

Expression of hepcidin and other iron-regulatory genes in human hepatocellular carcinoma and its clinical implications

Hsi-Huang Tseng · Jan-Gowth Chang ·
Yaw-Huei Hwang · Kun-Tu Yeh · Yao-Li Chen ·
Hsin-Su Yu

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Abstract

Purpose We aimed to assess expression of ten iron-regulatory genes in hepatocellular carcinoma (HCC) and its clinical implications.

Methods We used real-time polymerase chain reaction to measure ten iron-regulatory genes' mRNA and Perls' stain to assess iron stores in 50 HCCs and adjacent nontumor specimens. We compared the differences of gene expression and iron stores between tumor and nontumor specimens, and analyzed the relationships of gene expression with hepatic iron stores, patients' hemoglobin levels and clinicopathologic parameters.

Results Hepcidin, ceruloplasmin, transferrin, and transferrin receptor 2 were downregulated, while transferrin receptor 1 was upregulated in HCC. Hepcidin was markedly decreased in HCC but still correlated with hepatic iron stores. Iron-regulatory genes varied in their relationships of expression with clinicopathologic parameters.

Conclusions Altered expression of iron-regulatory genes in HCC may disturb patient's iron balance. Hepcidin may play a role in defending the body against HCC.

Keywords Iron · Hepcidin · Hepatocellular carcinoma · Anemia · Liver

H.-H. Tseng · Y.-H. Hwang
Institute of Occupational Medicine and Industrial Hygiene,
College of Public Health, National Taiwan University,
Taipei, Taiwan

J.-G. Chang
Departments of Medical Research and Laboratory Medicine,
Kaohsiung Medical University Hospital,
Kaohsiung Medical University, Kaohsiung, Taiwan

K.-T. Yeh
Department of Pathology,
Changhua Christian Hospital, Changhua, Taiwan

Y.-L. Chen
Division of General Surgery, Department of Surgery,
Changhua Christian Hospital, Changhua, Taiwan

H.-S. Yu (✉)
Department of Dermatology, Faculty of Medicine,
Kaohsiung Medical University,
100, Shi-chuan 1st Road, Kaohsiung, Taiwan
e-mail: dermyu@kmu.edu.tw

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. Patient prognosis is generally dismal, and nutritional problems commonly worsen the outcome. Most concerns about malnutrition in HCC patients focus on protein-calories deficiency, but little is known about the disturbance of iron metabolism.

Liver is the principal organ of iron metabolism. It serves for iron storage, and secretes transferrin (TF) to deliver iron in the blood. Recently, the importance of liver ascends by the discovery of iron hormone it secretes—hepcidin. Hepcidin binds the iron exporter ferroportin (FPN1) to block iron release from duodenocytes, macrophages, and hepatocytes, thus it is able to control serum iron levels (Nemeth et al. 2004). It rises with iron overload (Pigeon et al. 2001), inflammation and infection, and decreases with hypoxia and anemia (Nicolas et al. 2002). Deficiency of hepcidin leads to hemochromatosis (Nicolas et al. 2001; Roetto et al. 2003); overexpression of hepcidin results in severe iron

refractory anemia, and is implicated in anemia of inflammation (Roy et al. 2007; Weinstein et al. 2002).

Liver also expresses many other important iron-regulatory genes (Anderson and Frazer 2005). High-affinity transferrin receptor (TFR1) and low-affinity transferrin receptor (TFR2) take up iron-transferrin via endocytosis. Divalent metal transporter 1 (DMT1) escorts iron from endosome to cytoplasm. Ferritin locks iron and stores it. When iron is exported by FPN1, ceruloplasmin (CP), a liver-derived plasma protein, assists iron to be loaded onto TF. Hemochromatosis (HFE1), hemojuvelin (HFE2), and TFR2, all lying upstream of hepcidin expression, indirectly control systemic iron balance.

Studies on expression of iron-regulatory genes in liver diseases excluding hemochromatosis are limited. It has been reported that hepcidin mRNA is downregulated in alcoholic liver injury (Bridle et al. 2006; Ohtake et al. 2007) but not significantly affected by the presence of hepatitis B virus (HBV) or hepatitis C virus (HCV) infection (Aoki et al. 2005; Fujita et al. 2007) and cirrhosis (Bergmann et al. 2008). Hepcidin expression has been found reduced in rat model HCC (Holmstrom et al. 2006); recently its reduction in human HCC has also been reported (Kijima et al. 2008).

Our study aimed to examine the expression of hepcidin in human HCC, juxtaposing that of the other nine mentioned genes, and to assess its clinical significance.

Patients and methods

Patients and tissue specimens

Fifty HCC patients, who received operation at the Department of Surgery, Changhua Christian Hospital between November 2000 and March 2003, were included in this retrospective study (Tseng et al. 2009). The patients were 39 men and 11 women; their mean age was 54.5 years (SD 14.9 years, range 10–75 years). The mean presurgical hemoglobin level was 12.8 (SD 2.1 g/dl, range 9.1–18.8 g/dl). Twenty-nine (58%) patients were anemic. HCC specimens and adjacent nontumor tissue specimens were collected during hepatic resection, and were either freshly stored in liquid nitrogen or formalin-fixed and paraffin-embedded before use. Based on the WHO grading system (Hirohashi et al. 2000), the numbers of patients with well, moderately, and poorly differentiated HCC were 6, 29, and 15, respectively. According to pTNM staging system of the American Joint Committee on Cancer (American Joint Committee on Cancer 1997), the numbers of patients in stage I, II, IIIA, IIIB, and IV were 14, 17, 6, 3, and 10, respectively. Tumor size was defined as the greatest diameter of each tumor (mean \pm SD 5.4 \pm 3.3 cm, range 1.6–15.5 cm). This study

was approved by the Institutional Review Board of Changhua Christian Hospital.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from specimens by Trizol (Invitrogen). Five micrograms of the extracted RNA was reversely transcribed into cDNA in a final volume of 100 μ l. Random primers, oligo dT, and MMLV-RT (Promega) were used according to the manufacturer's protocol. Real-time PCR was performed on a LightCycler (Roche) and LightCycler-FastStart DNA Master SYBR Green kit (Roche) was used. The primer sequences of target genes and the reference gene hypoxanthine phosphoribosyltransferase 1 (HPRT) are given in Table 1. For ferritin, primers were designed to its subunit ferritin heavy polypeptide 1 (FTH1). The PCR cycling profile was one cycle of 95°C for 10 min, followed by 45 cycles of 95°C for 5 s, 60°C for 5 s, and 72°C for 9 s. Relative quantification of mRNA was determined by comparative Ct method (Schmittgen and Livak 2008). The mRNA level of tested gene was expressed as the amount relative to that of HPRT, and was calculated as $2^{-\Delta C_t}$, while the tumor/nontumor mRNA ratio was calculated as $2^{-\Delta\Delta C_t}$. We first measured the mRNA levels of each gene in 30 of the 50 tumor–nontumor pairs. For the genes showing a statistically significant difference between tumor and nontumor specimens, we further examined the remaining 20 pairs.

Perls' Prussian blue staining

Formalin-fixed, paraffin-embedded tissue sections (4 μ m thick) were deparaffinized and immersed in hydrochloric acid and potassium ferrocyanide solution. The presence of ferric iron was detected by its combination with ferrocyanide to form Prussian blue. Nuclear fast red was used as counterstain. Iron deposits were scored as 0 (absent at 400 \times magnification), 1 (visible at 400 \times magnification), 2 (visible at 100 \times magnification), 3 (visible at 25 \times magnification), or 4 (visible at 10 \times magnification) (Searle et al. 2002).

Statistical analysis

Paired Student *t* test was used to compare mRNA levels between tumor and nontumor specimens, while between unpaired groups, two-sample *t* test or ANOVA was used as appropriate. To do these, values were log-transformed to base 2 to obtain a normal distribution. Wilcoxon signed rank test was used to compare iron scores between tumor and nontumor specimens. Spearman rank correlation test was used for correlation analysis between variables. All statistical analyses were performed with SPSS 13.0 for

Table 1 Primer sequences of ten iron-regulatory genes

Gene	Forward 5' → 3'	Reverse 5' → 3'	Product size (bp)
CP	ATGGATGCTCAGCTGTCAGA	TAACATGCTTCCCACGGATA	116
DMT1	TCCACCATGACAGGAACCTA	GATGGCAATAGAGCGAGTCA	105
FPN1	ACAGCAGTCTACGGGCTGGT	CTGTACCACCAGCGAGGTCT	117
FTH1	GGAGAGGGAACATGCTGAGA	GCACACTCCATTGCATTACG	126
Hepcidin	GACCAGTGGCTCTGTTTTCC	CTCCTTCGCCTCTGGAACAT	111
HFE1	GATCATGAGAGTCGCCGTGT	ATGTGATCCCACCCTTTCAG	107
HFE2	CTGCCTACATTGGCACAACCT	CCTTGATGGAGAAGGAGAGC	66
TF	AGCAGAGACCACCGAAGACT	AGACAAACCCTCCATCCAAG	80
TFR1	GGTTGCAAATGCTGAAAGC	AAGGAAGGGAATCCAGGTGT	147
TFR2	GTGGACCGACACGCACTAC	TAGACGTCAGGGTCTCCAG	123
HPRT	GACCAGTCAACAGGGGACAT	CCTGACCAAGGAAAGCAAAG	132

HPRT reference gene

Windows and $P < 0.05$ (two-sided) was considered statistically significant.

Results

Comparison of expression of ten iron-regulatory genes between tumor and nontumor specimens

Hepcidin, CP, TF, and TFR2 were downregulated, while TFR1 was upregulated in HCC, as compared with those in nontumor specimens. There was no statistically significant difference in the expression of DMT1, FPN1, FTH1, HFE1, and HFE2 between tumor and nontumor specimens (Table 2). Notably, hepcidin expression in HCC was reduced to 3% of that in nontumor specimens; the magnitude of expression change far exceeded that of the other studied genes. Overall, the numbers of HCCs which had reduced expression of hepcidin, CP, TF, and TFR2 were 43 (86%), 36 (72%), 36 (72%), and 31 (62%), respectively. On the other hand, 32 (64%) HCCs had an increase of TFR1 expression.

Expression of hepcidin, CP, TF, TFR1, and TFR2 in nontumor specimens

By analyzing gene expression in nontumor specimens, we found hepcidin and TFR2 had a higher expression in men than in women (Table 3). Age, smoking, alcohol drinking, chronic viral hepatitis, and cirrhosis did not affect the expression of hepcidin, CP, TF, TFR1, and TFR2. There was also no significant difference in these genes' expression between HBV and HCV infection. Thus, the expression of these iron-regulatory genes was not affected by risk factors for HCC, but hepcidin and TFR2 expression was influenced by gender factor.

Table 2 Tumor/nontumor mRNA ratios of ten iron-regulatory genes

Gene	Mean	95% CI	<i>P</i> value
Hepcidin	0.03	0.01–0.08	<0.001
CP	0.25	0.10–0.51	<0.001
TF	0.36	0.21–0.62	<0.001
TFR2	0.35	0.19–0.67	0.002
TFR1	2.33	1.38–3.95	0.002
DMT1	1.62	0.50–5.29	0.942
FPN1	0.75	0.44–1.26	0.268
FTH1	0.96	0.41–2.27	0.935
HFE1	0.69	0.52–1.52	0.712
HFE2	1.17	0.52–2.63	0.689

Mean and 95% CI were calculated with log-transformed data and presented in antilog form as ratio scales

Expression of hepcidin, CP, TF, TFR1, and TFR2 in tumor specimens

By analyzing gene expression in tumor specimens, we found CP and TF were less expressed in larger tumor (≥ 5 cm); CP was expressed higher in the presence of HCV infection and cirrhosis (Table 4). However, expression of hepcidin, TFR1, and TFR2 was not affected by any of the parameters including age, gender, chronic hepatitis, cirrhosis, tumor size, histologic grade, and tumor stage.

Comparison of iron stores between tumor and nontumor specimens

Tumor and nontumor specimens differed in iron-staining intensity (Fig. 1a). Most of the nontumor specimens had a score of 2, whereas most of the tumor specimens had a score of 0 (Fig. 1b). In total, iron stores were decreased in

Table 3 Distribution of gene expression in nontumor specimens by demographic variables and risk factors for HCC

	<i>n</i>	Hepcidin	CP	TF	TFR2	TFR1
Age						
<60	25	5.15 ± 0.70	6.79 ± 0.35	8.36 ± 0.35	4.14 ± 0.38	-2.78 ± 0.41
≥60	25	6.79 ± 0.63	6.45 ± 0.53	8.09 ± 0.47	4.92 ± 0.54	-3.17 ± 0.81
Gender						
Men	39	6.62 ± 0.51 ^a	6.64 ± 0.36	8.40 ± 0.28	4.88 ± 0.38 ^a	-3.21 ± 0.55
Women	11	3.66 ± 0.96	6.52 ± 0.69	7.61 ± 0.87	3.28 ± 0.56	-2.15 ± 0.94
Smoking						
+	10	5.28 ± 0.72	6.35 ± 0.39	7.93 ± 0.51	4.02 ± 0.92	-3.21 ± 0.74
-	36	5.63 ± 0.53	6.69 ± 0.38	8.42 ± 0.35	4.51 ± 0.33	-3.07 ± 0.63
Alcohol						
+	13	6.26 ± 0.84	6.30 ± 0.38	8.30 ± 0.45	5.13 ± 0.88	-3.27 ± 0.79
-	36	5.90 ± 0.60	6.66 ± 0.41	8.17 ± 0.37	4.35 ± 0.33	-2.90 ± 0.61
Viral infection						
HBV+, HCV+	3	6.93 ± 2.06	7.44 ± 0.80	8.54 ± 1.17	3.82 ± 2.09	-1.47 ± 1.81
HBV+, HCV-	24	5.80 ± 0.70	6.80 ± 0.42	7.80 ± 0.43	4.68 ± 0.53	-2.44 ± 0.64
HBV-, HCV+	7	3.61 ± 1.43	6.08 ± 0.86	7.86 ± 1.01	3.68 ± 1.05	-4.74 ± 1.53
HBV-, HCV-	4	6.06 ± 1.01	7.61 ± 0.66	8.30 ± 0.42	4.84 ± 0.47	-0.73 ± 0.98
Cirrhosis						
+	26	5.92 ± 0.65	6.54 ± 0.48	8.42 ± 0.41	4.26 ± 0.36	-3.42 ± 0.75
-	19	5.42 ± 0.84	6.56 ± 0.51	7.87 ± 0.49	5.25 ± 0.57	-1.97 ± 0.62

Cases with missing data were not included in analysis. Values are relative (to HRPT) mRNA levels log-transformed to base 2 and presented as mean ± standard error

^a $P < 0.05$

78% (39 of 50) of HCCs. Wilcoxon signed rank test further confirmed a highly significant difference in iron stores between HCC and nontumor specimens ($P < 0.001$).

Correlation of hepcidin, CP, TF, TFR1, and TFR2 expression with hepatic iron stores and patients' hemoglobin levels

Hepcidin mRNA levels in both tumor and nontumor specimens correlated with hepatic iron stores (Fig. 2a, b) but not with hemoglobin levels (Fig. 2c, d), whereas CP, TF, TFR1, and TFR2 expression in both tumor and nontumor specimens did not correlate with either hepatic iron stores or hemoglobin levels.

Discussion

Our study shows that hepcidin, CP, TF, and TFR2 were downregulated, while TFR1 was upregulated in human HCC. Reduction of hepcidin expression has been seen in studies of rat model (Holmstrom et al. 2006) and human (Kijima et al. 2008) HCC. Both studies did not include CP and TF, but found TFR2 expression not significantly

altered. The rat model HCC also had an increased expression of TFR1 (Holmstrom et al. 2006).

TFR1 is ubiquitously expressed in all cell types. It has been constantly found to be increased in cancer cells, a phenomenon attributed to an increased iron demand by proliferating cancer cells (Kwok and Richardson 2002). It forms complex with HFE1, but the complex does not appear to have a direct iron-regulatory activity (Andrews and Schmidt 2007). On the other hand, TFR2 is expressed specifically in liver; its function might be more important to mediate hepcidin expression than to take up iron (Anderson and Frazer 2005). Deficiency of TFR2 as well as deficiencies of hepcidin, CP, and TF, which are liver-derived plasma proteins, leads to hemochromatosis (Pietrangelo 2006). In addition, patients with aceruloplasminemia usually present with microcytic anemia (Harris et al. 1995; Loreal et al. 2002), and patients with atransferrinemia commonly suffer from severe anemia (Beutler et al. 2000; Hayashi et al. 1993). Taken together, the upregulation of TFR1 may not have systemic impacts, but the downregulation of hepcidin, CP, TF, and TFR2 may disturb systemic iron balance and contribute to anemia in HCC patients.

These genes' expression was not affected by risk factors for HCC, as we observed in nontumor specimens. Although

Table 4 Distribution of gene expression in HCC specimens by demographic and clinicopathologic variables

	<i>n</i>	Hepcidin	CP	TF	TRF2	TFR1
Age						
<60	25	0.59 ± 0.98	4.84 ± 0.56	6.88 ± 0.41	2.91 ± 0.43	-1.39 ± 0.46
≥60	25	1.23 ± 0.75	4.45 ± 0.63	6.66 ± 0.49	3.16 ± 0.61	-2.12 ± 0.48
Gender						
Men	39	1.26 ± 0.74	4.79 ± 0.44	6.80 ± 0.34	3.19 ± 0.43	-1.84 ± 0.38
Women	11	-0.33 ± 0.93	4.11 ± 1.11	6.66 ± 0.81	2.48 ± 0.72	-1.46 ± 0.67
HBV						
+	32	1.26 ± 0.82	4.70 ± 0.46	6.86 ± 0.41	3.06 ± 0.44	-1.48 ± 0.40
-	11	0.29 ± 1.15	5.84 ± 0.68	7.37 ± 0.43	3.85 ± 0.60	-1.57 ± 0.70
HCV						
+	13	1.44 ± 1.03	6.32 ± 0.43 ^a	7.24 ± 0.44	4.13 ± 0.44	-2.37 ± 0.54
-	28	0.74 ± 0.74	4.51 ± 0.51	7.06 ± 0.42	3.26 ± 0.49	-1.25 ± 0.45
Cirrhosis						
+	26	1.20 ± 0.87	5.62 ± 0.32 ^a	7.41 ± 0.36	3.62 ± 0.43	-1.92 ± 0.46
-	19	0.80 ± 0.96	4.09 ± 0.73	6.30 ± 0.53	3.09 ± 0.58	-1.34 ± 0.49
Tumor size						
<5 cm	26	0.29 ± 3.95	5.59 ± 2.03 ^a	7.40 ± 1.59 ^a	3.36 ± 2.14	-1.75 ± 2.04
≥5 cm	24	1.57 ± 4.76	3.61 ± 3.49	6.07 ± 2.68	2.68 ± 3.06	-1.76 ± 2.71
Grade						
WD	6	2.26 ± 1.72	6.98 ± 0.53	7.46 ± 0.51	5.06 ± 0.34	-1.53 ± 1.06
MD	29	0.01 ± 0.77	4.65 ± 0.59	7.02 ± 0.39	2.95 ± 0.47	-1.89 ± 0.45
PD	15	2.10 ± 1.19	3.69 ± 0.66	5.99 ± 0.69	2.40 ± 0.74	-1.57 ± 0.59
Stage						
I, II	31	0.40 ± 0.72	4.94 ± 0.52	6.80 ± 0.40	3.58 ± 0.46	-2.07 ± 0.45
III, IV	19	1.73 ± 1.11	4.16 ± 0.70	6.71 ± 0.54	2.13 ± 0.56	-1.23 ± 0.47

Values are relative (to HPRT) mRNA levels log-transformed to base 2 and presented as mean ± standard error

WD well differentiated, MD moderately differentiated, PD poorly differentiated

^a *P* < 0.05

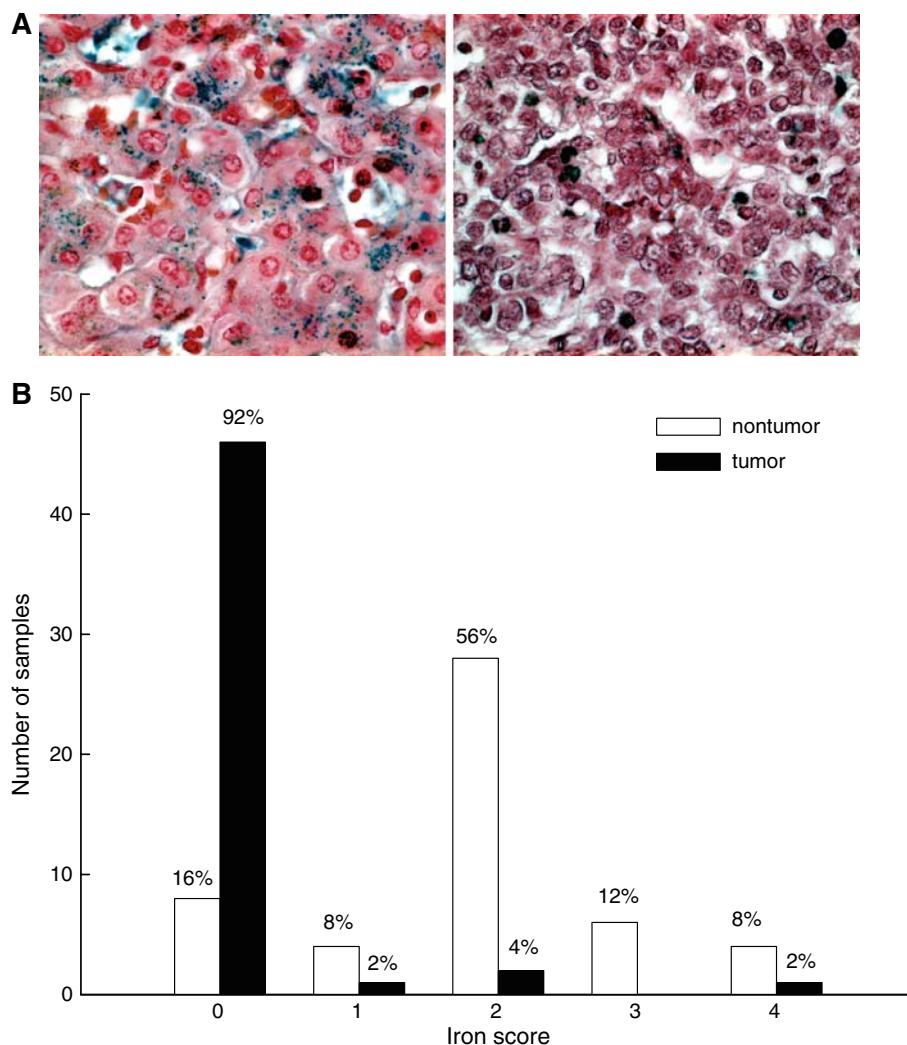
hepcidin mRNA was found downregulated upon alcohol exposure in animal models (Bridle et al. 2006; Ohtake et al. 2007), we did not find it associated with chronic alcohol consumption. Our findings agree with those from studies of human chronic liver diseases, that hepcidin mRNA was not altered in chronic hepatitis B and C (Fujita et al. 2007) or affected by HCV load (Aoki et al. 2005), and expression of hepcidin, TF, TFR1, and TFR2 was not altered in liver cirrhosis (Bergmann et al. 2008). These results suggest that the expression of iron-regulatory genes is not altered until carcinogenesis.

Under normal conditions, hepcidin is regulated by iron stores (Pigeon et al. 2001). Studies have shown that hepatic hepcidin mRNA correlated with hepatic iron stores in hemochromatosis (Bridle et al. 2003), chronic hepatitis C (Aoki et al. 2005; Fujita et al. 2007), cirrhotic livers (Bergmann et al. 2008), and nontumor part of the liver with primary or secondary carcinoma or cirrhosis (Detivaud et al. 2005), suggesting hepcidin expression is regulated by

iron stores even in the face of chronic injuries (Bridle et al. 2003). Similarly, we and Holmstrom et al. found hepcidin correlated with iron stores in HCC as well as in nontumor tissues (Holmstrom et al. 2006), despite that iron was depleted in HCC, a phenomenon reflecting active proliferation of neoplastic cells (Deugnier et al. 1993). The results further show that hepcidin remains to be regulated by iron stores even in hepatoma cells.

Under normal conditions, hepcidin is also regulated by anemia (Nicolas et al. 2002). However, the correlation of liver hepcidin with hemoglobin levels in patients with chronic hepatitis C is controversial (Aoki et al. 2005; Fujita et al. 2007). Although a positive correlation was noted between patients' hemoglobin levels and hepcidin expression in nontumor part of livers with cancers or cirrhosis (Detivaud et al. 2005), we found hepcidin expression in both tumor and nontumor tissues not correlated with hemoglobin levels. Considering that the tumor/nontumor mRNA ratio of hepcidin was far lower than those of the other

Fig. 1 Iron staining in HCCs and adjacent nontumor specimens. **a** A representative case showing iron abundant in nontumor (*left*) but scarce in matched tumor specimen (*right*) ($\times 400$). **b** Frequency distribution of iron scores in tumor and nontumor specimens



genes, or that of hepcidin in the rat HCC model, which used livers of rats without cancer as control (Holmstrom et al. 2006), we suspect that hepcidin might be increased in nontumor tissues we studied. Increase of hepcidin may result from an immune response to withhold iron against pathogens or cancer cells; in the extreme it may lead to anemia of inflammation (Andrews 2004; Roy et al. 2007). If so, both reduced expression of CP and TF in HCC, and increased expression of hepcidin in nontumor tissues, may contribute to anemia in HCC patients.

Anemia in HCC patients is commonly confounded by anemia resulting from chronic liver diseases. Fifty-eight percent of our patients were anemic. To what extent the anemia can be attributed to altered expression of iron-regulatory genes in HCC is unclear.

Some findings were unexpected, such as gender differences in hepcidin and TFR2 expression. Higher hepcidin expression in men than in women was noted in one (Fujita et al. 2007) but not other (Aoki et al. 2005; Detivaud et al. 2005) studies; however, higher hepcidin expression in

female than in male mice was also reported (Courselaud et al. 2004). Whether hepcidin and TFR2, a mediator of hepcidin expression, are regulated by species-related gender-linked factors or the gender differences in their expression were found by chance needs more validations.

Our results show expression of the five dysregulated genes in HCC was not correlated with histologic grade, agreeing with previous reports that hepcidin expression was not associated with tumor differentiation (Holmstrom et al. 2006; Kijima et al. 2008). The results suggest that morphologic grading may not be sensible enough to reflect expression patterns of some liver-specific genes; therefore, hepcidin, CP, TF, and TFR2 cannot be used as markers for HCC differentiation. The reason why expression of only CP and TF was negatively associated with tumor size needs further exploration.

There are limitations of our study. First, gene expression was not assessed at the protein level, because we did not have reliable antibody against hepcidin. Second, 90% of the tumor specimens had an iron score of zero; lack of variability

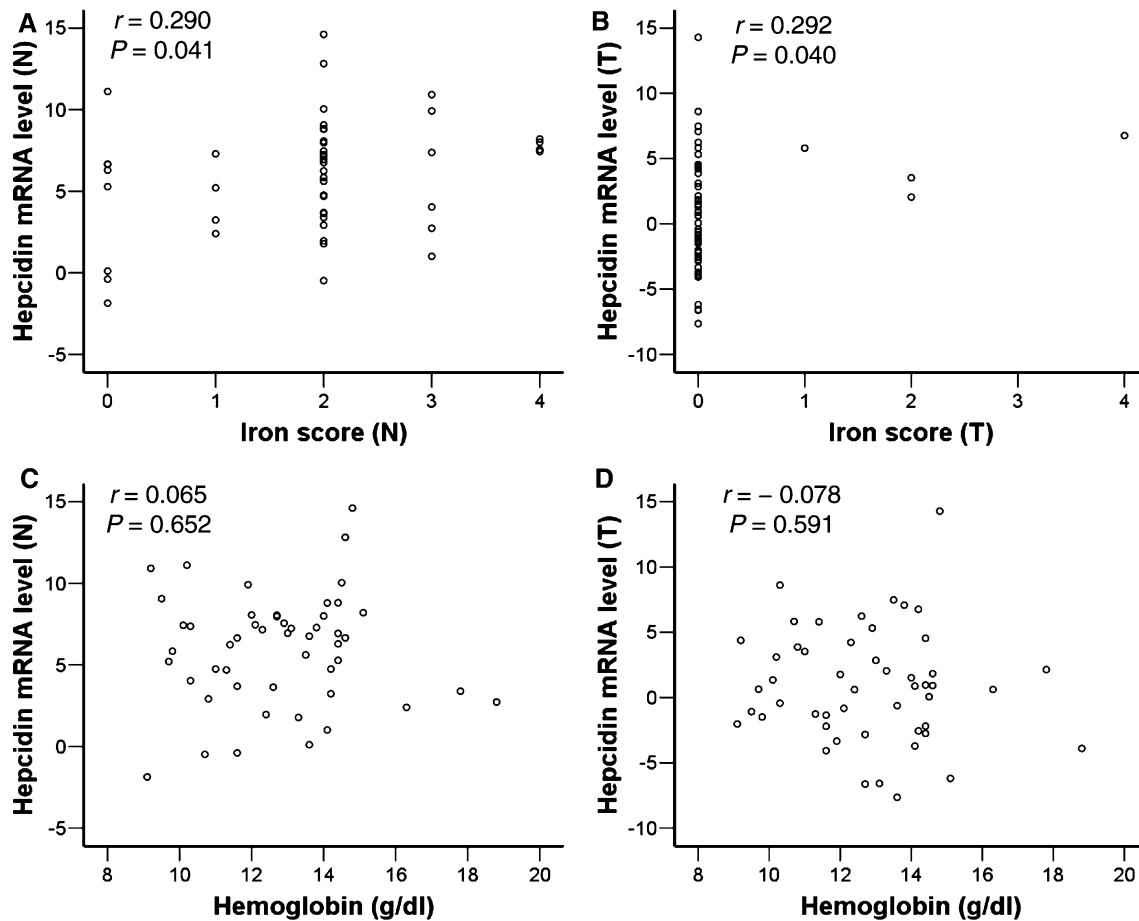


Fig. 2 Correlation of hepcidin mRNA levels with iron stores (a, b) and hemoglobin levels (c, d) in HCC patients. The hepcidin mRNA levels are relative (to HPRT) mRNA levels log-transformed to base 2. *N* nontumor, *T* tumor

in score ranking might decrease the power of correlation analysis. Third, the retrospective study design prevented us from assessing parameters such as serum iron indices or hepcidin levels to better understand patient clinical status regarding iron metabolism.

In conclusion, we have shown that the expression of iron-regulatory genes is not altered by chronic liver injuries until hepatocarcinogenesis, and the altered expression of iron-regulatory genes in HCC may disturb patient's iron balance. Our study provides insight into patient's iron disturbance at the basic level, and raises speculations on the possibility that hepcidin plays a role in defending the body against HCC.

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References

American Joint Committee on Cancer (1997) AJCC cancer staging manual. Lippincott-Raven, Philadelphia

- Anderson GJ, Frazer DM (2005) Hepatic iron metabolism. *Semin Liver Dis* 25:420–432. doi:10.1055/s-2005-923314
- Andrews NC (2004) Anemia of inflammation: the cytokine–hepcidin link. *J Clin Invest* 113:1251–1253
- Andrews NC, Schmidt PJ (2007) Iron homeostasis. *Annu Rev Physiol* 69:69–85. doi:10.1146/annurev.physiol.69.031905.164337
- Aoki CA, Rossaro L, Ramsamoj R, Brandhagen D, Burritt MF, Bowlus CL (2005) Liver hepcidin mRNA correlates with iron stores, but not inflammation, in patients with chronic hepatitis C. *J Clin Gastroenterol* 39:71–74
- Bergmann OM, Mathahs MM, Broadhurst KA et al (2008) Altered expression of iron regulatory genes in cirrhotic human livers: clues to the cause of hemosiderosis? *Lab Invest* 88:1349–1357. doi:10.1038/labinvest.2008.95
- Beutler E, Gelbart T, Lee P, Trevino R, Fernandez MA, Fairbanks VF (2000) Molecular characterization of a case of atransferrinemia. *Blood* 96:4071–4074
- Bridle KR, Frazer DM, Wilkins SJ, Dixon JL, Purdie DM, Crawford DHG et al (2003) Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homeostasis. *Lancet* 361:669–673. doi:10.1016/S0140-6736(03)12602-5
- Bridle KR, Cheung TK, Murphy TL, Walters MM, Anderson GJ, Crawford DHG et al (2006) Hepcidin is down-regulated in alcoholic liver injury: implications for the pathogenesis of alcoholic liver disease. *Alcohol Clin Exp Res* 30:106–112. doi:10.1111/j.1530-0277.2006.00002.x

- Courselaud B, Troade M-B, Fruchon S, Ilyin G, Borot N, Leroyer P et al (2004) Strain and gender modulate hepatic hepcidin 1 and 2 mRNA expression in mice. *Blood Cells Mol Dis* 32:283–289. doi:10.1016/j.bcmd.2003.11.003
- Detivaud L, Nemeth E, Boudjema K, Turlin B, Troade MB, Leroyer P et al (2005) Hepcidin levels in humans are correlated with hepatic iron stores, hemoglobin levels, and hepatic function. *Blood* 106:746–748. doi:10.1182/blood-2004-12-4855
- Deugnier YM, Charalambous P, Lequilleuc D, Turlin B, Searle J, Brissot P et al (1993) Preneoplastic significance of hepatic iron-free foci in genetic hemochromatosis: a study of 185 patients. *Hepatology* 18:1363–1369
- Fujita N, Sugimoto R, Takeo M, Urawa N, Mifuji R, Tanaka H et al (2007) Hepcidin expression in the liver: relatively low level in patients with chronic hepatitis C. *Mol Med* 13:97–104. doi:10.2119/2006-00057.Fujita
- Harris ZL, Takahashi Y, Miyajima H, Serizawa M, MacGillivray RT, Gitlin JD (1995) Aceruloplasminemia: molecular characterization of this disorder of iron metabolism. *Proc Natl Acad Sci USA* 92:2539–2543. doi:10.1073/pnas.92.7.2539
- Hayashi A, Wada Y, Suzuki T, Shimizu A (1993) Studies on familial hypotransferrinemia: unique clinical course and molecular pathology. *Am J Hum Genet* 53:201–213
- Hirohashi S, Ishak KG, Kojiro M, Wanless IR, Theise ND, Tsukuma H (2000) Hepatocellular carcinoma. In: Hamilton SR, Aaltonen LA et al (eds) World Health Organization classification of tumors, pathology and genetics of tumors of the digestive system. IARC Press, Lyon, pp 165–166
- Holmstrom P, Gafvels M, Eriksson LC, Dzikaite V, Hultcrantz R, Eggertsen G et al (2006) Expression of iron regulatory genes in a rat model of hepatocellular carcinoma. *Liver Int* 26:976–985. doi:10.1111/j.1478-3231.2006.01316.x
- Kijima H, Sawada T, Tomosugi N, Kubota K (2008) Expression of hepcidin mRNA is uniformly suppressed in hepatocellular carcinoma. *BMC Cancer* 8:167. doi:10.1186/1471-2407-8-167
- Kwok JC, Richardson DR (2002) The iron metabolism of neoplastic cells: alterations that facilitate proliferation? *Crit Rev Oncol Hematol* 42:65–78. doi:10.1016/S1040-8428(01)00213-X
- Loreal O, Turlin B, Pigeon C, Moisan A, Ropert M, Morice P et al (2002) Aceruloplasminemia: new clinical, pathophysiological and therapeutic insights. *J Hepatol* 36:851–856. doi:10.1016/S0168-8278(02)00042-9
- Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM et al (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 306:2090–2093. doi:10.1126/science.1104742
- Nicolas G, Bennoun M, Devaux I, Beaumont C, Grandchamp B, Kahn A et al (2001) Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proc Natl Acad Sci USA* 98:8780–8785. doi:10.1073/pnas.151179498
- Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I et al (2002) The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest* 110:1037–1044
- Ohtake T, Saito H, Hosoki Y, Inoue M, Miyoshi S, Suzuki Y et al (2007) Hepcidin is down-regulated in alcohol loading. *Alcohol Clin Exp Res* 31:2S–8S. doi:10.1111/j.1530-0277.2006.00279.x
- Pietrangelo A (2006) Hereditary hemochromatosis. *Annu Rev Nutr* 26:251–270. doi:10.1146/annurev.nutr.26.061505.111226
- Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P et al (2001) A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem* 276:7811–7819. doi:10.1074/jbc.M008923200
- Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J et al (2003) Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. *Nat Genet* 33:21–22. doi:10.1038/ng1053
- Roy CN, Mak HH, Akpan I, Losyev G, Zurakowski D, Andrews NC (2007) Hepcidin antimicrobial peptide transgenic mice exhibit features of the anemia of inflammation. *Blood* 109:4038–4044. doi:10.1182/blood-2006-10-051755
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3:1101–1108. doi:10.1038/nprot.2008.73
- Searle J, Leggett BA, Crawford DHG, Powell LW (2002) Iron storage diseases. In: MacSween RNM, Burt AD, Portman BC, Ishak KG, Scheuer PJ, Anthony PP (eds) Pathology of the liver, 4th edn. Churchill Livingstone, London, pp 257–272
- Tseng HH, Hwang YH, Yeh KT, Chang JG, Chen YL, Yu HS (2009) Reduced expression of C/EBPalpha protein in hepatocellular carcinoma is associated with advanced tumor stage and shortened patient survival. *J Cancer Res Clin Oncol* 135:241–247
- Weinstein DA, Roy CN, Fleming MD, Loda MF, Wolfsdorf JJ, Andrews NC (2002) Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. *Blood* 100:3776–3781. doi:10.1182/blood-2002-04-1260