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TMPRSS6 Gene Polymorphism and Serum Hepcidin in Iron Deficiency Anemia

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ABSTRACT

Background: Iron deficiency anemia (IDA) is a global health problem and common medical condition seen in everyday clinical practice. Hepcidin which is the key regulator of iron homeostasis, that down-regulates iron export by binding to ferroportin which is expressed on the surface of iron-releasing cells, leading to its degradation thus reducing plasma iron levels. Overexpression of hepcidin leads to iron deficiency anemia. Matriptase-2 (MT-2) which is encoded by transmembrane protease serine 6 (TMPRSS6) gene regulates hepcidin expression. **Objective**: Was to evaluate the role of TMPRSS6 gene polymorphisms and serum hepcidin level in IDA patients. **Subjects and methods**: This study was carried out on 30 patients with iron deficiency anemia and 30 age and sex matched individuals as control group. Patients were subdivided into (group 1) 14 patients with acquired iron deficiency anemia (IDA) and (group 2)16 patients with iron refractory iron deficiency anemia(IRIDA).TMPRSS6 gene single nucleotide polymorphisms(SNPS), (rs4820268)and(rs855791)were evaluated using real time – polymerase chain reaction (RT-PCR) while serum hepcidin level was measured by enzyme linked immunosorbent assay (ELISA). **Results**: There was a significant increase in frequency of the TMPRSS6 SNPs rs855791 and rs 4820268 separately in IRIDA group compared to IDA group,(P=0.003),(P=0.007) respectivelyand to control group(P=0.000) and (P=0.000) respectively.Also, there was highly significant increase in frequency of both mutations together in IRIDA group compared to IDA group (P=0.000) and to control group (P=0.000). In IDA group there was a significant increase in the frequency of TMPRSS6 SNPs rs 855791 when compared to control group (P=0.013), and a non-significant difference in frequency of SNPs rs 4820268 when compared to control group (P=0.092).Also, there was a non-significant difference in frequency of both mutations together in IDA group in comparison to control group (P> 0.05).There was a highly significant decrease in serum hepcidin level in IDA group and highly significant increase in hepcidin levels in IRIDA group compared to control group (P=0.000).In IRIDA patients group only there was highly significant increase in hepcidin level in those with mutation in both SNPs together (rs4820268 and rs855791) than patients with one mutation or with wild type(P=0.000).There was a significant decrease in Hb and iron and highly significant decrease in MCV, MCH and ferritin in IRIDA patients with mutation of both SNPs together (rs 4820268 and rs855791) compared to one mutation or wild type (P=0.015,0.016,0.002 ,0.000, 0.006 respectively).Also, there was highly significant decrease in Hb, MCV, MCH and ferritin in IDA patients with mutation of both SNPs together(rs 4820268 and rs 855791) compared to one mutation or wild type (P= 0.002,0.002 ,0.004, 0.009 respectively). In IDA patients there was a significant positive correlation between hepcidin and ferritin (P =0.034, r = 0.279),while there was a significant negative correlation between hepcidin and MCH in IRIDA group (P =0.032, r = -0.536).**Conclusion:** In IRIDA the rs4820268 and rs855791 mutations separately or in combination lack inhibitory effect on hepcidin and consequently iron profile and hemoglobin while in IDA although there was significant increase in frequency of rs855791 mutations, there was no effect on hepcidin which suggests that the rs 4820268 mutation is necessary for affecting hepcidin either alone or in combination with rs 855791 mutation. In IDA, there may be other mechanisms or mutations causing anemia.

Keywords: Iron deficiency anemia, TMPRSS6 gene polymorphism, Hepcidin

INTRODUCTION

Iron-deficiency anemia (IDA) is a major health problem and common medical condition. Is considered the most common cause of anemia IDA worldwide[(1)].

Hepcidin is the key regulator of iron homeostasis, controlling surface expression of the iron exporter ferroportin on enterocytes and macrophages. Hepcidin down-regulates iron

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export by binding to ferroportin expressed on the surface of iron-releasing cells, lead its degradation so reducing plasma iron levels. Levels of Hepcidin are regulated by systemic iron availability, hypoxia, iron demand for erythropoiesis, and inflammation. Hepcidin in activation leads to severe iron overload, whereas overexpression of hepcidin causes IDA[(2)].

Matriptase-2 (MT-2) that encoded by transmembrane protease serine 6 (TMPRSS6) gene that plays an important role in down-regulating expression of hepcidin. MT-2 cleaves haemojuvelin (HJV) leading to decrease in bone morphogenetic protein (BMP–SMAD) signaling. Different MT-2 mutations had been reported, affecting all the functional domains of the large ectodomain of the protein. In contrast to the low/undetectable hepcidin levels observed in IDA. In MT-2 mutation, serum hepcidin is high despite low iron status and accounts for the absent response to oral iron treatment[(3)].

A type of hereditary anemia has been described called iron refractory iron deficiency anemia(IRIDA),due to mutations in the *TMPRSS6* gene, mapping on chromosome 22. Hall marks of this disease are microcytic hypochromic anemia, low transferrin saturation, normal/high serum hepcidin values and partial slow response to intravenous iron injections[(4)].

Iron refractory iron deficiency anemia is a hereditary anemia that arise from defects in iron metabolism which lead to microcytic anemia that is resistant to treatment. Patients do not respond to oral iron treatment and partially respond to intravenous iron injection. Mutations inTMPRSS6 gene are the major cause of this disorder[(5)].

Diagnosis of IRIDA among iron deficiency and other microcytic anemias are a challenge for the clinicians. The current treatment of IRIDA is based on parenteral iron administration. In the future, suppression of hepcidin pathway might become an alternative therapeutic approach[(6)].

SUBJECTS AND METHODS

Subjects

This study included a total of thirty patients with iron deficiency anemia attending at Internal medicine clinic, Al-ZahraaUniversity Hospital. Thirty apparently healthy women, age matched, were selected as control group.Approval of the ethical committee and a written informed consent from all the subjects were obtained. This study was conducted between October 2013 to July 2015.

All patients were females to minimize differences caused by gender and their ages ranged from 22 years to 45years old (mean ± SD= 34.2 ± 5.86). Ages for control group ranged from 25 to 45 years old) (35.3± 6.37). Patients were divided into 2 groups:

Group1: Fourteen patients with Acquired iron deficiency anemia (IDA),diagnosedfrom history, they were newly diagnosed with anemia and were completely normal as evidenced from previous completely normal CBCs. At time of study they were not on iron, then advised to take iron. After 2 months of iron therapy great response was observed as evidenced by rise of hemoglobin and RBCs indices.

Group2: Sixteen patients with Iron refractory iron deficiency anemia (IRIDA), diagnosed from history of long-term iron therapy with no response to oral iron and partial improvement of anemia in response to parenteral iron therapy (many CBCs with persistent microcytic hypochromic anemia). At time of study they were not on iron therapy.

Inclusion criteria:

All patients had microcytic hypochromic anemia, low serum iron, ferritin, high TIBC and negative CRP.

Exclusion criteria:

Any patient with infection including helicobacter pylori, inflammation, bleeding including occult blood or other chronic diseases.

All patients and controlswere subjected to the following:

- Full medical history taking.
- Thorough clinical examination.

- Laboratory investigations including CBC, liver and kidney functiontests,serum iron, TIBC, ferritin, transferrin saturation,CRP, occult blood,and helicobacter pylori antigen in stool.
- Serum Hepcidin-25 was determined by enzyme linked immunosorbent assay (ELISA)
- Determination of TMPRSS6 gene SNPs (rs855791) and (rs4820268) was done usingreal time – polymerase chain reaction (RT-PCR).

Methods

Sampling

- Nine ml of venous blood were withdrawn aseptically from studied subjectsand divided into:
  - Five ml of blood were left to clot and centrifuged (for 15 minutes at 1000 ×g) and sera were separated for liver and kidney function testing, iron, ferritin, TIBC and CRP assay and 0.5 ml serum was stored at -40^0 C until analysis for hepcidin measurement by ELISA.
  - Two ml of blood were anticoagulated with EDTA for CBC.
  - Two ml of blood were anticoagulated with EDTA for TMPRSS6 SNPs (rs855791& rs4820268) real time-polymerase chain reaction (RT-PCR).
- Stool sample for occult blood, and Helicobacter pylori antigen.

MEASUREMENT OF HEPCIDIN-25:

Serum hepcidin-25 levels were measured using the commercially available human hepcidin ELISA kit (Glory Science Co; Ltd, USA) according to the manufacturer's instructions (with normal range: 7.5 - 150 µg/L)., using a complete set of ELISA reader model SLT 216687 (Italy).

GENETIC STUDY FOR DETECTION OF TMPRSS6 GENE SNPS (RS4820268) AND (RS855791) USING (RT-PCR).

DNA was extracted from peripheral blood. Samples were processed immediately. DNA extracts then was stored at -20º C for later use. Extraction was performed using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) and fully automated extractor (QIAcube -Qiagen, Hilden Germany). .The Kits are based on the selective binding of DNA to silica-based membrane in the presence of chaotropic salts. The cells were digested with Proteinase K at 55°C using an optimized digestion buffer formulation that aids in protein denaturation and enhances Proteinase K activity. Any residual RNA is removed by digestion with RNAase prior to binding samples to the silica membrane. The lysate was mixed with ethanol and Pure Link R genomic binding buffer that allows high DNA binding Pure Link R Spin column. The DNA binds to the silica-based membrane in the column and impurities were removed by thorough washing with Wash Buffers. The genomic DNA is then eluted in low salt Elution Buffer.

Testing DNA extract integrity:

The amount of DNA was measured spectrophotometrically for concentration and purity. All samples had an optical density (OD) 260/280 nm ratio >1.8, indicating high purity. The DNA integrity was tested on the Nanodrop. ND-1000 spectrophotometer (Germany) .

Amplification and Real-time PCR allelic discrimination: .

For Detection of TMPRSS6 gene SNPs (rs 4820268, rs 855791). Quantitative Real Time RT-PCR with sequence specific primers have been used. Real-time PCR allelic discrimination assays were designed using Taq-Man SNP Genotyping Assays (Applied Biosystems).

Table (1): Sequence of used primers of SNPs rs 4820268 and rs 855791**.**

	rs 4820268	**rs 855791**
Forward	5' TGT AAA ACG ACG GCC AGT 3'	5' TGA CCT CAG GTG TTC CGT C 3'
Reverse	5' CAG GAA ACA GCT ATG ACC 3'	5' AGG CTT CAG CAG GCT GAT G 3'

The TaqMan SNP Genotyping Assay: Each TaqMan probe contains:

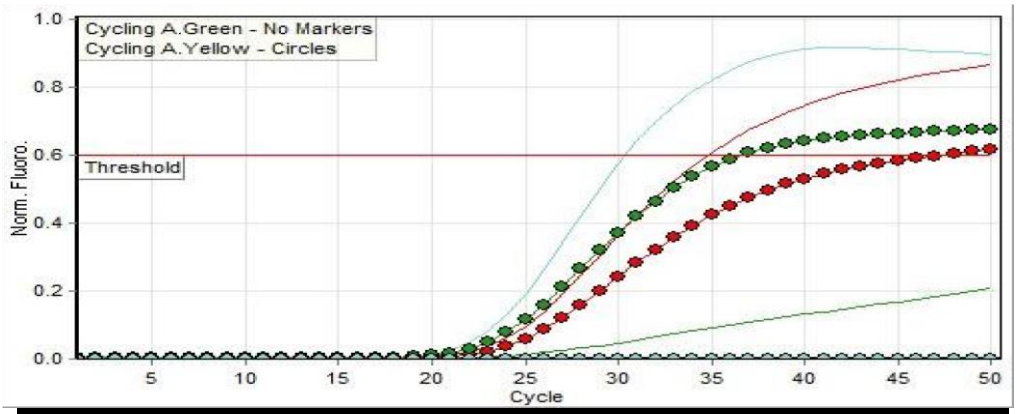
- A reporter dye at the 5′end of each probe.VIC® dye is linked to the 5′end of the Allele 1 probe and FAM™ dye is linked to the 5′end of the Allele 2 probe.
- A non-fluorescent quencher (NFQ) at the 3′end of each probe.

During PCR, each TaqMan probe anneals specifically to its complementary sequence between the forward and reverse primer sites. The increase in fluorescence signal occurs when probes that have hybridized to the complementary sequence are cleaved. Thus, the fluorescence signal generated by PCR amplification indicates which alleles are present in the sample. The table below shows the correlation between fluorescence signals and sequences in a sample

Table (2): The correlation between fluorescence signals and sequences in a sample.

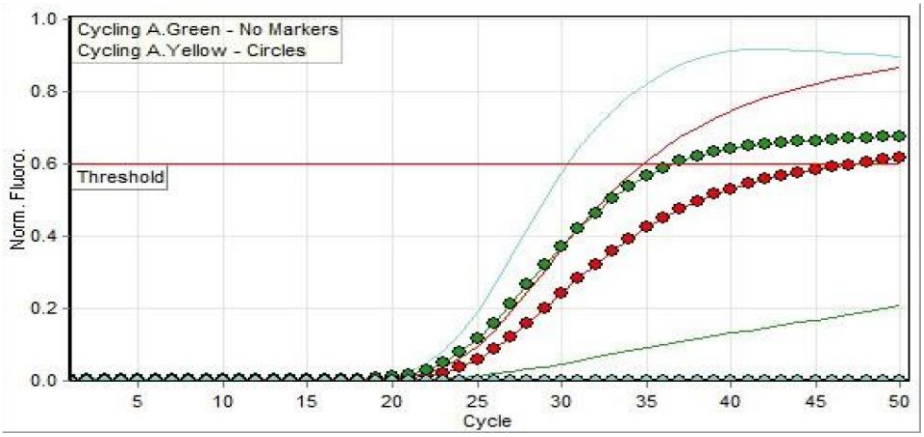
A substantial increase in…	Indication
VIC-dye fluorescence only	Homozygosity for Allele 1
FAM-dye fluorescence only	Homozygosity for Allele 2
Both VIC- and FAM-dye fluorescence	Allele 1-Allele 2 heterozygosity

Allelic Discrimination Plate Read and Analysis After PCR amplification, an endpoint was performed using an Applied Biosystems Real-Time PCR System. The SequenceDetection System (SDS) Software uses the fluorescencemeasurements made during the plate read to plot fluorescence. Values were based on the signals from each well. The plotted fluorescencesignals indicate which alleles were in each sample.



Color	Genotype	Cycling A.Green	Cycling A.Yellow
	Heterozygous	Reaction	Reaction
	Wild Type	No Reaction	Reaction
	Mutant	Reaction	No Reaction

Fig.(1),Table (3): Allelic data for Cycling A. Green, Cycling A. Yellow of rs 4820268 (yellow for Wild and green for mutant).



Color	Genotype	Cycling A.Green	Cycling A.Yellow
	Heterozygous	Reaction	Reaction
	Mutant	No Reaction	Reaction
	Wild Type	Reaction	No Reaction

Fig. (2), Table(4): Allelic data for Cycling A. Green, Cycling A. Yellow of rs 855791 (yellow for mutant and green for wild).

Statistical analysis:

Data were collected, revised, coded and entered to the statistical package for social science (SPSS) version 17 and **Qualitative data** were presented as number and percentages while **quantitative data** were presented as mean, standard deviation and ranges. The comparison between groups with qualitative data were done by using ***Chi-square test and Fisher exact test*** was used only when the expected count in any cell was found less than 5. The comparison between two groups with quantitative data and parametric distribution were done by using ***Independent t-test. Mann-Whitney test*** was used when the data were non parametric or data were not normally distributed. The comparison between 3 groups with quantitative normally distributed data were done by **ANOVA test**, not normally distributed data were compared by using **Kruskal-wallis** test. Pearson correlation coefficients were used to assess the significant relation between two quantitative parameters. The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant as the following:

- **P > 0.05:**Non significant
- **P < 0.05:** Significant
- **P < 0.01:** Highly significant.

RESULTS

There was non-significant difference in frequency of SNP rs 4820268 (heterozygous and homozygous) in IDA group when compared to control group (p=0.092). On the other hand, IRIDA showed highly significant increase in frequency of SNP rs 4820268 when compared to control group (p=0.000) and highly significant increase in frequency of rs4820268 when compared to IDA group (p=0.007) Table (5), Fig. (3).

Table (**5**) :Frequency `of SNP rs 4820268 in group 1 and group 2 compared to control group.

	Wild TT		Heterozygous CT		Homozygous CC					
	No.	%	No.	%	No.	%	overall	P 1	P2	P3
Group 1 (n= 14)	8	57.1	2	14.3	4	28.6	0.000	0.092	0.001	0.007
Group 2 (n=16)	3	18.8	5	31.3	8	50				
Control (n=30)	20	66.7	10	33.3	0	0.0				
Total (n=60)	31	51.7	17	28.3	12	20				

P> 0.05: *non-significant.* ***P<0.05:*** *significant.* ***P<0.01:*** *highly significant*

Overall: Comparison between the three groups.
P1: Comparison between control group and group I.
P2: Comparison between control group and group II.
P3: Comparison between group I and group II.

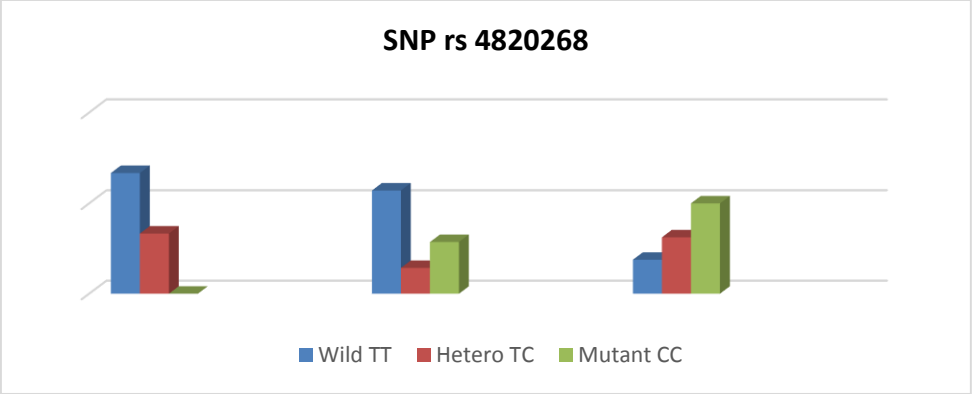


Fig. (3):Frequency of SNP rs 4820268 genotypes in group 1 and group 2 compared to control group.

As regard SNP rs 855791, there was a significant increase in frequency of mutations (heterozygous and homozygous) in IDA group compared to control group (p=0.013) and highly significant increase in frequency of mutations (heterozygous and homozygous) in IRIDA group compared to control group (p=0.000) and compared to IDA group (p=0.003).Table (6), Fig. (4).

Table(6): Frequency of distribution of SNP rs 855791 genotypes in group 1 and group 2 compared to control group.

	Wild CC		Heterozygous CT		Homozygous TT					
	No.	%	No.	%	No.	%	overall	P1	P2	P3
Group 1 (n= 14)	11	78.6	1	7.1	2	14.3	0.000	0.00	0.013	0.003
Group 2 (n= 16)	4	25	2	12.5	10	62.5				
Control (n=30)	28	93.3	2	6.7	0	0				
Total (60)	43	71.7	5	8.3	12	20				

P> 0.05: *non-significant.* ***P<0.05:*** *significant.* ***P<0.01:*** *highly significant*

Overall: *Comparison between the three groups.*
P1: *Comparison between control group and group I.*
P2: *Comparison between control group and group II.*
P3: *Comparison between group I and group II.*

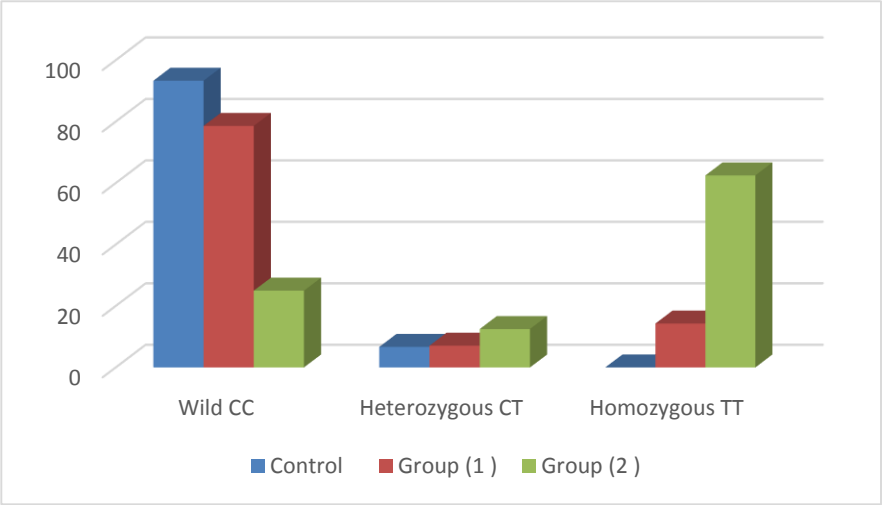


Fig. (4):Frequency of SNP rs 855791 genotypes in group 1 and 2 compared to control group

In the present study there wasnon-significant difference in frequency of both polymorphisms in IDA group compared to control group(P> **0.05).**while there was highly significant increasein frequency of both mutations in IRIDA group compared to control group (p=0.000) . Also, there was highly significant increase in frequency of both mutations in IRIDA group compared to IDA group(p=0.001).Fig. (5)

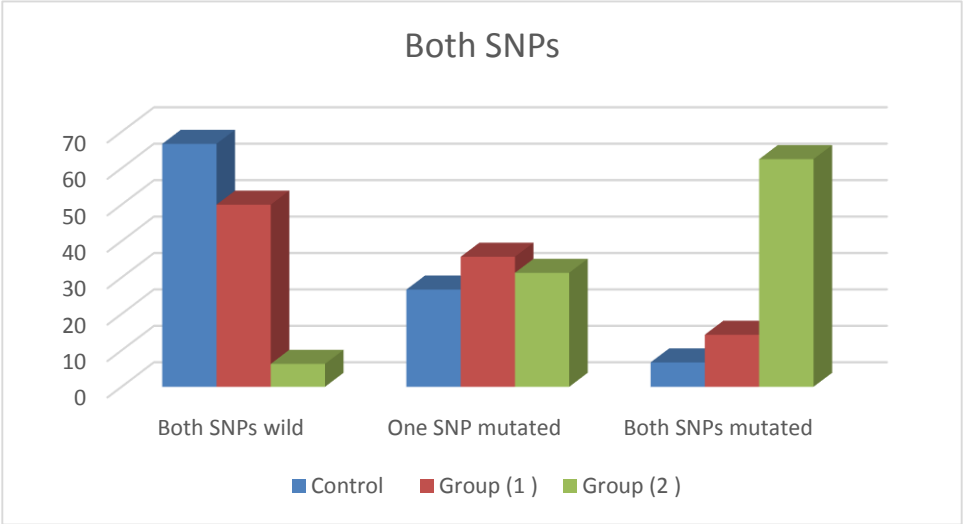


Fig. (5):The frequency of distribution of mutations in both SNPs (rs 4820268 and rs 855791) in group 1, group 2 compared to control group.

The Comparison between patients with different SNPs genotypes as regard all studied parameters revealed that,there was highly significant decrease in Hb,(P= 0.002) MCV (P= 0.002) and MCH (P= 0.004) in patients with mutations in both SNPs together (rs 4820268 and rs 855791) and single mutation of only one SNP compared to those with wild genotype for both SNPs in IDA group. Table (7)**.**

Table(7): Comparison between patients with mutations in both SNPs together (rs4820268 and rs855791), mutation in only one SNP and wild both SNPs as regard results of CBC in group IDA group.

Group (1) IDA (n=14)	Both SNPs wild(n=7)		One SNP mutated(n=5)		Both SNPs mutated(n=2)		Anova Test	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	F	P-value
WBCs ($X10^9$/L)	7.7±0.89	6.7-9.6	7.3±0.63	6.8-8.5	8.8±0.92	8.2-9.5	2.161	0.162
Hb (g/dl)	9.1±0.55	8.5-9.9	7.9±0.64	7.8-9.3	6.9±0.21	6.8-7.1	12.385	0.002
RBCs ($X10^{12}$ /L)	4.3±0.34	3.9-4.9	4.6±0.63	3.5-4.9	4.7±0.28	4.5-4.9	0.523	0.607
HCT	28±2.1	22-32	24±1.76	22-30	21±1.0	20-22	4.448	0.108
MCV(fl)	67±2.57	65-71	62±3.49	61-68	56.5±2.12	55-58	11.107	0.002
MCH (pg)	22.2±2.1	19.6-25.5	19.1±1.24	18.3-20.9	16.8±0.78	16.3-17.4	9.361	0.004
MCHC	26±2.16	22-29	25±2.35	20-26	23±7.07	18-28	1.084	0.372
RDW	20±1.89	16-222	19.5±1.6	17-21	20.5±0.71	20-21	0.484	0.629
PLT ($X10^9$/L)	328±51.3	247-289	310±46.5	247-368	318±56.5	278-358	0.016	0.985

P> 0.05:*non significant.* ***P<0.05:****significant.****P<0.01:****highly significant*

In IRIDA patients There was a significant decrease in Hb (P=0.015), HCT (P= 0.037) and highly significant decrease in MCV(P=0.002) and MCH (P=0.000) in patients with mutations in both SNPs together (rs 4820268 and rs 855791) and with single mutation of only one SNP compared to those with wild both SNPs together in IRIDA group , Table (8).

Table(8): Comparison between patients with mutations in both SNPs together (rs4820268 and rs855791), mutation in only one SNP and wild both SNPs as regard results of CBC in IRIDA group.

Group(2)(IRIDA) (n=16)	Both SNPs wild(n=1)		One SNP mutated(n=5)		Both SNPs mutated(n=10)		Anova Test	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	F	P-value
WBCs ($X10^9$/L)	7.5±0	7.5	6.8±1.34	5.8-9.3	6.8±1.25	5.7-9.3	0.041	0.960
Hb(g/dl)	10.1±0	10.1	8.9±0.79	8.6-10.5	7.5±1.10	6.1-9.6	5.947	0.015
RBCs ($X10^{12}$ /L)	4.1±0	4.1	4.1±0.29	3.8-4.5	4.48±0.17	4.24.7	5.514	0.214
HCT	32±0	32	27±1.56	25-32	23.5±1.34	19-27	6.593	0.037
MCV(fl)	75±0	75	69±5.81	56-70	58±3.81	52-63	10.896	0.002
MCH (pg)	24.3±0	24.3	21.3±1.25	20.1-23.2	19±1.61	16-25	16.517	0.001
MCHC	28±0	28	25±1.14	20-28	26±2.58	20-28	1.419	0.277
RDW	16.8±0	16.8	17.5±1.55	16.8-23	19.5±1.87	16.8-23	0.372	0.697
PLT ($X10^9$/L)	298±0	298	283±43.4	214-341	275±39	214-352	0.113	0.894

P> 0.05:*non significant.* ***P<0.05:****significant.****P<0.01:****highly significant*

There was highly significant decrease in ferritinin patients with mutations in both SNPs together (rs 4820268 and rs 855791) and with single mutation of only one SNP compared to those with wild genotype for both SNPs in IDA (P=009), Table (9)**,**

Table(9): Comparison between patients with mutations in both SNPs together (rs4820268 and rs855791), mutation in only one SNP and wild both SNPs as regard results of iron profile in IDA group.

Group(1) IDA (n=14)	Both SNPs wild (n=7)		One SNP mutated(n= 5)		Both SNPs mutated(n=2)		Kruskulwallis test	
	Median	Range	Median	Range	Median	Range	K	P-value
Iron (µg/dl)	14	8-24	15	3-15	21	16-26	3.633	0.163
Ferritin (µg/dl)	10.4	7.2-12.3	6.1	5.1-9.5	3.9	3.3-4.6	9.528	0.009
TIBC (µg/L)	421	412-487	434	416-498	424	415-433	0.914	0.429
Transferrin saturation	3.39	1.6-5.7	3.09	0.6-3.6	4.9	3.8-6	4.200	0.122

P> 0.05:*non significant.* ***P<0.05:*** *significant.* ***P<0.01:****highly significant.*

There was significant decrease in iron(P=0.016) and, highly significant decrease in ferritin (P=0.006) and highly significant increase in TIBC(P=0.005) in patients with mutations in both SNPs together (rs 4820268 and rs 855791) and with single mutation of only one SNP compared to those with wild genotype for both SNPs in IRIDA group , **Table(10).**

Table(10) :Comparison between patients with mutations in both SNPs together (rs4820268 and rs855791), mutation in only one SNP and wild both SNPs as regard results of iron profile in IRIDA group.

Group (2)IRIDA (n=16)	Both SNPs wild(n=1)		One SNP mutated(n=5)		Both SNPs mutated(n=10)		Kruskulwallis test	
	Median	Range	Median	Range	Median	Range	K	P-value
Iron (µg/dl)	21	21	10	8-12	6.5	3-10	8.295	0.016
Ferritin (µg/dl)	13	13	9.3	8.5-10	5.2	3.2-8.8	10.27	0.006
TIBC (µg/L)	406	406	421	410-433	471.5	419-493	8.085	0.005
Transferrin saturation	5.17	5.17	2.4	1.9-3.6	1.3	0.6-2.14	9.053	0.011

There was non-significant difference in hepcidin in patients with mutations in both SNPs together (rs 4820268 and rs 855791) compared with those with single mutation of one SNP and those with wild genotype for both SNPs in IDA group. On the other hand,there was highly significant increase in hepcidin in patients with mutations in both SNPs together (rs 4820268 and rs 855791) and in patients with single mutation of only one SNP compared with patients with wild genotype for both SNPs together in group (2) (IRIDA), Fig (6).

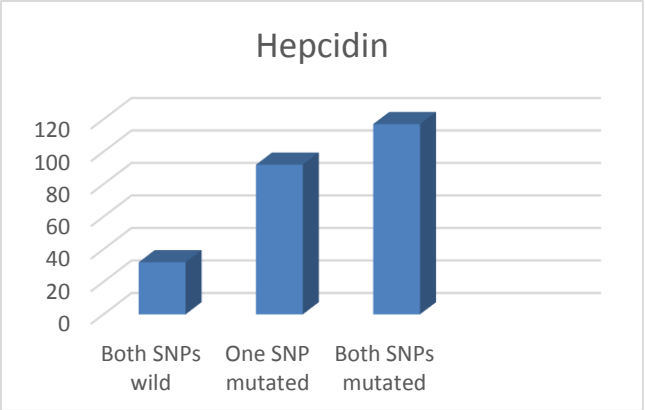


Fig. (6):Comparison between patients with mutations in both SNPs together (rs4820268 and rs855791), mutation in only one SNP and wild both SNPs as regard results of hepcidin in IRIDA group.

Our results also showed significant positive correlation between hepcidin and ferritin in IDA group(P=0.034, r=0,279) Fig. (7).While there was a significant negative correlation between hepcidin and MCV in IRIDA group (P=0.032, r= - 0,536). Fig(8)

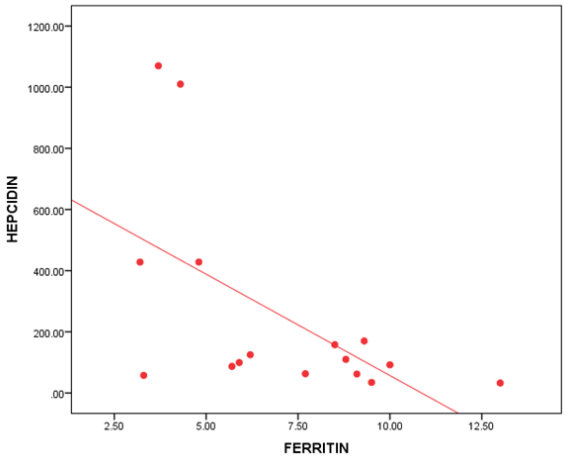


Fig (7): Correlation between serum hepcidin and ferritin in group (1).

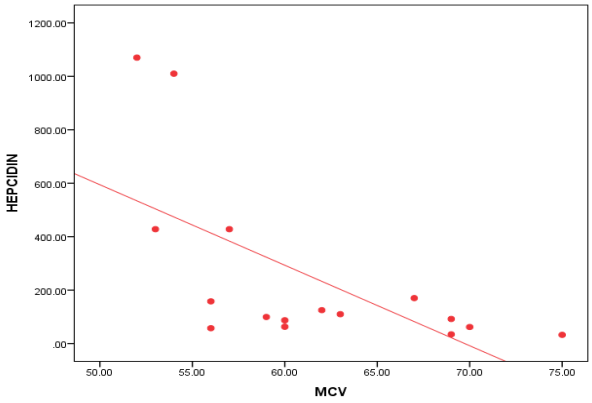


Fig (8): Correlation between serum hepcidin and MCV in group (2).

DISCUSSION

Iron deficiency anemia originates primary from dietary, blood loss and other environmental factors. Many discoveries concerning iron metabolism disorders revealed that there is a genetic contribution to the development of iron deficiency. In particular, TMPRSS6 genepolymorphisms have been implicated as influencing iron metabolism in human studies[7].

Hepcidin the key hormone which regulates systemic iron homeostasis. Hepcidin inhibits the transportation of iron across the gut mucosa, thus preventing excess iron absorption, also it maintains iron levels within normal limits in the body and inhibits iron transport out of macrophages[8].

MT-2 is encoded by TMPRSS6 gene that regulates hepcidin expression. Polymorphisms of TMPRSS6 leads to diminshed ability of MT-2 to down-regulate hepcidin production which persist elevated in spite of iron deficiency, preventing iron stores refilling leads to IRIDA. Iron-refractory iron deficiency anemia is an autosomal recessive hereditary disorder of iron metabolism characterized by hypochromic microcytic anemia unresponsive to oral iron treatment, low transferrin saturation and inappropriate normal to high levels of hepcidin[9].

In the present study, there was a highly significant decrease in hepcidin in IDA group compared to control group (p=0.001) and highly significant increase in hepcidin in IRIDA group compared to control group (p=0.001) and in

IRIDA group compared to IDA group (p=0.001). As hepcidin level increases also in anemia of chronic disease, inflammatory conditions and, these diseases were excluded.

The highly significant increase in hepcidin in IRIDA group compared to IDA groupwhich may be attributed to mutations in TMPRSS6 gene which is the cause of IRIDA resulting in loss of or altered inhibitory effect of MT-2 on hepcidin secretion leading to normal to high hepcidin irrespective to low iron, while in IDA group , hepcidin decreased to increase iron absorption.

Parallel to our results those obtained by ***Pasricha et al.*** who found that serum hepcidin markedly decreased in IDA and itcould be used as a diagnostic test of iron deficiency[10].

The results of the present study were in accordance with ***Choi et al.***who found that serum hepcidin levels were significantly lower in children with ID and IDA than that in patients in the control group[11].

In agreement with the present study results obtained by ***Elgari et al.***who found that serum hepcidin levels were significantly lower in children with IDA as compared to children in the normal control group. Reduced serum hepcidin is an essential part of the physiological response to an iron deficiency anemia and could be a useful indicator of IDA[8].

Hepcidin has the ability to limit ferroportin-dependent iron export from enterocytes and macrophages. The inappropriately elevated hepcidin levels in IRIDA provide insight into the iron refractory features of the disorder. The inappropriate hepcidin excess in IRIDA can explain the development of systemic iron deficiency as a result of impaired absorption of dietary iron, the failure to achieve a hematological response to oral iron therapies, and the sluggish and incomplete utilization of parental iron that require processing by macrophages before the iron can be used in erythropoiesis[12]**.**

In concordance with our study, the results obtained by***Bregman et al.***whofound thathepcidin levels were significantly higher in non-responders to oral iron therapy versus responders, so, normal to high hepcidin levels could predict non responsiveness to oral iron in patients with IDA[13].

Transmembrane protease serine 6, is likely to be involved in iron metabolism through its pleiotropic effect on hepcidin concentrations. Recently, genome-wide association studies have identified common variants in the TMPRSS6 gene to be linked to anemia and low iron status. The strongest association was found between lower hemoglobin and iron and SNPs rs4820268 and rs855791[14]**.**

In the present study, there was non-significant difference in frequency of SNP rs 4820268 (heterozygous and homozygous) in IDA group compared to control group (p=0.092). On the other hand, IDA group showed highly significant increase in frequency of SNP rs 4820268 compared to control group (p=0.000). There was highly significant increase in frequency of rs4820268 (p=0.007) in IRIDA group compared to IDA group.

In agreement with the present study, results obtained by ***Bataret al.***and ***Beutler et al.***who found that the TMPRSS6 SNPs rs 4820268 was not likely to be associated with iron deficiency anemia[15],[16].

In concordance with the present study, the results obtained by ***Erika et al.***and***Keskin and Yenicesu,*** who found that there was highly significant increase in frequency of SNP rs4820268 in subjects with IRIDA compared with healthy controls[17,19].

As regard SNP rs 855791, there was significant increase in frequency of mutations (heterozygous and homozygous) in IDA group compared to control group (p=0.013) and highly significant increase in frequency of mutations (heterozygous and homozygous) in IRIDA group compared to control group (p=0.000) and compared to IDA group (p=0.013).

In concordance with the present study, the results obtained by ***Delbini et al.***who found that there was highly significant increase in frequencies of SNP rs855791 in subjects with IRIDA compared with healthy controls[18].

In agreement with the present study, the results obtained by ***Anetal.,*** who identified a significant increase of rs 855791 mutation in iron deficiency anemia than in normal individuals[19].

In agreement with the present study results reported by ***Gonçalveset al.,*** who found an increase in frequency of heterozygous and homozygous rs855791 in women with IDA more than in control group. Sequencing analysis of TMPRSS6 gene in IRIDA patients revealed the presence of rs855791[20].

Parallel to our results those obtained by ***Pei et al.,*** who found that theTMPRSS6 rs855791 CC genotype (wild) is less frequent in reproductive age women with IDA than in healthy women and suspected that the wild genotype (CC) for rs855791 plays a protective role against IDA, especially for those with menorrhagia. It is likely that women with the CC genotype increase the iron absorption from the intestine through decreased hepcidin production and then can offset the menstrual losses. For those with CC genotypes, diet modification may be enough to keep iron balance; otherwise, long-term iron replacement till menopause may be considered[21].

Keskin and Yenicesu ,reported that the TMPRSS6 polymorphism rs 855791 resulted more frequent in anemic patients than in health, suggesting their possible contribution in the occurrence of IDA. This effect would be attributed to reduced ability of TMPRSS6 polymorphisms to down-regulate hepcidin production that persists elevated despite iron deficiency, preventing iron stores refilling[9].

In concordance with the present study, the results obtained by ***Erika et al.,*** who found that the TMPRSS6 SNP rs855791 resulted more frequent in anemic patients than in healthy controls, suggesting their possible contribution in the refractoriness to oral iron.This effect would be imputable to reduced ability of TMPRSS6 polymorphisms to down-regulate hepcidin production, which persists elevated despite iron deficiency, preventing iron stores refilling[17].

In the current study, there was highly significant increase in frequency of distribution of mutations of both polymorphisms together (rs 4820268 and rs 855791) (p=0.002) in all patients compared to control group. On the other hand, there was non-significant difference in frequency of both polymorphisms in IDA group compared to control group while there was highly significant increase (p=0.000) in frequency of both mutations in IRIDA group compared to control group. Also, there was highly significant increase (p=0.000) in frequency of both mutations in IRIDA groupcompared to IDA group.

In the present study gathering both SNPs (4820268 and 855791), in all patients, Hb (p=0.000), MCV (p=0.000) and MCH (p=0.000) were highly significant decrease in patients with double mutations in both SNPs (rs4820268 and rs855791), compared to those with single mutation and those with wild genotype of both SNPs. In IDA group , Hb (p=0.002), MCV (p=0.002) and MCH (p=0.000) were highly significant decrease in patients with double mutations in both SNPs (rs4820268 and rs855791), compared to those with single mutation and those with wild genotype of both SNPs. In IRIDA patients Hb (p=0.015), MCV (p=0.002) and MCH (p=0.000) were highly significant decrease in patients with double mutations in both SNPs (rs4820268 and rs855791), compared to those with single mutation and those with wild genotype of both SNPs.

In the present study gathering both SNPs (4820268 and 855791),in all patients, iron (p=0.024), ferritin (p=0.000) and TS (p<0.020) were significantly lower and hepcidin (p=0.002) was highly significant higher in patients with double mutations in both SNPs (rs4820268 and rs855791), compared to those with single mutation and those with wild genotype of both SNPs. In group (1), ferritin (p=0.009) was highly significant lower and hepcidin was not significantly differ in patients with double mutations in both SNPs (rs4820268 and rs855791), compared to those with single mutation and those with wild genotype of both SNPs. In group (2), iron (p=0.016), ferritin (p=0.006) and TS (p=0.011) were significantly lower and hepcidin (p=0.005) was highly significant in patients with double mutations in both SNPs (rs4820268 and rs855791), compared

to those with single mutation and those with wild genotype of both SNPs.

Bataret al., and ,Gichohi-Wainainaet al., reported that, rs4820268 and rs855791 mutations were associated with lower Hb and ferritin concentrations in patients than in wild genotype in all populations . It is proposed that association of rs4820268 mutations with lower iron and erythrocyte traits might simply be secondary to that of rs855791 as based on gene expression analysis, rs4820268 is in linkage disequilibrium with rs855791.[15],[14]

In the present study there was non-significant correlation between hepcidin and the other studied parameters in all patients. On the other hand, there was positive correlation between hepcidin and ferritin in IDA group, while there was negative correlation between hepcidin, and MCV and ferritin in IRIDA group.

In agreement with the present study, the results obtained by ***Elgari et al.,*** who found positive correlation between hepcidin and ferritin in patients with iron deficiency anemia. Reduced serum hepcidin is an essential part of physiological response to an iron deficiency which signals that increased iron is needed.[8]

In Conclusion: our study showed that in IRIDA the rs4820268 and rs855791 mutations separately or in combination lack inhibitory effect on hepcidin and consequently iron profile and hemoglobin while in IDA although there was significant increase in frequency of rs855791 mutations, there was no effect on hepcidin which suggests that the rs4820268 mutation is necessary for affecting hepcidin either alone or in combination with rs855791 mutation. In IDA, there may be other mechanisms or mutations causing anemia.

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