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EXPLORING TYPE II TRANSMEMBRANE SERINE PROTEASES IN IRON DEFICIENCY ANEMIA THROUGH GENE EXPRESSION ANALYSIS

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ABSTRACT

Background: Inadequate iron levels necessary for effective haemoglobin synthesis lead to the common dietary problem known as iron deficiency anaemia (IDA). The challenging regulation of iron metabolism depends on hepcidin and the matriptase-2 enzyme, which is linked to the TMPRSS6 gene. By limiting the formation of hepcidin through the BMP-SMAD signalling pathway, TMPRSS6 plays a critical role in maximising iron absorption. Disruptions in this pathway result in IDA, an uncommon hereditary disorder marked by inadequate iron absorption. It is necessary to comprehend these pathways in order to treat and prevent iron deficiency anaemia. It is a rare genetic disorder characterised by insufficient absorption of iron.

Objective: Iron deficiency anaemia is associated with hepcidin and the expression of the TMPRSS6 gene.

Methodology: 40 people between the ages of 16 and 60 participated in a case control study (20 cases and 20 controls). TMPRSS6 with particular primers used for quantitative PCR (qPCR) investigation of gene expression between patients and controls throughout a 12-month period from November 2024 to November 2025. ANOVA, Pearson correlation, multiple linear regression, and the Mann Whitney U test were among the statistical methods used to examine the relationship between gene expression levels in IDA patients and controls.

Results: According to the study, patients with iron deficiency anaemia (IDA) had lower TMPRSS6 gene expression than healthy controls, as evidenced by a larger ΔCT value. Though there was no statistically significant difference ($p = 0.099$), fold change analysis confirmed that this downregulation occurred.

Conclusion: The work highlights the critical role that the TMPRSS6–hepcidin pathway plays in controlling iron homeostasis. Gaining a better understanding of these molecular alterations may aid in the identification of future therapeutic targets and better diagnostic markers for the treatment of iron-related illnesses.

KEYWORDS: Bone Morphogenetic Protein-Suppressor of Mothers against Decapentaplegic (BMP-SMAD), Hepcidin, Iron Deficiency Anaemia (IDA), Iron Refractory Deficiency Anaemia (IRIDA), PCR (Polymerase Chain Reaction) & Transmembrane Protease Serine 6 (TMPRSS6).

1. INTRODUCTION:

Healthy red blood cells, or haemoglobin levels are abnormally low in anaemia, a common blood condition. Iron supplements are typically used to treat iron-deficiency anaemia (IDA), a common type of anaemia. However, there are instances where taking iron supplements does not increase haemoglobin levels, indicating that the body is not adequately absorbing the iron. This anaemia may be caused by changes in hepcidin, a hormone that regulates iron absorption. The TMPRSS6 gene encodes the matriptase-2 enzyme, which is crucial for maintaining the body's iron balance and is involved in the negative regulation of hepcidin. Matriptase-2 helps to maintain iron homeostasis by controlling hepcidin hormone levels. Modifications in the TMPRSS6 gene may cause an uncommon form of anaemia associated with these disorders. The TMPRSS6 gene, IDA & hepcidin are the specific topics of this study.

It is crucial to comprehend the functions of matriptase-2 and hepcidin in iron metabolism because their interactions are not well understood.[1]

Hepcdin is the primary hormone that controls the body's iron balance and is essential for preserving iron homeostasis. It operates by binding to ferroportin, a protein that is present on the surface of cells that release iron into the blood. Ferroportin is broken down when hepcidin binds to it, releasing less iron into the plasma. This process helps control the total iron levels in the blood. Iron deficiency anaemia, which reduces haemoglobin

formation and slows oxygen delivery, is brought on by an excess of hepcidin. Matriptase-2 (MT-2), an enzyme that precisely regulates hepcidin production, is also encoded by the TMPRSS6 gene. Matriptase-2 is a regulator that influences hepcidin synthesis, which in turn influences the body's metabolism and availability of iron. Understanding the relationship between hepcidin, ferroportin, and matriptase-2 is necessary to comprehend how the body regulates its iron levels and cures iron-related diseases. [2,3]

TMPRSS6 regulates hepcidin by inhibiting the BMP-SMAD signaling pathway. When there is iron deficiency or increased demand for erythropoiesis (red blood cell production), the expression of TMPRSS6 increases. Matriptase-2, an enzyme, cleaves hemojuvelin (HJV), a membrane-bound protein that acts às a crucial co-receptor for BMP signaling. Since the BMP-HJV-SMAD pathway promotes the transcription of hepcidin, the cleavage of HJV by

TMPRSS6 results in reduced hepcidin synthesis. This relationship can be summarized as follows:

- Increased TMPRSS6 expression leads to decreased BMP-SMAD signaling, which results in lower hepcidin expression and increased iron absorption.
- Conversely, decreased or defective TMPRSS6 expression causes persistent BMP-SMAD activation, leading to increased hepcidin expression and reduced iron absorption.

The inverse relationship between TMPRSS6 and hepcidin is central to maintaining iron balance. Experimental study group have consistently shown that loss of TMPRSS6 function increases hepatic hepcidin mRNA expression, while enhanced TMPRSS6 activity suppresses hepcidin production. 3,4

In the present study we explored the innovative expression patterns of the matriptase gene, focusing on TMPRSS6 gene expression òn blood samples with iron deficiency and healthy controls taken from individuals. This investigation showing significant potential for àdvancing our understanding of these genetic factors.

2. METHODOLOGY

2.1 Patient selection

The study was còndúcted at Medicine and Biochemistry depàrtment of S N. Medical College and H S K Hospital and Research Centre, Bagalkot & BLDE (Deemed to be University), Vijayapura, Karnataka, India. Clearance from the Institutional Ethics Committee (IEC) was obtained from both the institutions BLDE (Du)/IEC/1068/2023-24, SNMC/IECHSR/2023/A-81/1.1) and this was conducted within the period of November 2024 to October 2025. Written informed consent was also taken from the patients prior to the collection of blood samples.

2.2 Inclusion Criteria

Iron deficiency anemia patients characterized by nutritional deficiency between the age following 16-60 years, attending medicine OPD were inclusion. Subjects in the same age group without anemia were referred as healthy controls.

2.3 Exclusion Criteria

Patients with anemia due to chronic inflammation like trauma, PIH, pregnancy, excessive menorrhagia, anemia due to bleeding, iron overload, hemolytic anemia, on iron therapy and systemic illness and giving no consent for the study will be excluded.

2.4 Sample size calculation

In the present study sample size wàs calculated to 40 samples & divided as 20 nutritional IDA Cases and 20 Controls. Patients involved were from Medicine OPD under the inclusion and exclusion criteria selected. Blood sample was collected total around 3ml in K2-EDTA tube under aseptic precautions & stored at -80^{0}C.

2.5 RNA isolation and quantification

Total cellular RNA was extracted from human blood samples using Qiagen Cat. 181033042. Next, the amount of extracted total RNA was measured using,

2.5.1 RT-PCR

Total RNA was quantitatively converted to single-stranded cDNA in a single 20µL reaction using the cDNA transcription reversible kit. Random primers, specially designed RT buffer, and dNTPs are included in the kit.

First, 5x prime script buffer (2µL), prime script enzyme I (0.5µL), oligo dT primers (0.5µL), random primers (2µL), and RNase-free water (4µL) were combined to create 10µL of 2X RT master mixes. Ten microlitres of the RNA sample were added to this mixture. The PCR was set up to do one-step heat cycles at 25^{0}C for 10 minutes, 37^{0}C for 120 minutes, 85^0 C for 5 minutes, and 4^{0}C hold. [6,7]

Table 1: The β-actin and TMPRSS6 primer sequences utilised in qPCR tests. [8]

Primer	sequence (5'-3')
TMPRSS6 Forward	AACGGCAGCGACGAAGAGCA
TMPRSS6 Reverse	TCACACTGCGGGTTGGGCTT
β-actin Forward	AGGCCCAGAGCAAGAGAGGTA
β-actin Reverse	TCTCCATGTCGTCCCAGTTG

2.5.2 qPCR
For real-time PCR analysis employing SYBR® Green 1 dye, Power SYBR® Green PCR master mix was utilised. MBW 3µL, Rox dye 0.2µL, template DNA 1µL, forward and reverse primers 0.4µL each, and TB green primer 5µL. One phase of initial denaturation at 95^0C for two minutes, forty cycles at 95^0Cfor fifteen seconds, and a final elongation at 72^0C for five minutes comprised the PCR cycle.[6,7]

2.6 Analysis
The qPCR based formula was used to assess TMPRSS6 gene expression results for fold variation based on Ct values,
ΔCT = CT(target) – CT(β-actin) was already computed.[9, 10]

3. RESULTS
A total of 20 patients with Iron deficiency anemia and 20 healthy controls were studied for TMPRSS6 gene expression along with β-actin as a house keeping gene.

Table 2: Comparative Analysis of Biochemical, Hematological, and Molecular Parameters Between Study Groups.

Parameter	Controls(20)	Cases(20)	P value
Hemoglobin (g/dl)	14.08 ± 1.41	7.62 ± 1.47	<0.0001**
Serum ferritin (ng/mL)	95.89 ± 31.92	27.66 ± 8.25	<0.0001**
Serum Hepcidin (ng/mL)	190.41 ± 59.35	366.53 ± 67.34	0.12
ΔCT for TMPRSS6	8.74 ± 2.55	10.17 ± 3.35	0.099

3.1 The mean hemoglobin concentration were decreased from values 14.08 ±1.41 g/dL in the Control to 7.62 ± 1.47 g/dL in the Cases. This reduction showed statistical significance ($p < 0.0001$). Systemic cellular iron reserves were significantly compromised in the cases. The mean Serum Ferritin level for the Control group were 95.89 ±31.92 ng/mL were reduced in the Cases group 27.66 ± 8.25 ng/mL. This reduction in tissue iron stores proved to be highly statistically significant ($p < 0.0001$), validating absolute iron deficiency as a primary clinical marker. The mean Hepcidin value calculated across the Controls 190.41 ± 59.35 ng/ml, while an elevated mean of 366.53 ± 67.34 ng/mL in the Cases. Comparative research revealed that even though the average was significantly greater in the patient's condition (p-value of 0.12). This was not statistical significance ($p > 0.05$). This result is mostly attributed to the massive intra-group variation and high standard deviation profile observed within the control group.
The qPCR based TMPRSS6 were examined in 20 cases with IDA patients and 20 normal subjects. The fold chain variation changes showed that TMPRSS6 gene is downregulated in all the iron deficient cases. This study shows that TMPRSS6 downregulation does play important role in iron metabolism.

Table 3: Comparison of ΔCT values between control and IDA groups

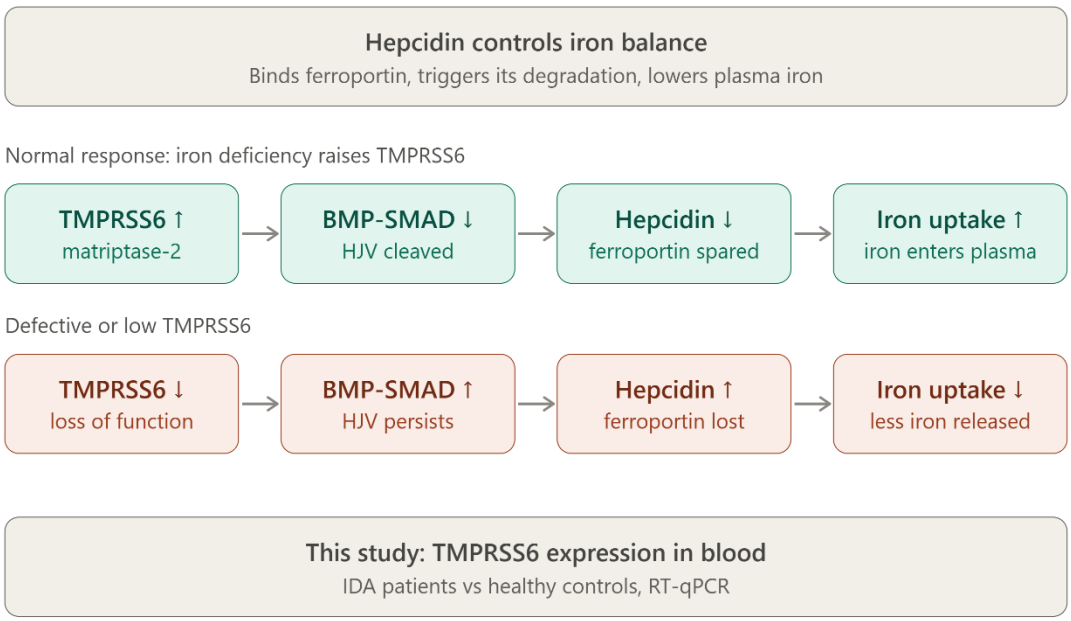
Group	N	Mean ΔCT	SD	Median ΔCT	p-value*
Control	20	8.735	2.555	8.093	0.099
IDA	20	10.171	3.347	10.319	

3.2 Table 3, shows healthy Control group with mean Δ Ct of **8.74 ±2.55**, whereas the Cases group demonstrated an elevated mean value of **10.17 ± 3.35**, resulting a foldchange of 0.37, approximately a 2.7 fold downregulation. This baseline difference indicates a down-regulation tendency of TMPRSS6 activity within the symptomatic group since larger Δ Ct values indicate an underlying drop in initial beginning of mRNA transcripts. Nevertheless, a Mann-Whitney statistical analysis showed a p-value of 0.099, suggesting that there is no statistical significance. Our results suggest that iron deficiency anemia regulates TMPRSS6 expression, and in turn TMPRSS6 has a functional role on hepcidin regulation.

4. DISCUSSION
Iron deficiency anemia (IDA) is one of the commonest nutritional disorders worldwide, & it is particularly prevalent in India, especially among women and children. While nutritional deficiency is the primary cause of IDA, some patients do not respond well to òral iròn therapy, even when they are receiving adequate supplementation and is often linked to abnormalities in iron regulatory pathways, particularly mutations or changes in the expression of the TMPRSS6 gene.

The goal of the current study was to compare the expression of the transmembrane serine protease gene (TMPRSS6) in healthy controls and individuals with iron deficient anaemia.[1,2]
One of the most widespread nutritional conditions in the world, iron deficiency anaemia (IDA) is particularly prominent in India, particularly among women and children. While nutritional deficiency is considered the major cause, a subset of patients demonstrates poor response to oral iron therapy despite adequate supplementation. The type II transmembrane serine protease gene (TMPRSS6) expression in iron deficient anaemia was assessed in this study and compared with healthy controls.[1,2]
The findings of the study demonstrated reduced expression of the TMPRSS6 gene in patients with IDA compared to healthy individuals. The higher mean ΔCT value observed in the IDA group indicated lower gene expression levels. Therefore, downregulation is observed in this study supporting the biological mechanism underlying genetic cause.
This observation is concordant with the work of **Al-Sheikh et al. (2020)**[3], who evaluated the genetic profile of female patients with severe iron imbalances. They reported that both acquired IDA and hereditary IRIDA groups exhibited significantly lower TMPRSS6 RNA transcription levels compared to healthy control cohorts. Similarly, **Guillem et al. (2008)**[4] highlighted that structural and expression-level anomalies in TMPRSS6 disrupt homeostatic intestinal absorption, cementing the gene's status as a critical checkpoint for microcytic anemia development. Furthermore, the mechanistic pathways driving this transcriptional suppression align with observations by **Meynard et al. (2013)**[5], who demonstrated that secondary systemic factors, such as underlying inflammatory cytokines, can actively suppress TMPRSS6 expression, compounding iron-restricted erythropoiesis. Taken together, our results reinforce that monitoring TMPRSS6 expression via quantitative Delta CT profiling offers powerful diagnostic utility in distinguishing classic iron deficiencies from transcriptionally driven, iron-refractory states.
Previous studies conducted in different populations have also reported associations between TMPRSS6 mutations or reduced activity and refractory iron deficiency anemia. Research by Finberg et al. first identified TMPRSS6 mutations as a causative factor in IRIDA, highlighting the gene's importance in iron metabolism. Likewise, more molecular research has demonstrated that impaired TMPRSS6 function results in elevated hepcidin expression and microcytic hypochromic anemia resistant to oral iron treatment. The present findings are in accordance with these reports and suggest that altered TMPRSS6 expression may also contribute to iron refractory states in the Indian population.[6]
Thus, the TMPRSS6–hepcidin axis represents a critical regulatory pathway in iron metabolism and a potential therapeutic target in disorders of iron overload and iron insufficiency.

Figure 1: Graphical Abstract of the study.



5. CONCLUSION

In Iron deficiency anaemia showed different gene expression, with higher hepcidin levels and higher TMPRSS6 expression than controls. These modifications are indicative of compensating mechanisms by the body to increase

iron absorption and mobilisation under deficient conditions. The inverse relationship of hepcidin and TMPRSS6 highlights their important regulatory functions in iron homeostasis. The understanding of these molecular patterns not only clarifies the pathophysiology of iron deficiency anaemia while also pointing out possible treatment targets, opening the path for more precise and effective treatments in the future.

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