Molecular study of hepcidin in chronic hepatitis C genotype 4 patients treated with pegylated interferon/ribavirin with vitamin D

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Introduction
We aimed to find out an association between hepcidin and chronic hepatitis C virus (HCV) patients treated with pegylated interferon-α/ribavirin (PEG-IFN-α/RBV) with vitamin D supplements.
Patients and methods
Seventy-three chronic HCV patients who were enrolled in the study received the standard of care therapy consisting of PEG-IFN/RBV+vitamin D_3. All patients were subjected to vitamin D and hepcidin level assessment and HCV RNA detection using quantitative reverse transcription PCR for 48 weeks of treatment.
Results
We classified our patients retrograde into responders and nonresponders to PEG-IFN-α2b+RBV therapy. Among responders, the mean level of vitamin D after therapy was 65.48 versus 9.30 ng/ml among nonresponders. Among responders, serum hepcidin, serum ferritin, and hepcidin/ferritin ratio were significantly elevated ($P<0.001$, <0.001, and $P=0.036$, respectively) after vitamin D therapy compared with nonresponders ($P=0.06$, 0.76, and 0.428, respectively). There was a significant increase in hepcidin, ferritin, hepcidin/ferritin ratio in responders compared with nonresponders after treatment ($P<0.001$, <0.001, and $P=0.05$, respectively). There was a negative correlation between hepcidin and vitamin D in responders, with a statistically significant difference ($r=-0.977$ and $P<0.001$) compared with nonresponders ($r=0.367$ and $P=0.046$).
Conclusion
In naive patients infected with HCV genotype 4, treatment with interferon α2b and ribavirin and vitamin D_3 significantly increased the rate of viral response. No correlation was found between hepcidin expression, hepcidin serum level, and vitamin D level before start of therapy.

Keywords:
hepatitis C virus, hepcidin, interferon, iron overload, ribavirin, vitamin D

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Introduction

Iron is an essential element for all living organisms and is required in many metabolic processes, but excess iron can be harmful to the organism, due to the generation of oxygen radicals [1].

Therefore, iron homeostasis must be tightly regulated in all organisms. Hepcidin, a peptide hormone, showed an important role in iron homeostasis [2,3]. It binds to the iron exporter ferroportin [4]. Hepcidin level increased with inflammation [5], an effect believed to be dependent on cytokine production [6]. Iron accumulation in the liver, where hepcidin is exclusively synthesized, is common in chronic liver diseases [7], especially in hepatitis C virus (HCV) infection (increased hepatic iron concentration is present in 10–36% of patients) [8,9]. It is even more common among patients with end-stage liver disease due to hepatitis C [10,11]. Excess iron deposition in the liver is known to be hepatotoxic, exacerbating liver injury [12], and results in resistance to interferon (IFN)-based therapy in patients with chronic hepatitis C (CHC) [13,14].

Vitamin D deficiency is very common (92%) among patients with chronic liver disease, and at least one-third of them suffer from severe vitamin D deficiency (<12 ng/ml) [15]. A low serum vitamin D level can be related to severe fibrosis and low responsiveness to

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IFN-based therapy in genotype 1 CHC [16]. Adding vitamin D to conventional pegylated/ribavirin (PEG/RBV) therapy for naive, genotype 1 patients with chronic HCV infection significantly improves sustained virological response (SVR) [17]. Few studies examined the relationships between hepcidin and disorders of mineral metabolism. Vitamin D is considered as a new regulator of iron metabolism. Vitamin D administration in healthy volunteers lowered serum hepcidin by 50% compared with baseline and persisted for 72 h [18].

In our study we, aimed to find out an association between hepcidin level and chronic HCV patients treated with PEG-IFN-α/RBV and vitamin D supplements.

Patients and methods

This prospective study included 73 chronic HCV patients (35 male and 38 female) who were enrolled from the Hepatology Outpatient Clinic In the National Hepatology and Tropical Medicine Research Institute, and Internal Medicine Outpatient Clinic.

The study was conducted according to the principles of the Declaration of Helsinki. Institutional Review Board (IRB) study approval was obtained through the (NHTMRI) IRB office before the start of the study, and a signed informed consent was obtained from all study patients at the point of recruitment. None of the enrolled patients had hepatitis B virus, hepatitis delta virus, or HIV coinfections. All patients were subjected to physical examination and biochemical markers analysis, including serum aspartate aminotransferase, alanine transaminase, albumin hepcidin, ferritin, and transferrin levels. Quantitative HCV was measured by means of real-time reverse transcription PCR using TaqMan before the start of treatment and at 12, 24, and 48 weeks of treatment with a lower limit of detection of 50 IU/ml at baseline. Liver fibrosis staging was assessed according to the METAVIR scoring system [19].

Study medications

Chronic HCV patients were treated with PEG-IFN-α2b (Peg-Intron-MDS, white house station NJ, USA) at a dose of 1.5 μg/kg by means of subcutaneous injection once weekly plus oral RBV (1000–1200 mg/day), the dose of which was determined according to patient's weight (<75 kg=1000 mg/day; ≥75 kg=1200 mg/day; rebetol, MDS), in two separate oral doses after meals in the morning and at night, plus vitamin D_3 (cholecalciferol) (Vidrop; Medical Union Pharmaceuticals, Ismailia, Egypt) 15 000 IU/week for a total duration of 48 weeks. Patients with complete early virological response or partial early virological response at week 12 continued treatment including PEG-IFN-α2b, RBV, and vitamin D until week 24 [20]. Patients with undetectable HCV viremia at week 24 continued their treatment until 48 weeks.

HCV RNA extraction and quantitative PCR detection

HCV RNA was extracted from 140 μl serum using the QIAamp Viral RNA Mini Kit (QIAgen, Hilden, Germany). Absolute quantitation of the concentration of HCV RNA was based on an external standard curve (HCV standards, IU/ml) in the presence of an internal positive control. Internal positive control was added to a mixture of lysis buffer and sample material during RNA extraction of clinical blood samples. The TaqMan assay was carried out using the AgPath-ID One-Step RT-PCR kit (Applied Biosystems, Foster City, California, USA). One-Step RT-PCR kit includes an enzyme mixture, buffer, and detection enhancer for one-step quantitative reverse transcription PCR according to the instruction of the manufacturer.

Quantitative reverse transcriptase PCR

First strand synthesis was carried out following the manufacturer's protocol for Moloney-murine leukemia virus reverse transcriptase with 5 μg of total RNA and 250 ng of random primer. The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystems) using gene-specific primers for hepcidin (forward 5′-TTCGCCTCTGGAACATGG-3′; reverse 5′-AGCCTGACCAGTGGCTCTGT-3′; gene bank accession number NG_011563.1), and GAPDH (forward 5′-CCTCTACTGGCGCTGCCAAGGCT-3′; reverse 5′-GTCCACCACTGACACGTTGG-3′; gene bank accession number NT_009759.16) for 40 cycles (95°C/45 s, 60°C/45 s, and 72°C/45 s). Changes in the expression of target gene were normalized relative to the mean critical threshold (C_t) values of GAPDH housekeeping gene using the fx1 method.

Hepcidin level assessment

Iron status assessment for each patient was carried out using biochemical tests, including estimation of ferritin (ng/ml) and transferrin (mg/dl) using enzyme-linked immunosorbent assay (ELISA) (Diagnostic automation Inc.). Hepcidin serum level was measured at weeks 0, 6, 12, 24, and 48. Human hepcidin assay was carried out using ELISA kit

(DRG International Inc., Springfield, New Jersey, USA). The concentration of the samples can be read directly from the standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted or reported as more than 80 ng/ml. Undetectably low hepcidin values (<5.4 ng/ml) were defined as '0' [21].

Vitamin D level assessment

Vitamin D serum level was measured at weeks 0, 6, 12, 24, and 48. Human 25-hydroxyvitamin D [25(OH)D] assay was carried out using ELISA kit (DRG International Inc.). The range of the kit is 0.5–150 ng/ml. Vitamin D level is considered deficient if less than 20 ng/ml, insufficient if between 20 and 30 ng/ml, and normal if more than 30 ng/ml [22].

Statistical analysis

Results were presented as mean±SD. One-way analysis of variance and Tukey's multiple comparison post-hoc tests were performed. *P* value less than 0.05 was considered significant.

Results

We classified our patients retrograde into responders and nonresponders (for patients without 2-$\log_{10}$ drop in HCV RNA levels at week 12, treatment was discontinued, including PEG-IFN-α2a, RBV, and vitamin D). Patients characteristics are presented in Table 1.

The mean level of vitamin D in responders was insufficient at the start of therapy (13.38±4.26 ng/ml) and in nonresponders it was 8.25±11.25 ng/ml. The mean level of vitamin D in responders was 65.48 ng/ml and in nonresponders it was 9.30 ng/ml, after 48 weeks of therapy (Fig. 1).

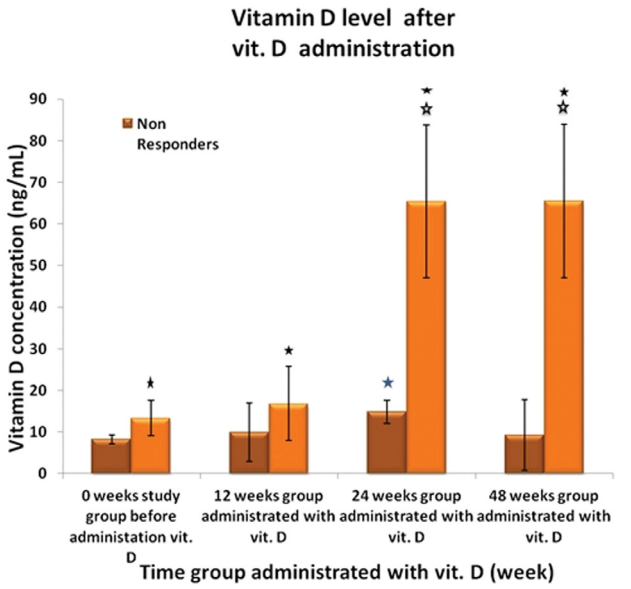
In responders, serum hepcidin, ferritin, and hepcidin/ferritin ratio were significantly elevated ($P<0.001$, <0.001, and $P=0.036$) after vitamin D administration compared with nonresponders ($P=0.06$, 0.76, and 0.428) (Fig. 2).

Table 1 Patients' characteristics in the study

	Responders (*n*=50)	Nonresponders (*n*=10)	*P* value
Age	45.73±9.5	42.16±9.29	0.147
Albumin	4.17±0.36	4.156±0.4	0.887
ALT	57.9±6.53	35.3±6.68**	<0.001
AST	54.13±4.82	58.5±3.19**	<0.001
Ferritin	193.74±9.81	156.6±3.7**	<0.001
Hb	11.25±1.1	12.6±1.4**	<0.001
hepcidin (ng/ml)	5.94±1.8	7.617±1.3**	<0.001
HCV RNA	1 047 707± 1 157 808	108 894± 1 339 694*	0.005
Vitamin D	13.38±4.26	8.25±11.25	0.023

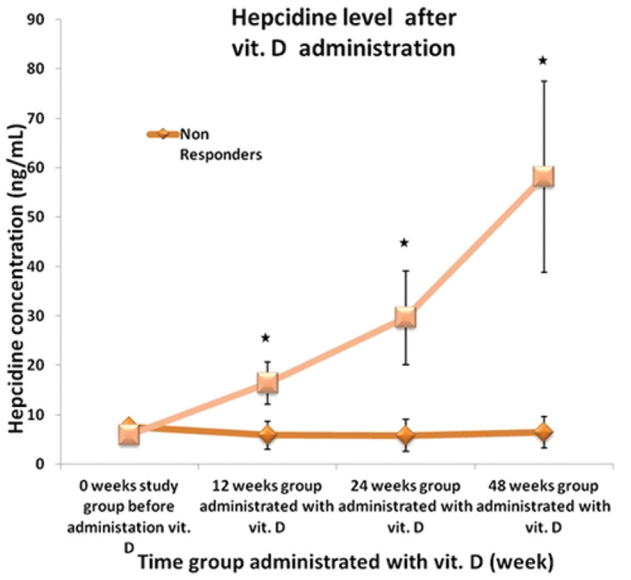
ALT, alanine transaminase; AST, aspartate aminotransferase; Hb, hemoglobin; HCV, hepatitis C virus.
*$P<0.05$ significant;
**$P<0.001$ highly significant.

Figure 1



Vitamin D level in responders and nonresponders before and after standard of care therapy.

Figure 2



Hepcidin level after vitamin D in all patients.

Table 2 Serum hepcidin, ferritin, and hepcidin/ferritin ratio in responders and nonresponders

	Responders (mean±SD)			Nonresponders (mean±SD)		
	Before SOC therapy	After SOC therapy	*P* value	Before SOC therapy	After SOC therapy	*P* value
Serum hepicidin (ng/ml)	5.9±1.8	58.1±19.36	<0.001	7.6±1.13	6.4±3.2	0.06
Serum ferritin (ng/ml)	193.7±98.1	408.6±69.7	<0.001	156.6±37.2	154.6±69.7	0.76
Hepicidin/ferritin ratio	0.031±0.018	0.14±0.277	0.036	0.049±0.03	0.046±0.04	0.428

SOC, standard of care.

Table 3 Correlation of different biochemical markers in responders and nonresponders at week 48

	Vitamin D		Hepcidin		Ferritin	
	Responders	Nonresponders	Responders	Nonresponders	Responders	Nonresponders
Albumin	–0.092 (*r*)	–0.22	–0.89	0.10	0.147	–0.003
	0.62 (*P*)	0.909	0.639	0.957	0.437	0.988
ALT	0.158	0.237	0.135	0.20	0.177	–0.18
	0.403	0.207	0.476	0.915	0.350	0.925
AST	0.008*	0.226	0.21	–0.17	0.209	–0.121
	0.967	0.229	0.914	0.930	0.267	0.523
Ferritin	–0.211	–0.086	–0.207	–0.94	1	1
	0.263	0.652	0.271	0.622		
	–0.104	–0.0.127	–0.127	–0.222	–0.238	–0.732*
Hb	0.584	0.503	0.502	0.239	0.205	<0.001
Hepcidin	–0.977*	0.367	1	1	–0.207	–0.094
	<0.01	0.046			0.271	0.622
HCV RNA	0.259	0.246	0.260	–0.130	–0.43	–0.134
	0.166	0.190	0.166	0.494	0.823	0.480

ALT, alanine transaminase; AST, aspartate aminotransferase; Hb, hemoglobin; HCV, hepatitis C virus.

Table 4 Correlation between hepcidin gene reverse transcription PCR (gene expression) and serum hepcidin level at different periods of treatment

	r^2	*P* value
Hepcidin_week 0	0.653**	<0.001**
Hepcidin_week 12	0.502**	<0.001**
Hepcidin_week 24	0.528**	<0.001**
Hepcidin_week 48	0.353*	0.016*

*$P<0.05$ significant. **$P<0.001$ highly significant.

The mean levels of serum hepcidin, serum ferritin, and hepcidin/ferritin ratio in responders and nonresponders before and after therapy are shown in Table 2.

A negative correlation was found between hepcidin and vitamin D in responders with statistical significance (r=–0.977 and $P<0.001$) compared with nonresponders (r=0.367 and P=0.046) (Table 3).

Table 4 shows a highly significant correlation between hepcidin gene expression measured by means of quantitative reverse transcription PCR and serum level of hepcidin at different time intervals starting from week 0 (before start of therapy), except for the correlation with serum hepcidin level measured 48 weeks after therapy, which was significant ($P<0.05$).

No correlation was found between levels of hepcidin, vitamin D, and hepcidin gene expression and fibrosis stage (Table 5).

Discussion

The current standard treatment for chronic HCV is administration of PEG-IFNα+RBV for 48 weeks as recommended for patients with HCV genotype 4 [23].

Although this treatment is effective, the SVR approaches 50% based on various retrospective and prospective trials [24].

In our work, we aimed to study the association between PEG-IFNα+RBV for 48 weeks and iron metabolism parameters in the form of hepcidin gene expression and serum hepcidin levels during different periods of treatment. In addition to the recommended standard of care (SOC), those patients were given vitamin D supplementation to enhance their response to SOC.

Synthesis of hepcidin is increased with iron overload and decreased with anemia. Moreover, it may be induced by infection, hypoxia, and inflammatory responses, which result in rapid plasma iron decrease [25].

Table 5 Relations between levels of hepcidin, vitamin D, and hepcidin gene expression and fibrosis stage at week 0

	F1			F2			F3			
	Median	25th percentile	75th percentile	Median	25th percentile	75th percentile	Median	25th percentile	75th percentile	*P* value
qRT-PCR	0.90	0.43	1.31	1.20	0.51	1.40	0.72	0.39	1.71	0.696
Hepcidin_week 0	8.80	5.20	11.60	10.00	6.10	15.30	8.90	3.15	15.25	0.882
Vitamin D_week 0	3.70	2.00	5.50	3.00	2.00	6.75	5.00	2.00	9.25	0.767

qRT-PCR, quantitative reverse transcription PCR.

To our knowledge, this was the first study to provide insights into the molecular mechanisms of hepcidin dysregulation in CHC among Egyptian patients.

Our study showed a low expression of hepcidin in most of the patients with CHC. This is in agreement with some previous observations [16–18].

The reduction of hepcidin gene expression has been suggested to be the main reason for iron overload in CHC infection [24].

Sikorska *et al.* [24] found that 31 Polish patients with CHC (genotype 1) had lower hepcidin mRNA expression and more frequently iron deposits in hepatocytes compared with patients with chronic hepatitis B.

Fujita and colleagues found that total iron score correlated positively with transaminase activity, histological grading, and staging in CHC patients. Interestingly, in this study, baseline iron metabolism alterations were more pronounced in non-SVR than in SVR to IFN/RBV treatment, thus suggesting the association between iron deposition and resistance to anti-HCV therapy. This observation has been further confirmed in another study conducted by Peyssonnaux and colleagues showing inadequate hepcidin expression in chronic HCV, which could be restored by eradication of the virus [25,26].

In our study no correlation was found between hepcidin expression and aspartate aminotransferase or alanine transaminase at any point of treatment or even before start of therapy. In this study, liver biopsies from 66 patients (36 hepatocellular carcinoma and 30 CHC) and normal human liver biopsies obtained from 20 healthy liver transplant donors (a control group) were analyzed. The expression of hepcidin mRNA is decreased in liver tissues of CHC patients and more suppressed in the liver tissues of patients with hepatocellular carcinoma, suggesting that hepcidin expression appears to be appropriately responsive to iron status and disease progression in cirrhosis and hepatocarcinogenesis [27].

Our study showed that hepcidin serum level had significantly increased in response to SOC treatment when hepcidin level was compared with that before therapy up to 48 weeks of treatment (end of treatment response). There was a significant positive correlation between serum hepcidin level and hepcidin expression before start of therapy. In other words, serum hepcidin level depended on hepcidin expression. Previous data showed that hepcidin expression was dependent on serum iron concentration [24,26,27].

Recent results have shown hepcidin deregulation in CHC and have suggested the pivotal role of this hormone in the pathogenesis of iron overload [28].

In a study by Marzouk and colleagues, following 24 weeks of PEG-IFN/RBV therapy, hepcidin levels significantly increased to be higher than that of the control group, whereas iron and hepcidin/ferritin ratio increased, but their levels were still significantly lower compared with the control group, and this was not related to the virological response. In contrast, both baseline and follow-up ferritin levels were significantly higher in CHC patients than in the control group. In conclusion, serum hepcidin and hepcidin/ferritin ratio were significantly lower in CHC patients than in HCV-negative controls. Following antiviral therapy, both hepcidin and hepcidin/ferritin ratio were elevated irrespective of the virologic response [28].

Adding vitamin D supplements (2000 IU/day) to standard PEG/RBV therapy for patients with HCV genotypes 2–3 significantly improves viral response (viral clearance) from 42 to 86% for genotype 1 and from 77 to 95% for genotype 2/3. The addition of vitamin D is cheap and without side effects [17].

Many studies showed reasonable results of the possible role of vitamin D supplementation in augmenting the response to SOC therapy [29].

In the current study, 97.5% of patients showed marked deficiency in $25(OH)D_3$ levels. These data match with previously published trials that showed a remarkable

vitamin D deficiency in their studied populations [15,17,28].

After vitamin D supplementation, there was a significant elevation in serum level of vitamin D [25 $(OH)D_3$] among different treatment periods, which is in agreement with other studies [29].

In our study, no correlation was found between 25 (OH)D levels and fibrosis stage at baseline assessment of the studied population, and no significant difference was observed. These data run parallel to those reported earlier by Ladero and colleagues [30,31], who found that baseline 25(OH)D level is not associated with fibrosis stage. However, another study by Arteh and colleagues declared a strong negative association between serum 25(OH)D levels and fibrosis scores.

Southern and colleagues have retrospectively shown that vitamin D supplementation improves SVR in patients with HCV genotype 2–3, with mild-to-moderate fibrosis. Vitamin D improved response due to its immunomodulator effect together with its potential effect on modulating the effect of hepcidin [32].

Bell demonstrated that 1, 25$(OH)2D_3$ is a direct inducer of the antimicrobial peptide cathelicidin in human monocytes, keratinocytes, and neutrophils, suggesting a potential effect of 1, 25$(OH)2D_3$ in modulating the expression of other antimicrobial peptides, including hepcidin [33].

This effect was reported earlier in a pilot study conducted by Bacchetta *et al.* [34]. In a pilot study with healthy volunteers, supplementation with a single oral dose of vitamin D (100 000 IU vitamin D_2) increased serum levels of 25(OH)D from 2762 ng/ml before supplementation to 4463 ng/ml after supplementation (P=0.001).

This response was associated with a 34% decrease in circulating levels of hepcidin within 24 h of vitamin D supplementation (P=0.05). These data show that vitamin D is a potent regulator of the hepcidin–ferroportin axis in humans [34].

Conclusion

Hepcidin serum level had significantly increased in response to SOC treatment when compared with that before therapy up to 48 weeks of treatment (end of treatment response). There was a significant positive correlation between serum hepcidin level and hepcidin expression before start of therapy. Sample size of our retrograde study was a limitation of this study and we recommend another study with higher number of patients. Finally, no correlation was found between hepcidin expression, hepcidin serum level, and vitamin D level before start of therapy. We recommended augmenting hepcidin expression or hepcidin-enhancing agents as an effective therapy in the treatment of CHC patients, including those with existing IFN resistance.

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Conflicts of interest
There are no conflicts of interest.

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