

A Key Regulator For Iron Homeostasis in chronic hepatitis C.

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Abstract

Background: Hepcidin is a small, cysteine-rich cationic peptide produced by hepatocytes. There is a single human hepcidin gene; whose essential role in iron homeostasis was confirmed by identifying homozygous frameshift or nonsense mutations in affected individuals with severe Juvenile hemochromatosis. IL-6 may be the mediator of hepcidin induction by inflammation. Hypoferremia is a common response to systemic infections or generalized inflammatory disorders, anemia of chronic disease occurs in patients with acute and chronic immune activation and represents an important clinical problem.

Aim of the work: The study will attempt to determine the hepatic hepcidin expression levels in patients with chronic hepatitis C virus infection.

Patients and methods: Fifty patients with chronic hepatitis C virus infection (CHCV), their age between (20- 55) years, selected from the National Hepatology and Tropical Medicine Research Institute were included in this study, before interferon and Ribavirin therapy, and ten healthy individuals were included to serve as controls. All the patients and controls were subjected to the following history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigations. CBCs and serological assay for serum ferritin, iron, transferrin (s-TFR) levels, Liver biopsy for hepcidin mRNA levels and iron deposits in liver by (PCR) polymerase chain reaction. All subjects gave written informed consent for enrolment in the study, which was approved by the Research Ethical Committee of the General Organization for Teaching Hospitals and Institutes. Liver biopsy was taken from healthy subjects during abdominal surgery.

Results: Our results revealed that hepatic hepcidin expression is considered highly valid marker in case of CHCV infection.

Conclusion: Our study concluded that there's a highly significant inverse correlation between hepcidin versus liver iron, serum iron and serum transferrin but there's no significant correlation versus ferritin.

Recommendations: Hepcidin measuring and manipulating hepcidin levels will, in the future, have a role in diagnosing and treating any number of iron related disorders. Hepcidin itself has antimicrobial properties of uncertain importance so that careful clinical trials will be required to define appropriate indications of hepcidin antagonists.

Key Words: Hepcidin an iron-regulatory hormone, anaemia of chronic disease, inflammation, cytokine IL-6, CHCV.

Introduction

Patients with CHCV infection frequently have serum and hepatic iron overload. Recently, hepcidin, exclusively synthesized in the liver, is thought to be a key regulator for iron

Homeostasis. It is induced by infection and inflammation. Hepcidin expression levels in chronic liver diseases (CLD) were strongly correlated with either the serum ferritin concentration or degree of

iron deposits in the liver , Hcpidin indices were significantly lower in HCV patients than in HBV patients (Fujita , et al., 2007).

Infection was associated with hypoferremia , providing a partial explanation for the common finding of anemia in patients with chronic infection (Locke , et al ., 1932) .The anemia associated with infection was indistinguishable from the anemia of inflammation, and established that hypoferremia resulted from reticuloendthelial sequestration of iron and interruption of intestinal iron absorption . (Cartwright and Wintrobe, 1952), cytokines have been shown to modulate the expression of iron transport and storage proteins (Ludwiczek , et al ., 2003). Erythropoietic activity is known to affect iron homeostasis through regulation of the liver iron regulatory hormone hepcidin (Chung, et al 2010).

Both mice and humans as experimental models have shown that IL-6 acts directly on hepatocytes to stimulate hepcidin production (Nemeth et al, 2003) . Hepcidin , in turn , acts as a negative regulator of intestinal iron absorption and macrophage iron release. Increased levels of hepcidin, the master regulator of homeostasis, contribute to the diversion of iron underlying the anemia of chronic disease. Yet hepcidin levels are low in anemia of chronic disease with concomitant true iron deficiency (Theurl, 2011). Iron metabolism and erythropoiesis are closely linked. About 20 mg of iron is required daily by the bone marrow to efficient hemoglobin synthesis in developing erythrocytes. Iron supply for erythrocyte precursors comes predominantly from iron recycling of senescent erythrocytes within the reticuloendothelial system (splenic macrophages and hepatic Kupfer cells, essentially) and iron absorption from the diet by the duodenal enterocytes (Andrews, and Schmidt, 2007).

Iron is an essential element for all living organisms , being a requirement in a wide range of metabolic processes including DNA synthesis , oxygen transport, and energy production, but excess iron can be harmful and in part, through the generation of free oxygen radicals (Hentze et al ., 2004). The regulatory pathway is the iron-signaling pathway: when iron increases, hepcidin is physiologically induced, allowing decreased circulating iron levels, therefore limiting iron toxicity. The major contribution of hemojuvelin (HJV), transferring receptor 2 (TfR2; a liver-specific homologue of TfR1) and HFE (an atypical major histocompatibility class 1 like molecule) in the iron-sensing pathway have been high-lighted by studies of inherited defects: gene mutation of these three liver-enriched membrane proteins is associated with human hereditary hemochromatosis, an iron-overload disease characterized by increased iron absorption, and tissue iron deposition (Vaulont, et al 2005).

Iron homeostasis must be tightly regulated in all organisms . The importance of the peptide hormone hepcidin in iron homeostasis as a negative regulator of iron release into the system by duodenal enterocytes. (Nicoras, et al ., (2002) & Ganz , (2003). Iron accumulation in the liver, where hepcidin is exclusively synthesized, is common in patients with CLD (Bisceglie, et al ,1992), especially in patients with CHCV infection (Boucher, et al., (1997) & Metwally, et al., (2004).

Patients and Methods

Fifty patients with CHCV infection, whose age was (20-55) years, were selected from the National Hepatology and Tropical Medicine Research Institute for this study, before Interferon and Ribavirin therapy, and ten healthy individuals were included to serve as controls. All patients have anti-HCV antibodies, HCV RNA in serum, evidence of chronic hepatitis on liver biopsy, elevated levels of aminotransferase above the upper limit, serum albumin,

bilirubine, and prothrombine time within normal limit with negative history of drug abuse, non reactive HBsAg, with exclusion of other chronic disease and pregnancy. There was no clinical signs of decompensated liver disease. All the patients and controls were subjected to the following history, clinical examination, and abdominal ultrasonography. Collection of blood samples. A 5 ml whole blood was drawn by venipuncture into edeta coated tube. Serum ferritin levels were estimated using Accu Bind ELISA kit supplied by Monobind Inc. product code 2825-300, according to manufacturers instruction R3. Serum soluble transferring receptor (s-TFR) levels were measured using ELISA kit supplied by Bio Vendor Research and Diagnostic products Cat. No. RD 194011100 according to manufacturers instruction R4. Serum iron level was measured using colorimetry method by kit supplied by Stan Bio USA cat. No. (REF 0370-110).R5 (Bohne, et al., 2012).

Real Time PCR (qRT-PCR) for quantitative expression of Hepcidin Anti Microbial Peptide (HAMP): The RNA isolation: Liver biopsy core was homogenized by small probe ultrasonic homogenizer (ART-MICCRA-Germany) and total RNA was isolated with RNA easy Mini Kit (Qiagen) and further analyzed for quantity and quality with Beckman dual spectrophotometer (USA). The RNA integrity and the GAPDH-RNA ratio were used as the quality control. All samples included in this study had a GAPDH-RNA ratio

more than 1.7. A1000 ng of the total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit from Applied Biosystem. Briefly, 1mg of total RNA, 1mL of oligo dT-primer, and 2mL dNTPs were incubated at 65 °C for 5 min, then 10 mL of a cDNA synthesis mixture were added and this mixture was incubated at 50 °C for 50min. The reaction was terminated by adding 1mL of RNaseH and incubating the mixture at 37 °C for 20 min. The DNA was subsequently amplified with the TaqMan Universal PCR Master Kit (No AmpErase UNG) in a 48-well plate

using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95 °C for enzyme activation followed by 40 cycles of 15 seconds at 95 °C, 20 seconds at 55 °C and 30 second at 72 °C for the amplification step. Changes in the expression of target gene (HAMP) were measured relative to the mean critical threshold (CT) values of GAPDH housekeeping gene (HAMP/GAPDH), by the $\Delta\Delta C_t$ method. The primers and Universal Probe Library (Hs 00221783-ml-Applied Biosystem, USA) used in the qRT-PCR evaluation were specific for target gene. We used 1 uM of both primers and 0.2 uM from UPL. The primers structure for HAMP was: Forward primer (5'-CCACTTCGTGATGATTCTGC) and Reverse primer (5'-TACCTCCACCATGCCAAGT). For the GAPDH housekeeping, we used the Forward primer (5'-GCAATTATTCCCCATGAACG) and Reverse primer (5'-GGGACTTAATCAACGCAAGC-3') RI.

5-Liver iron concentration (LIC)

LIC was evaluated in liver tissues as described previously (R2). The fresh liver tissue fragment (approximately 15 mm) was weighed and dried at 85 °C for 2 hours in decontaminated quartz vessel and then digested in concentrated nitric oxide (2ml). The resulting solution was then diluted to 5ml with deionized water. Quantitative analysis was performed by inductively coupled plasma-atomic emission spectrometry to determine hepatic iron content {Little, (1998)& Hikawa, et al., (1996)& Looker, El., (1995) and Barry and Sherlock, (1971)}.

Statistical analysis : Analysis of data of all patients was done by IBM computer using SPSS (Statistical Program for social science version 12) as follows: Description of quantitative variables as mean, SD and range. Description of qualitative variables as number and percentage. Chi-square test was used to compare qualitative variables between groups. Fisher exact was used instead of chi-square when one or more

expected cell less than or equal 5. Pearson Correlation co-efficient test was used to rank different variables versus each others positively or inversely. Unpaired t-test was used to compare quantitative variables, in parametric data (SD< 50% mean). ROC Curve (receiver operator characteristic curve was used to find out the best cut off value, and validity of certain variable), (Miller, et al., 1992).

RESULTS

The study included 50 patients with CHCV infection and 10 healthy volunteers.

The mRNA expression level of Hecpidin, were highly significant different, between patients and the controls group (p< 0.001) by using unpaired t-test, table (1).

The liver iron concentration (LIC) was evaluated in CLD and there’s highly significant difference between the patients in comparison to the controls (group), (p< 0.001) by using unpaired t-test, table (1).

The mean serum levels of iron were significantly higher in patients than in the controls group, (p< 0.001) table (1), by using unpaired t-test.

We found that the mean serum levels of ferritin were significantly higher in patients compared to the controls group, (p< 0.001) table (1) by using unpaired t-test.

We found that the mean serum levels of soluble transferrin receptor were highly significant as regard the controls group, (p< 0.001) table (1) by using unpaired t-test.

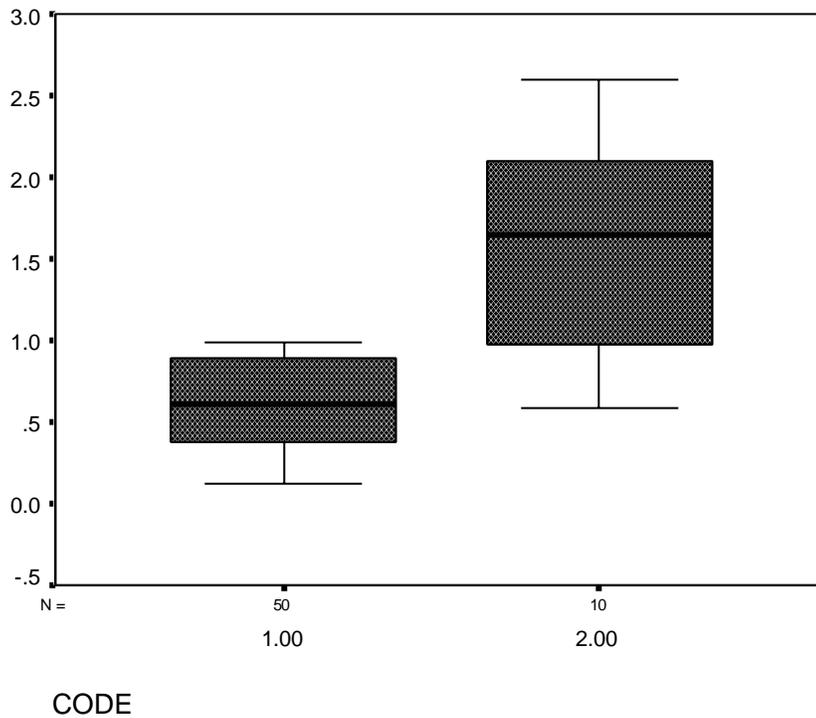
In correlation between Hecpidin versus [liver iron, serum iron, serum ferritin, and soluble serum transferring (s-TFR)] among cases table (2) shows highly significant inverse correlation between Hecpidin versus liver iron, s.iron and s-TFR by using correlation co-efficient test, but ther’s no significant correlation versus ferritin, (p> 0.05).

In correlation between Hecpidin versus [liver iron, s-iron , s-ferritin, and s-TFR] among controls: table (3) shows no significant inverse correlation by using correlation co-efficient test (p> 0.05).

Table (4) and Fig (5) illustrates the validity of hecpidin in cases of CHCV infection before interferon and ribavirin therapy, shows that Hecpidin is considered highly valid marker in cases of HCV detection, the best cut off = 1.09 with 100% sensitivity, 99% specificity, 97% positive predictive value, 100% negative predictive value and 100% accuracy, by using (ROC) receiver operator characteristic curve.

Variables	Cases N=50	Controls N=10	t	P
Hecpidine	0.62±0.22	1.9±0.48	12	<0.001 HS
Liver iron	87±12	28.7±9	13	<0.001 HS
Iron	104.9±11	70.5±10	9	<0.001 HS
Ferritin	299.4±42	112±32	13	<0.001 HS
S-TFR	2.95±0.9	1.19±0.35	5.9	<0.001 HS

This table shows highly significant difference between both groups as regard different variables by using unpaired t-test. {p<0.001 by unpaired t-test}.



**Fig (1): Quantitative expression of liver hepcidin mRNA in CHCV infection patients and controls.(code:- 1.00= patients &2.00=controls).
Hepcidin indices were significantly lower in CHCV patients.**

Table (2) Correlation between hepcidin versus different variables among cases of CHCV infection.

Variables	Hepcidine	
	r	P
Liver iron	-0.56	<0.001HS
Iron	-0.45	<0.001HS
Ferritin	-0.25	>0.05
S-TFR	-0.55	<0.001HS

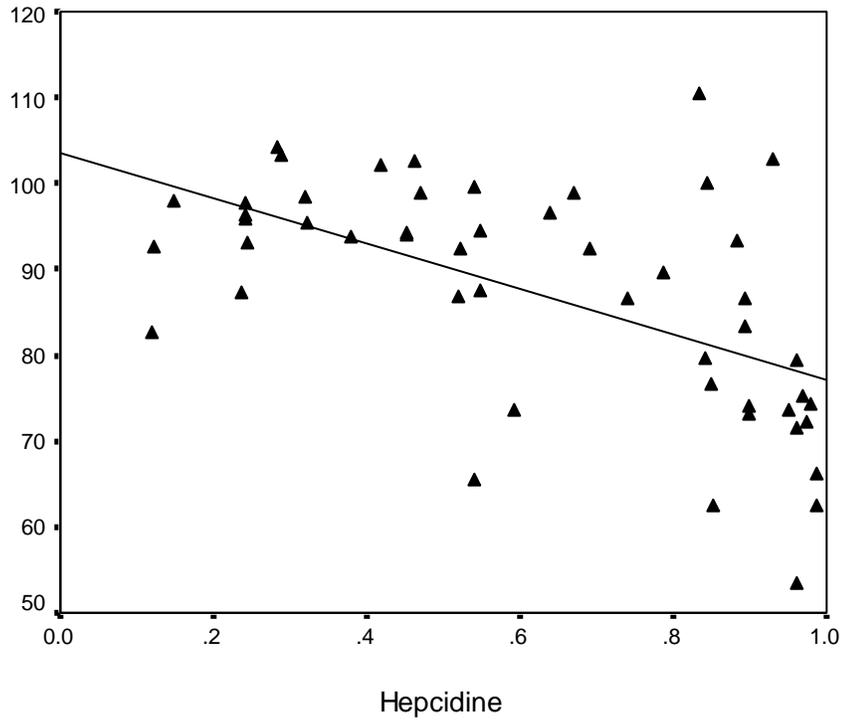
This table shows highly significant inverse correlation between hepcidin versus liver iron, iron and s-TFR by using correlation co-efficient test. There was no significant correlation versus ferritin.

Table (3) Correlation between hepcidin versus different variables among controls

Variables	Hepcidine	
	r	P
Liver iron	-0.16	>0.05
Iron	-0.25	>0.05
Ferritin	-0.11	>0.05
S-TFR	-0.09	>0.05

This table shows no significant inverse correlation between hepcidin versus different variables by using correlation co-efficient test.

Fig (2): Hepcidin mRNA , liver iron inverse correlation in CHCV patients .



Fig(3):Hepcidin mRNA, serum iron inverse correlation in CHCV patients.

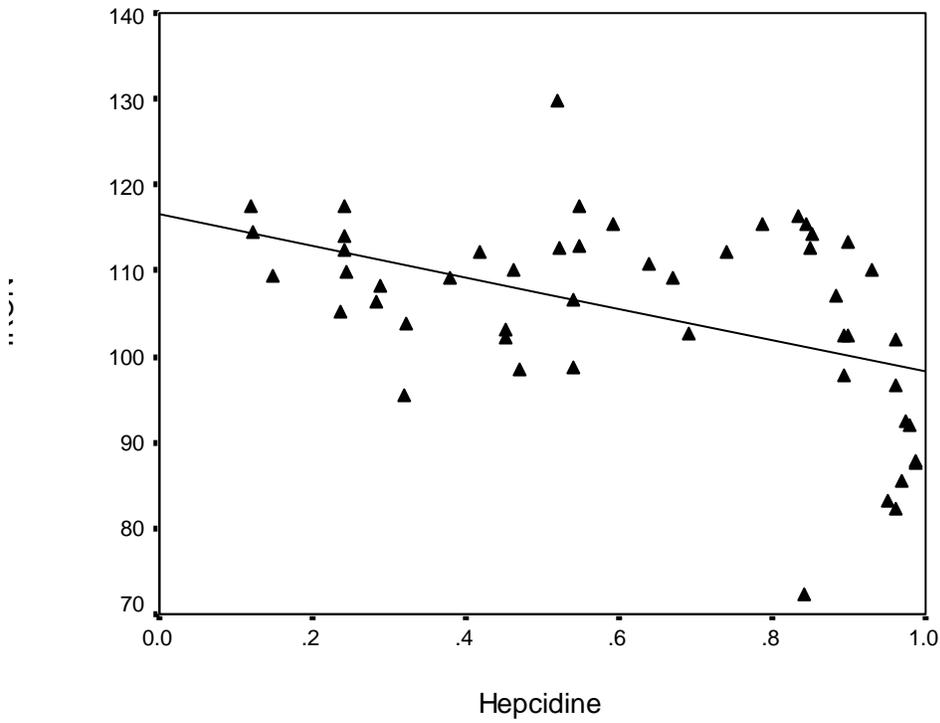


Fig (4) Hepcidin mRNA , serum Transferrin (s-TFR) inverse correlation in CHCV patients

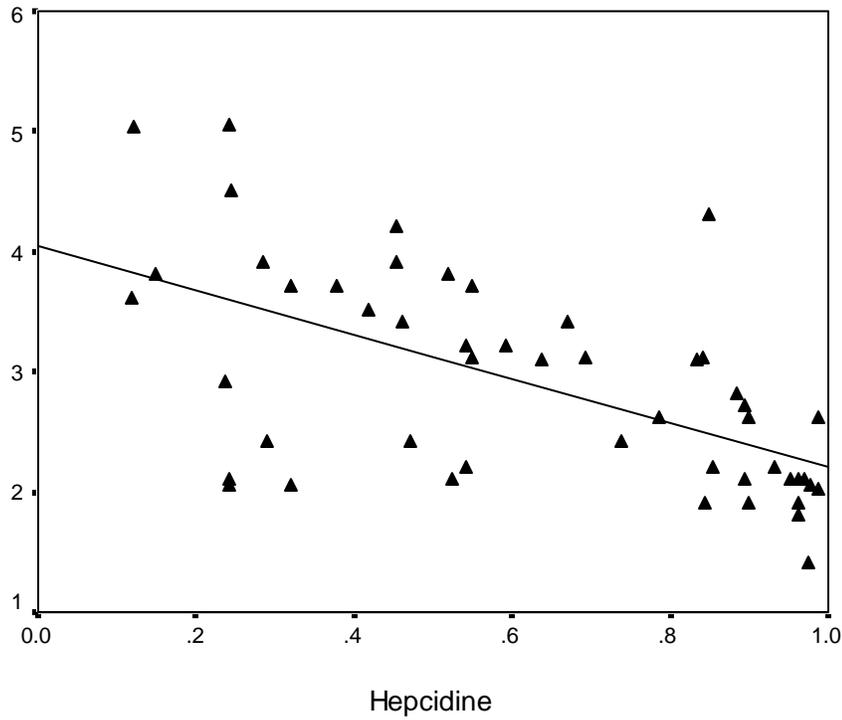
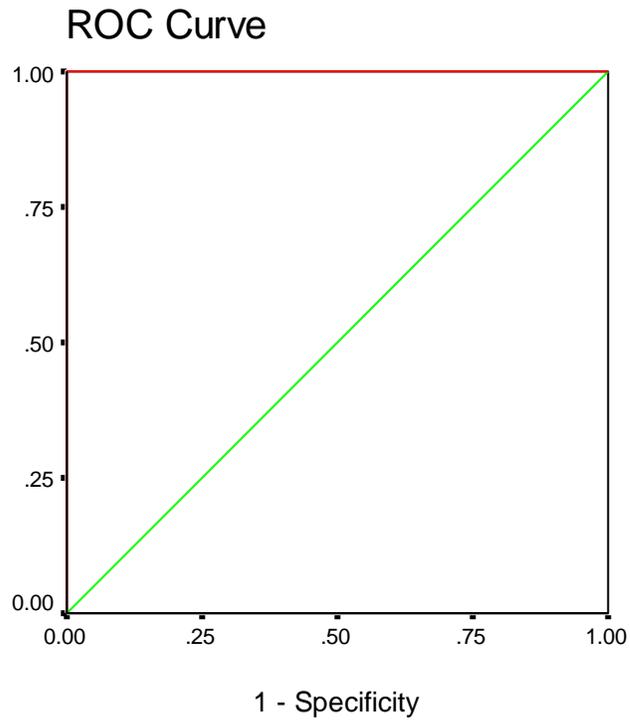


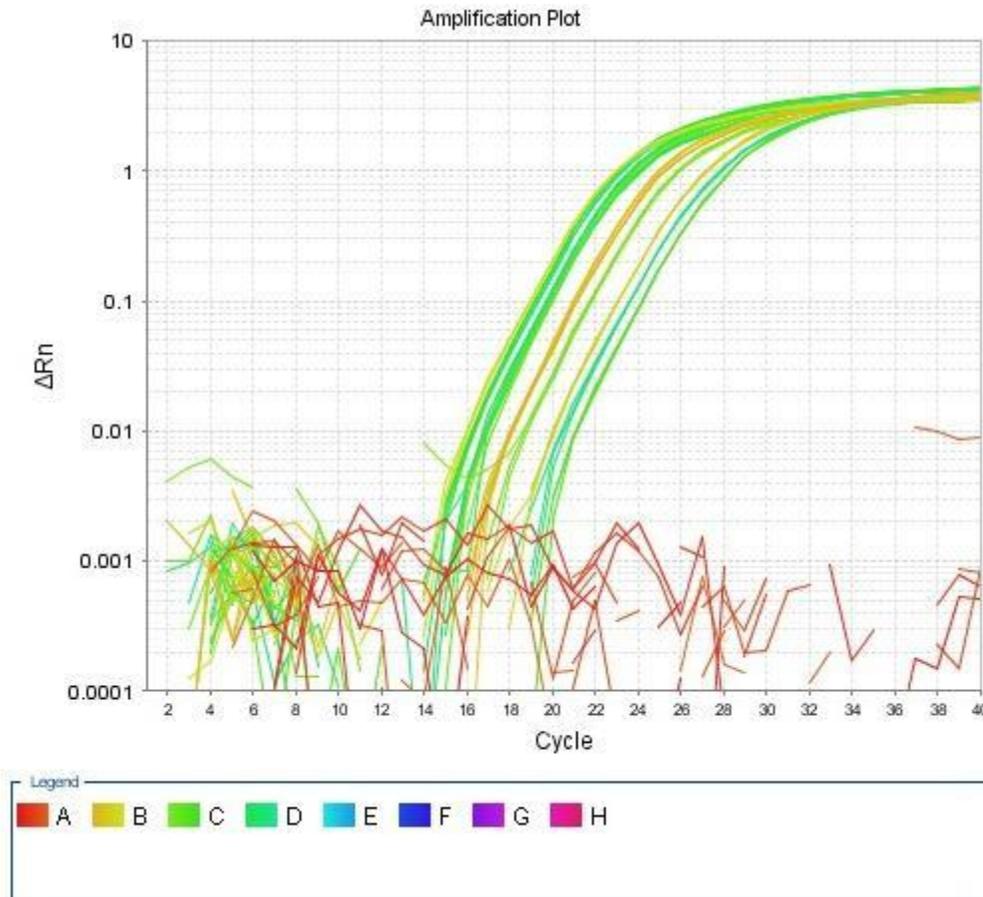
Table (4) Validity of mRNA hepcidin expression in case of CHCV infection.

Variables	%
Best cut off= 1.09	
Area under the curve	0.100
Sensitivity	100%
Specificity	99%
PPV	97%
NPV	100%
Accuracy	100%

This table shows that hepcidin can be considered highly valid marker in case of CHCV infection.

Fig(5):Illustrate the validity of hepcidin gene expression in CHCV patients.





Hepcidin amplification plot by Real-Time PCR in CHCV patients and controls.
(Code: above and left the curve revert controls gp- ϵ -below and right the curve revert patients gp.)

Discussion

Increased levels of hepcidin, the master regulator of iron homeostasis, contribute to the diversion of iron underlying the anemia of chronic disease. Yet hepcidin levels are low in anemia of chronic disease with concomitant true iron deficiency (Theurl, et al., 2011). Hepcidin indices were significantly lower in HCV patient (Fujita, et al., 2007).

In the present study, we found that (qRT-PCR) hepcidin levels in the liver tissue of CHCV were significantly lower than controls.

Theurl, et al., (2011) found that hepcidin levels are low in anemia of chronic disease with concomitant true iron deficiency. Fujita, et al., (2007) postulated that hepcidin indices were significantly lower in HCV

patients. Nicolas, et al., (2002) showed that hepcidin mRNA decreased in mice with anemia caused by bleeding or hemolysis or in mice with hypoxemia due to decreased ambient oxygen, these finding in agreement with our results.

Weinstein, et al. (2002) postulated that autonomous overexpression of hepcidin mRNA expression was seen in large hepatic adenomas associated with iron-refractory anemia. {Park, et al. (2001)& Hunter, et al. (2002)} observed that structurally, hepcidin is similar to disulfide-rich antimicrobial peptides such as those produced in the fat body of insects (the equivalent of the vertebrate liver) in response to infections. Nemeth et al. (2003) observed that in vitro stimulation of fresh human hepatocytes with

a panel of cytokines showed strong induction of hepcidin mRNA by IL-6 but not IL-1a or TNF-a, and these results in agreement with our results that mRNA hepcidin expression in HCV infection.

Cartwright and Wintrobe went on to show that the anemia associated with infection was indistinguishable from the anemia of inflammation {Cartwright and Wintrobe, (1952)& Cartwright, (1966)}. Others correlated the anemia of inflammation with elaboration of inflammatory cytokines and ascribed changes in iron metabolism to the effects of cytokines (Means, 1995). Cytokines have been shown to modulate the expression of iron transport and storage proteins (Ludweiczek, et al. 2003). IL-6 acts directly on hepatocytes to stimulate hepcidin production. Hepcidin, in turn, acts as a negative regulator of intestinal iron absorption and macrophage iron release (Leudweiczok, et al.2003). Nemeth, Rivera, and colleagues showed that IL-6 induced hepcidin expression in hepatic cells (Nemeth, et al. 2003).{Pigeon, et al. (2001)& Llyin, et al. (2003)}observed that hepcidin mRNA expression was increased in mice with diet, induced or genetically induced iron over load. At least three major immunity driven mechanisms contribute to anemia of chronic disease (ACD) among which the retention of iron within the mononuclear phagocytic system with subsequent development of hypoferrremia, along with a limited availability of iron for erythroid progenitor cells, are of pivotal importance. {Weiss, et al., (2005)& Spivak, (2002)}postulated that this diversion of iron traffic is induced via regulatory effects of cytokines on iron uptake and release by macrophages, {Ludwiczek, et al.(2003)& Yang, et al.(2002)} and by the activity of the iron and cytokine inducible liver peptide hepcidin (Ganz, 2005), those results are in correlation with our results.

Nemeth, Rivera and colleagues, (2004) have shown that IL-6 is not involved in the regulation of hepcidin in response to iron. Furthermore, their data suggest that hepcidin levels are not simply responding to

increased iron stores. Their results are against our results.

In human volunteers, urinary hepcidin levels were boosted soon after a single dose of oral iron, which should have no significant effect on iron stores. Perhaps the serum iron level, known to increase transiently after iron ingestion, might itself be the signal to induce hepcidin expression. Alternatively, the signal might relate to the degree of iron saturation of serum transferrin (Nemeth, et al. (2004).Also Krause, et al.(2000)& Park, et al. (2001)& Pigeon, et al. (2001) found that hepcidin is produced primarily by the liver in response to stimuli known to modulate tissue iron stores and serum iron availability. Krause, et al. (2000)& Park, et al. (2001) observed that it circulates in the plasma, is filtered by the kidney and accumulates in urine. And these finding in agreement with

Our results that there's highly significant difference in liver iron concentrations between the patients in comparison to controls, also serum ferritin and s-TFR levels were significantly higher in patients than controls, and these results also correlated with

Aoki, et al., (2005) showed that among patients with hepatitis C there was a significant correlation of hepcidin mRNA expression in the liver with hepatic iron concentration and serum ferritin.

In the present work there's highly inverse correlation between hepcidin versus liver iron , serum iron and serum transferrin.. This is in accordance with data showing that hepcidin deficiency leads to persistence of FPN1(feroportin) on the cell membrane and iron overload {Nicolas, et al. (2001)& Viatte, et al. (2005)& Lesbordes-Brion, et al.(2006)}. Whereas administration of synthetic hepcidin leads to a rapid, sustained decrease in serum iron (i.e., hypoferrremia), {Delaby, et al.(2005)& Rivera, et al. (2005)}. Thus, overall, hepcidin expression both restricts iron absorption and macrophage iron release, both activities reduce body iron stores and limite iron available for erythropoiesis. Nemeth, et al. (2004) comment was serum iron level,

known to increase transiently after iron ingestion, might itself be the signal to induce hepcidin expression. Alternatively, the signal might relate to the degree of iron saturation of serum transferrin. {Nicolas, et al.(2002)& Roy, et al.(2007)& Viatte, et al. (2006)}explained that the increased serum ferritin- indicative of increased macrophage iron stores-and decreased serum iron/transferrin saturation-indicative of decreased macrophage iron recycling-typical of this disorder suggest a condition of hepcidin excess.

In the present work, hepcidin is considered highly valid marker in cases of CHCV infection, the results is in agreement with Felming, (2008) who discovered that there's increasingly evidence that the major physiological regulator of body iron stores and the availability of serum iron is the peptide hormone hepcidin.

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المفتاح المنظم لعملية التوازن بين البناء و الهدم لعنصر الحديد فى حالات الالتهاب الكبدى الفيروسى سى المزمن

سعدية فريد, سامح سيف قسم الأمراض المتوطنة المعهد القومى للكبد و الأمراض المتوطنة , ليلى راشد , دينا
صبرى

قسم الكيمياء الحيوية الطبية كلية الطب جامعة القاهرة

الخلفية: هيبسدين هو أحد الأحماض الأمينية الصغيرة, الغنية التعلق بالبيبتيدات السلبية التاين المنتجة من الخلايا الكبدية, يوجد هيبسدين فردى بشرى له دور هام فى عملية التوازن بين البناء و الهدم لعنصر الحديد المثبت أنه مرتكز الدورة متمائل الصفات أو له تحول عبثى فى الأشخاص المصابة إصابة خطيرة بداء التلوث الدموى.

إنترليوكين 6 هو نوع من السيتوكين الخاص بالالتهابات ويمكن أن يكون عامل وسيط للتحفيز فى إنتاج الهيبسدين نتيجة الالتهابات الكبدية, الإصابة بنقص الحديد فى الدم هى الإستجابة العادية لعدوى الأجهزة أو نتيجة للاختلالات الناتجة عن الالتهابات الشاملة, أنيميا الامراض المزمنة تحدث فى المرضى ذات النشاط المناعى الحاد و المزمن وتمثل أهم المشاكل الإكلينيكية.

الهدف من البحث: دراسة لقياس مقدار التعبير الجينى للهيبسدين الكبدى فى المرضى المصابين بالالتهاب الكبدى الفيروسى سى المزمن.

طريقة البحث: شملت الدراسة عدد 50 مريض من مرضى الالتهاب الكبدى الفيروسى سى المزمن أعمارهم ما بين (20-55) عاماً و العينات منتقاة من مرضى المعهد القومى للكبد و الامراض التوطنة, البحث يجرى جمع العينات فيه فى فترة التحضير لتناول عقار الانترفيرون و الريبافيرين و عدد 10 من الأشخاص الأصحاء كعينات ضابطة كلاً من المرضى و العينات الضابطة خضعوا للفحوص الطبية مع أخذ السيرة الذاتية للمرضى و عمل الموجات الصوتية على منطقة البطن.

و قد أخذت عينات الدم للمرضى لعمل الفحوصات الروتينية والسيرولوجى لقياس نسب الفيرتين, الحديد و الترانسفيرين مع أخذ عينة نسيج كبدية لقياس نسبة التعبير الجينى للهيبسدين فى الكبد و أيضاً لقياس درجة ترسيب الحديد بالكبد بال(بى سى آر) بتفاعل البلمرة المتسلسل العدى.

النتائج: لقد أسفرت نتائج هذه الدراسة عن وجود تعبير جينى عالى للهيبسدين الكبدى ذو دلالة إحصائية عالية فى حالات الالتهاب الكبدى الفيروسى سى المزمن.

الخلاصة: التعبير الجينى للهيبسدين فى الكبد له علاقات عكسية واضحة مع كلاً من درجة ترسيب الحديد فى الكبد و نسبة الحديد و الترانسفيرين فى الدم, بينما لا توجد علاقة هامة مشتركة بين الهيبسدين و الفيرتين.