automated ABI Sequencer (Apical scientific SdnBhd No. 7-1 to 7-3, Jalan SP 2/7, Taman SerdangPerdana, Seksyen 2, 43400 Seri Kembangan, Selangor, Malaysia).

Table 1: Sequence of *HAMP* gene oligonucleotide primers.

Gene	Primer sequence	Product size	T_m
HAMP-(1)-F *HAMP*-(1)-R	5′-GTGTGTCTGTGACCCCGTCT-3′ 5′-ACTGGGCTCTCACCTGTTGT-3′	305bp	59.5^{o} C 57.5^{o} C
HAMP-(2)-F *HAMP*-(2)-R	5′-ATCCTCTGCACCCCCTTCT -3′ 5′-CACTGTTGCGCTCACCATC-3′	105bp	57.1^{o} C 57.1^{o} C
HAMP-(3)-F *HAMP*-(3)-R	5′-CACAGCCCATGTTCCAGAG-3′ 5′-ACACTCGGCAGAGAGAAAGG-3′	247bp	57.1^{o} C 57.5^{o} C

RESULTS

P.Cys70Arg Effect on Protein Stability

Stability of wild-type *HAMP* protein was found 11.54 kcal/mol by using BioEdit software. *HAMP* p.Cys70Arg divulged protein to less stable conformation with 13.47 kcal/mol, so the energy difference (ΔG) between mutated and wild-type was -1.93 kcal/mol which showed a decrease in stability (Figure 1).

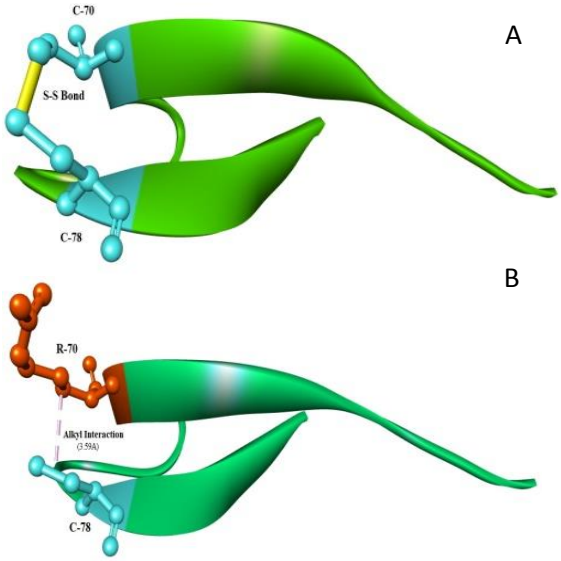

Figure 1: Three dimensional model of HAMP protein.
(A) Wild type structure showing S-S bond in yellow colour (B) p.Cys70Arg showing disrupted S-S bond (in red colour) and mutated cysteine (in blue colour).

Clinical Manifestations

Diagnosis of β-thalassemia major (β-TM) patients included in this study was made from the age of 2 months to 5 years, after which these clinically diagnosed patients receive a first blood transfusion. In more than 90 % of cases, patients receive their first blood transfusion in the same age of diagnosis, but some patients receive transfusion after three months to one year of diagnosis. Average age for the transfusions of blood and diagnosis was 1.3 year and 1.2 year, respectively.

Ferritin level was found to be higher in eight β-TM patients from a maximum value of 1376 ng/ml to 1245 ng/ml concluding that level of ferritin protein in blood is proportional to iron surplus when compared with normal individuals. The level of different blood parameters was found considerably variable among different β-TM patients (Table 2).

The most common blood group in β-TM patients was B^+ (Figure 2a). Some complications were found connected with β-TM patients like the presence of HCV antigens, lymphadenopathy, hepatomegaly, jaundice, ascites, oedema, splenomegaly and typical pale color of skin due to the anemia (Figure 2b).

Variant Analysis with SacII Restriction Enzyme

PCR was performed for *HAMP* gene exon 3 and the proposed band of 247bp was obtained. After performing PCR, samples were treated with SacII restriction enzyme but no β-TM patient was found mutated with C70R mutation (Supplementary Figure 1).

Sequencing of HAMP Gene Exon 3 for C70R Mutation

The sequence of β-TM patients complemented perfectly with *HAMP* exon 3 reported sequences which were taken from NCBI Reference Sequence NG 011563.1. Amino acid sequence alignment of patient samples verses normal ones did not reveal previously reported p.Cys70Arg variant (Supplementary Figure 2).

Table 2: Blood parameters for 106 β-TM patients.

Parameter		Gender	Reference range	< Reference range	> Reference range	=Reference range
BCs	b	M	13.5-17.5 g/dL	63 (100%)	00 (0%)	00 (0%)
	b	F	12.0-15.5 g/dL	111 (100%)	00 (0%)	00 (0%)
	bA	M & F	95%-98%	164 (92.7%)	1 (0.6%)	12 (6.8%)
	bA2	M & F	1.5%-3.5%	21 (11.9%)	72 (40.7%)	84 (47.5%)
	bF	M & F	<2.2%	23 (13.0%)	00 (0%)	154 (87.0%)
WBCs		M & F	3500-10,500 cells/mcL	18 (10.2%)	55 (31.1%)	104 (58.8%)
Platelets		M & F	150,000-450,000 cells/mcL	42 (23.7%)	19 (10.7%)	116 (65.5%)
Ferritin		M	12-300 ng/mL	3 (2.7%)	108 (97.3%)	00 (0%)
Ferritin		F	12-150 ng/mL	4 (6.1%)	62 (93.9%)	00 (0%)

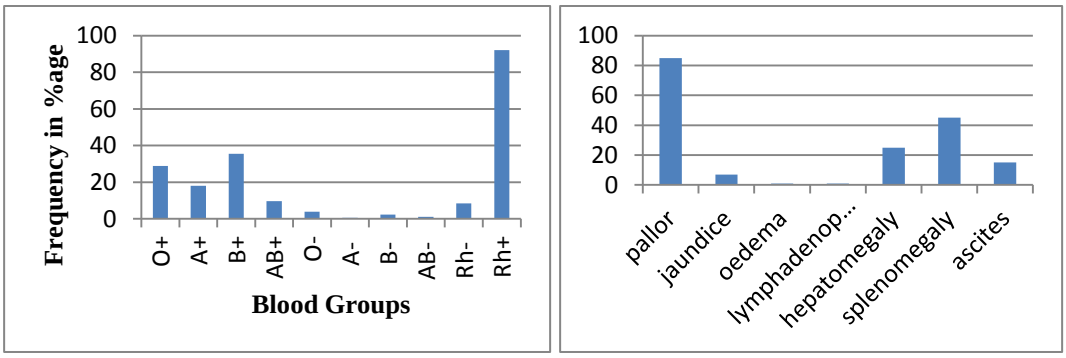

Figure 2: Clinical data of β-TM patients
(A) Prevalence of different blood groups (B) Common maladies.

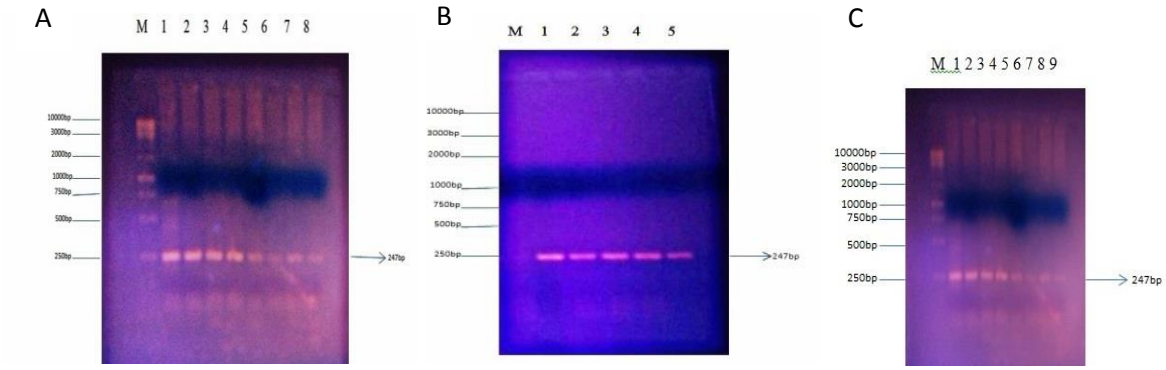

Figure 3: PCR product banding pattern of HAMP exon 3 of β-TM patient samples.
M; 1kb DNA ladder (A) Lane 1; A1, Lane 2; A2, Lane 3; A3, Lane 4; A4, Lane 5; A5, Lane 6; A6, Lane 7; A7, Lane 8; A8 (B) Lane 1; A101, Lane 2; A102, Lane 3; A103, Lane 4; A104, Lane 5; A105, Lane 6; A106 (C) M; 1kb DNA ladder, Lane 1; N1, Lane 2; N2, Lane 3; N3, Lane 4; N4, Lane 5; N5, Lane 6; N6, Lane 7; N7, Lane 8; N8, Lane 9; N9.

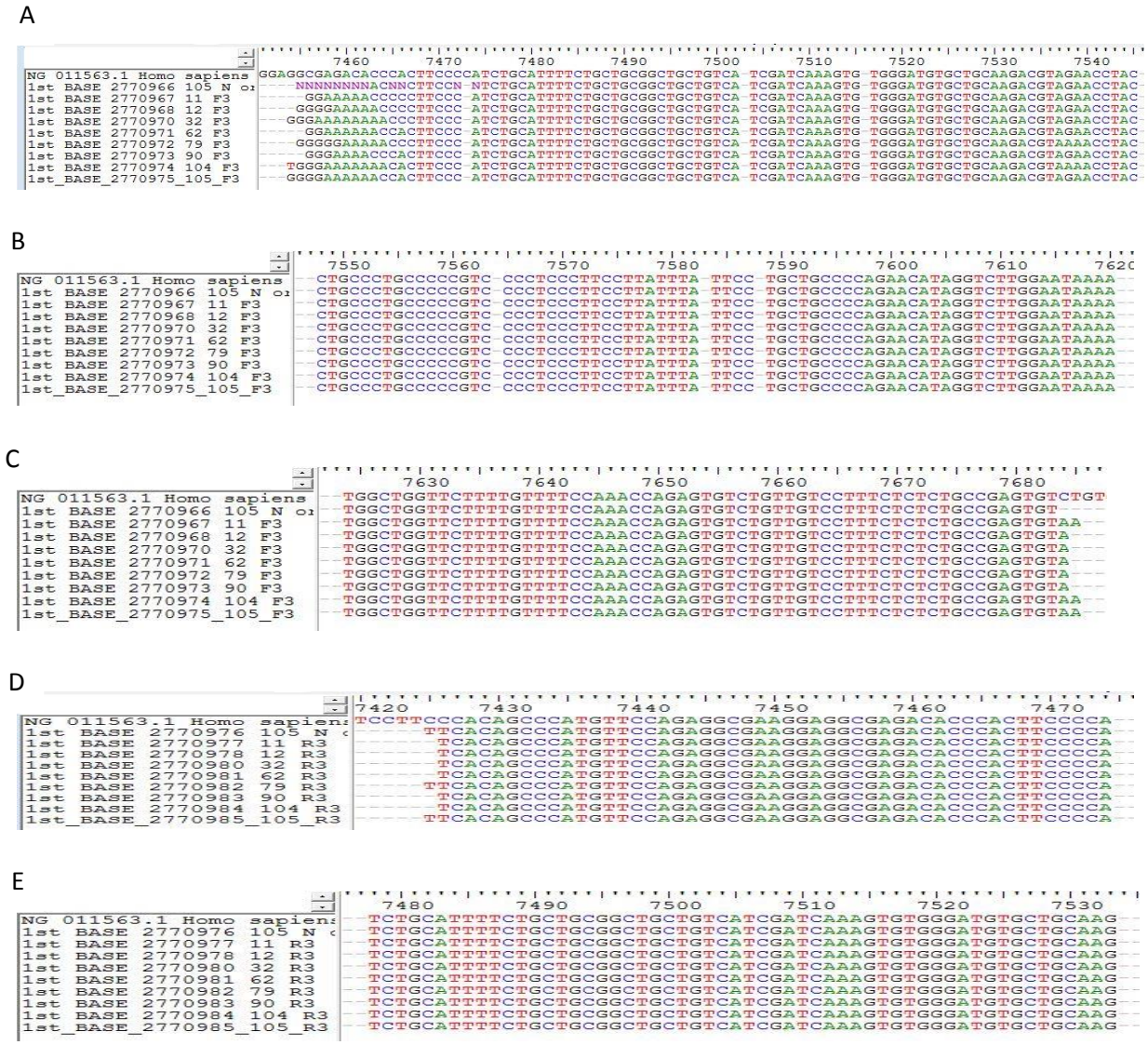

Figure 4: Sequence alignment of *HAMP* exon 3.
Lane 1; NCBI Reference sequence NG 011563.1, Lane 2; N1, Lane 3; A1, Lane 4; A2, Lane 5; A3, Lane 6; A4, Lane 7; A5, Lane 8; A6, Lane 9; A7, Lane 10; A8 from nucleotide (A) 7460-7540 (B) 7550-7620 (C) 7630-7680 (D) 7420-7470 (E) 7480-7530.

DISCUSSION

Iron dysregulation and deposition in body organs is the main cause of elevated mortality rate among β-thalassemia major patients (Musallam et al. 2014). Hepcidin regulates cellular iron through ferroportin which is the only iron exporter protein and regulates iron concentration in the plasma and the intestine (Ganz and Nemeth 2012). Hepcidin is key regulator of iron homeostasis, as its deficiency causes iron overload in hereditary hemochromatosis, hepatitis C and anemias, whereas its overloading causes iron deficiency anemia, chronic kidney disease and inflammation. *HAMP* is synthesized in the liver by lymphocytes, hepatocytes and monocytes (Nemeth et al. 2004).

Genes playing a critical role for iron homeostasis are *HFE* and *HAMP*. *HAMP* p.Cys70Arg missense variant that disrupts the final conformation of the polypeptide, affects one of the eight evolutionary conserved cysteines. Eight cysteines in HAMP form four disulphide bonds which indicate their role for the stability of the protein. In mature peptide, neutral amino acid cysteine is substituted with basic amino acid arginine (Roetto et al. 2003). Previously regular catalogue of C70R mutation in open reading frame was studied in Italy population (Roetto et al. 2004). Cysteine mutated with arginine could disrupt S-S bond between third and sixth cysteine, paving light on molecular pathogenesis of HH.

In Lahore region of Punjab, Pakistan, there was no previous work on *HAMP* gene mutational analysis and its role in iron dysregulation. First time in Lahore, screening of p.Cys70Arg variant was performed. Level of fetal hemoglobin (HbF) and HbA among thalassemia patients was found in a range from 99%-0.02% (Figure 1, 2). HbF level declines as a child grows and with the passage of time, it diminished (Keikhaei et al. 2017). Level of platelets was decreased from normal range (150,000-450.000/µl) in thalassemia patients as compared to controls (29800/µl) indicating that immune level in these patients disturbed to greater extent (Table 2) as indicated previously (Musallam et al. 2014). Brdises was found in only 1 patient while pallor had been found most common among anemic patients. Out of these 8 patients, 1 was dead at the age of 25 months after 1 month of our blood collection.

CONCLUSION

Based on the results obtained from the current study, it is concluded that *HAMP* p.Cys70Arg variant is not prevalent among population of Lahore region of Pakistan. Clinical data implicates that the prevalence of pallor, splenomegaly and hepatomegaly was high among β-TM patients as compared to normal ethnically matched controls. Moreover, sequencing of all the three exons of the *HAMP* gene along with its intervening sequences will increase our knowledge regarding the causes of iron overload. Furthermore, it will also provide a better understanding of hemochromatosis that leads to plausible genetic therapies.

ACKNOWLEDGEMENT

We are thankful to all patients, their family members and Sundas Foundation for their co-operation. We especially acknowledge Azizah Ziaudin for suggestons in manuscript writing. The research was supported financially by the School of Biochemistry and Biotechnology, University of the Punjab, Lahore, Pakistan.

CONFLICT OF INTEREST

Authors have declared no conflict of interest.

AUTHORS CONTRIBUTION

AK performed the experiments and wrote the original manuscript. MSM performed the bioinformatics analysis. SI conceived the original idea and supervised the work. All authors edited and reviewed the final version of manuscript.

REFRENCES

Ahmad KA, Ahmann JR, Migas MC, Waheed A, Britton RS, Bacon BR, Sly WS, Fleming RE. 2002. Decreased liver hepcidin expression in the Hfe knockout mouse. Blood Cells Mol Dis. 29(3):361-366.

Al-Khabori M, Al-Riyami AZ, Al-Farsi K, Al-Huneini M, Al-Hashim A, Al-Kemyani N, Daar S. 2014. Validation of a non-invasive pulse CO-oximetry based hemoglobin estimation in normal blood donors. Transfus Apher Sci. 50(1):95-98.

Badial PR, Oliveira Filho JP, Cunha PHJ, Cagnini DQ, Araújo Jr JP, Winand NJ, Borges AS. 2011. Identification, characterization and expression analysis of hepcidin gene in sheep. Res Vet Sci. 90(3):443-450.

Camaschella C. 2013. Iron and hepcidin: a story of recycling and balance. Hematology 2013, the American Society of Hematology Education Program Book. 2013(1):1-8.

Ganz T. 2003. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood. 102(3):783-788.

Ganz T, Nemeth E. 2012. Hepcidin and iron homeostasis. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 1823(9):1434-1443.

Haq H, Uddin N, Khan SA, Ghaffar S. 2020. Prognostic markers in Chronic Lymphocytic Leukaemia-A flow cytometric analysis. Pakistan Journal of Medical Sciences. 36(3):338.

Kawabata H. 2018. The mechanisms of systemic iron homeostasis and etiology, diagnosis, and treatment of hereditary hemochromatosis. Int J Hematol. 107(1):31-43.

Keikhaei B, Slehi-fard P, Nojoumi S-A, Khosravi A. 2017. Relationship between serum ferritin and hemoglobin levels determined by cardiac and hepatic T2 MRI in beta-thalassemia intermedia and major patients. Frontiers in biology. 12(4):311-315.

Krause A, Neitz S, Mägert H-J, Schulz A, Forssmann W-G, Schulz-Knappe P, Adermann K. 2000. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett. 480(2-3):147-150.

Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. 2011. Hepcidin in human iron disorders: diagnostic implications. Clin Chem. 57(12):1650-1669.

Lou D-Q, Nicolas G, Lesbordes J-C, Viatte L, Grimber G, Szajnert M-F, Kahn A, Vaulont S. 2004. Functional differences between hepcidin 1 and 2 in transgenic mice. Blood. 103(7):2816-2821.

McDonald CJ, Wallace DF, Crawford DH, Subramaniam VN. 2013. Iron storage disease in A sia-P acific populations: The importance of non-HFE mutations. J Gastroenterol Hepatol. 28(7):1087-1094.

Musallam KM, Cappellini MD, Daar S, Karimi M, El-Beshlawy A, Graziadei G, Magestro M, Wulff J, Pietri G, Taher AT. 2014. Serum ferritin level and morbidity risk in transfusion-independent patients with β-thalassemia intermedia: the ORIENT study. Haematologica. 99(11):e218.

Musallam KM, Cappellini MD, Daar S, Karimi M, El-Beshlawy A, Graziadei G, Magestro M, Wulff J, Pietri G, Taher AT. 2014. Serum ferritin level

and morbidity risk in transfusion-independent patients with β-thalassemia intermedia: the ORIENT study. Haematologica. 99(11):e218-e221.

MWer S, Dykes D, Polesky H. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16:1215.

Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. 2004. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 306(5704):2090-2093.

Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S. 2002. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. The Journal of clinical investigation. 110(7):1037-1044.

Pietrangelo A. 2015. Genetics, genetic testing, and management of hemochromatosis: 15 years since hepcidin. Gastroenterology. 149(5):1240-1251. e4.

Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loréal O. 2001. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem. 276(11):7811-7819.

Roetto A, Daraio F, Porporato P, Caruso R, Cox TM, Cazzola M, Gasparini P, Piperno A, Camaschella C. 2004. Screening hepcidin for mutations in juvenile hemochromatosis: identification of a new mutation (C70R). Blood. 103(6):2407-2409.

Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, Loukopoulos D, Camaschella C. 2003. Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet. 33(1):21-22.

Roetto A, Totaro A, Cazzola M, Cicilano M, Bosio S, D'ascola G, Carella M, Zelante L, Kelly A, Cox T. 1999. Juvenile hemochromatosis locus maps to chromosome 1q. The American Journal of Human Genetics. 64(5):1388-1393.

Sato K, Taniai M, Kato K, Kato T. 2020. Relationship between the Induced Iron Overload Model and Hepatic Erythropoiesis in Xenopus laevis. Zoolog Sci. 37(1):1-9.