Modulation of Iron-Regulatory Genes in Human Hepatocellular Carcinoma and Its Physiological Consequences

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Hepatocellular carcinoma (HCC) commonly develops in patients with underlying chronic liver disease. Additionally, the tumorous lesions of HCC patients are consistently characterized by the lack of iron accumulation even when arising in iron-loaded liver. However, the molecular mechanism leading to this observed phenomenon is currently poorly understood. In this study, all tumorous tissues from 24 HCC patients with chronic HBV infection were stained negative for iron when histologically assessed by Perls' Prussian blue stain, whereas excess iron deposits were present in 17 of the 24 adjacent non-tumorous liver tissues. To elucidate the concerted regulation of iron homeostasis in these patients, we studied the gene expression profiling of 42 relevant iron-regulatory genes in the tumorous and adjacent non-tumorous liver tissues of these HCC patients along with 10 normal liver controls. Expression for most of the iron-regulatory genes, including hepcidin, transferrin receptor 2 (TfR2), transferrin (Tf), ceruloplasmin (Cp) and iron regulatory protein 1 (IRP1), were significantly down-regulated in the tumorous tissues of these patients compared to the adjacent non-tumorous liver tissues and normal liver controls. On the other hand, expression of hepcidin, TfR2, ferroportin 1 and DMT1 were significantly up-regulated in iron-loaded non-cirrhotic non-tumorous liver tissues as compared with normal liver controls. Hence, the reduction of hepcidin expression within the iron-depleted tumorous lesions likely reflects the physiological consequence of the obligate demand for iron in the rapidly growing neoplastic cells, whereas the up-regulation of hepcidin expression in the iron-loaded adjacent non-tumorous liver tissues is likely a physiological response. Exp Biol Med 234:693–702, 2009

Key words: iron homeostasis; hepatocellular carcinoma; hepcidin; iron-regulatory genes; hepatic iron deposition

Introduction

Iron is an essential cofactor for a wide variety of vital cellular processes. However, free cellular iron has been postulated to be involved in cancer initiation through generating reactive oxygen species (ROS) which may induce DNA mutations (1). Furthermore, iron could promote cancer progression by being a mandatory cofactor for ribonucleotide reductase, an enzyme that is essential for DNA synthesis during cell proliferation (2).

The liver is the major site for storage of iron (3) and a central regulator of systemic iron homeostasis (4). Recent studies have revealed that the liver achieves its important role in intestinal iron absorption and iron release from sites of storage through secreting a peptide hormone named hepcidin (4). The observation of severe tissue iron overload in hepcidin-deficient mice (5) and severe iron deficiency in transgenic mice overexpressing hepcidin (6) further confirm its critical role in iron homeostasis. Importantly, nonsense mutations of the hepcidin gene have been reported for patients with severe juvenile hemochromatosis (7). Hereditary hemochromatosis (HH) is an inherited iron disorder characterized by excess iron deposits throughout the body, particularly liver tissues. In patients with HH, despite significant hepatic iron loading, expression of hepcidin could not be induced and further suggests that the

This work was supported by grants from the National Medical Research Council of Singapore (NMRC), Biomedical Research Council of Singapore (BMRC) and the Singapore Millennium Foundation (SMF).

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Received July 22, 2008.
Accepted February 23, 2009.

DOI: 10.3181/0807-RM-227
1535-3702/09/2346-0693$15.00

dysfunction of hepcidin is associated with hepatic iron overload (8, 9). Over the past decade, major advances have been made in defining the central role of hepcidin in orchestration of iron metabolism, and also providing a link between iron metabolism and inflammation and innate immunity (10).

Being the first organ that receives intestinal absorbed iron from the portal circulation, the liver is also a principal target for iron toxicity (3). It is well documented that in patients with HH there is a markedly increased risk of hepatocellular carcinoma (HCC) (11), indicating that excess iron could be considered as a potential carcinogen in the liver. A recent study in a murine model further demonstrated that the direct hepatocarcinogenic effect of free iron is mediated by ROS induction and oxidative damage (12). On the other hand, Turlin *et al*. and Chapoutot *et al*. independently demonstrated that iron deposition at the non-tumorous liver tissues of HCC patients were significantly increased when compared with matched controls with similar chronic liver disease but without HCC (13, 14), indicating that hepatic iron overload may be associated with HCC.

In general, neoplastic cells have an obligate demand for iron to accommodate their higher proliferation rate. Paradoxically, in HH patients who developed HCC and in HCC-bearing rats loaded with iron, the proliferative pre-neoplastic and neoplastic lesions lack iron accumulation in spite of iron loading in the adjacent non-tumorous liver tissues (15, 16). However, the underlying mechanism of this well-recognized histological anomaly of HCC is still unresolved.

In this retrospective study, all 24 HCC patients studied had associated underlying chronic HBV infection. We observed two independent histological phenomena in these patients: the prevalence of iron loading in the non-tumorous liver tissues, and the lack of iron accumulation in the tumorous lesions even when arising in the iron-loaded surrounding non-tumorous liver tissues. We investigated the regulation of iron homeostasis in these patients through gene expression profiling of all relevant iron-regulatory genes in the tumorous and adjacent non-tumorous liver tissues of these patients along with 10 normal liver controls, with the aim of elucidating the molecular mechanism for the lack of iron accumulation in HCC liver tissues.

Materials and Methods

Tissue Specimens. All tissue samples were provided by the National Cancer Centre Tissue Repository. Written informed consent was obtained from all patients. All the HCC patients studied had a chronic HBV infection without HH background, although the presence of HFE mutations was not studied. Samples were assigned consecutively for Affymetrix analysis and real-time RT-PCR. For Affymetrix analysis in conjunction with Perls' iron staining, 24 pairs of tumorous and adjacent non-tumorous liver tissues were surgically obtained from patients undergoing partial hepatectomy for treatment of HCC (20 males and 4 females; ages 56 ± 14). For real-time RT-PCR analysis, tumorous and adjacent non-tumorous liver tissues were obtained from an independent cohort of 13 HCC patients (11 males and 2 females; ages 62 ± 14). In addition, histologically normal liver controls were obtained from non-tumorous liver tissues resected from 10 patients with metastatic colorectal adenocarcinoma.

Affymetrix GeneChips. The global changes of gene expression in 24 pairs of tumorous and adjacent non-tumorous liver tissues from HCC patients, as well as 10 normal liver controls, were analyzed on HG-U133-A and -B GeneChips (Affymetrix) according to the manufacturer's recommended procedures (17). The statistical algorithms implemented in Affymetrix GCOS software were used for analysis. The average signal intensity for all the genes was normalized to 100. In a comparison expression analysis, each probe pair on the experimental array (tumorous tissues) was compared with the corresponding probe pair on the baseline array (paired non-tumorous tissues) and generated an associated 'Change' to determine the relative expression of transcripts (Increase, No Change, Decrease).

To monitor the expression changes of iron-regulatory genes, the signal intensity of a total of 42 genes that are either directly involved in iron metabolism or essential components constituted with iron, based on the literature search, were retrieved from Affymetrix GeneChips database. Other iron-related genes, for example hephaestin, lactotransferrin, IL6, BMP2 and BMP4 with an absent call (i.e. signal intensity less than 20) in over 70% of the samples studied as determined by the detection algorithm incorporated in the Affymetrix GCOS software, were excluded from our study. Furthermore, signal intensities were transformed to log2-base and imported to Partek Genomics Suite software (Partek Inc.) to perform principal component analysis (PCA), aimed at finding patterns in the data.

Real-Time RT-PCR. Total RNA was reverse transcribed using oligo(dT) primers with Superscript II reverse transcriptase (Invitrogen) and employed for real-time PCR. Quantification of hepcidin, TfR2 and β-actin expression was performed on a RotorGene real-time PCR machine (Corbett Research, Australia) using QuantiTech™ SYBR Green PCR kit (Qiagen). The sequences of primer sets were as follows: 5′-CCTGACCAGTGGCTCTGTTT-3′ and 5′-TGGGGCAGCAGGAATAAATA-3′ for hepcidin; 5′-ACCCCAGCACAGATATCCAC-3′ and 5′-CCTTTCACCACAGACCACCT-3′ for TfR2; 5′-ACTGGAACGGTGAAGGTGAC-3′ and 5′-AGAGAAGTGGGGTGGCTTTT-3′ for β-actin. Standard curves of each gene were generated independently by 10× serial dilution of template DNA. The relative copy number of each sample was calculated according to the corresponding standard curve using the software RotorGene version 4.6. Normalization was performed in each sample by dividing the copy number of hepcidin or TfR2 by that of β-actin. The relative expression levels were calculated by arbitrarily designating the lowest normalized value to 1.

Histological Evaluation and Semi-Quantitation of Hepatic Iron Content by Iron Staining. Paraffin-embedded tissue sections were stained with hematoxylin-eosin and Perls' Prussian blue. The degree of fibrosis was evaluated by a modified Knodell (Ishak) scoring system with a score of 0 to 6 (18). Iron deposition was assessed in tumorous and non-tumorous liver tissues away from the growing edge of the tumor. After considering the possible variation of the distribution of iron within the tissues, non-tumorous liver tissues in the same block containing the tumor as well in a separate block available from random non-tumorous liver tissues of the hepatectomy specimens were used in the assessment. The pattern and degree of iron deposition were assessed by a pathologist who performed the analyses without prior knowledge of the results obtained from gene expression profiling. The hepatic iron contents in the liver sections were graded according to Searle *et al*. (19). Iron deposition was semi-quantitatively graded on a scale of 0 to 4 based on the magnification at which iron granules were readily discernible: Grade 0 (absent), iron granules absent/barely discernible at ×400; Grade 1 (scarce), barely discernible at ×250 but easily discernible at ×400; Grade 2 (mild), discrete granules resolved at ×100; Grade 3 (moderate), discrete granules resolved at ×25; and Grade 4 (severe), massive iron granules visible at ×10 or with the naked eye.

Statistical Analysis. The statistical differences between groups were analyzed using Wilcoxon Signed-Ranks test for paired samples and Mann-Whitney U test for unpaired samples. χ^2 test was used to compare qualitative variables. Pearson's Correlation was employed to study the relationship between two continuous variables. Two-tailed P values of < 0.05 were considered to be statistically significant.

Results

Modulation of Iron-Regulatory Gene Expression in HCC. We employed Affymetrix GeneChips to identify differentially expressed genes between tumorous and adjacent non-tumorous liver tissues from 24 HCC patients associated with chronic HBV infection. Hepcidin gave the highest fold change among the down-regulated genes in HCC as determined by the ratio of median signal intensity of tumorous tissues to adjacent non-tumorous liver tissues (70 versus 2818 intensity units; $P < 0.0001$). This result was further confirmed by real-time RT-PCR using 13 independent pairs of HCC samples. Consistently, the median relative expression level of hepcidin in the tumorous tissues of HCC patients was significantly lower than that in the paired adjacent non-tumorous liver tissues (14 versus 3060 arbitrary units; $P = 0.0005$).

Since hepcidin is a principal hormonal regulator of iron homeostasis, its drastic reduction in HCC could bear potential physiological relevance especially with reference to the overall cellular iron status. We therefore specifically focused our subsequent analysis on the expression of the 42 iron-regulatory genes made available on Affymetrix gene chips for the 24 pairs of tumorous and adjacent non-tumorous liver tissues using Changes Algorithm, a method dedicated to the comparison of paired samples. These results are summarized in Table 1. The expression of 25 out of 42 iron-regulatory genes gave consistent modulation. Their expression was either increased or decreased in tumorous tissues in relationship to their corresponding paired non-tumorous liver tissues, in over 50% (12/24) of the HCC patients studied. Twenty of the differentially expressed iron-regulatory genes, including several key regulators such as hepcidin, transferrin receptor 2 (TfR2), transferrin (Tf), ceruloplasmin (Cp) and iron regulatory protein 1 (IRP1), were consistently reduced in expression in the tumorous tissues of patients with HCC as compared with adjacent non-tumorous liver tissues. In addition, the significant down-regulation of TfR2 expression in HCC (Table 1) was further validated by real-time RT-PCR using 13 independent pairs of HCC samples, and this demonstrated that the expression level of TfR2 in the tumorous tissues of HCC patients was significantly lower than that in the paired adjacent non-tumorous liver tissues (183 versus 863 arbitrary units; $P = 0.0002$).

On the other hand, the expression of ferritin H chain and ribonucleotide reductase (RRM2) was up-regulated in tumorous tissues, implicating the high demand for iron in proliferative neoplastic cells. We also detected the elevated expression of lipocalin-2 (LCN2), a non-transferrin-bound iron (NTBI) trafficking protein, in tumorous liver tissues (Table 1).

The identification of differentially expressed genes was based on the relative gene expression between two sample groups. To rule out the possibility that the observed reduced expression of the majority of the iron-regulatory genes in HCC was simply a consequence of the relative increase in gene expression of the non-tumorous liver tissues to iron loading or inflammatory stimuli, we employed normal liver controls as an additional baseline for determining the changes in gene expression between tumorous and non-tumorous liver tissues. Principal component analysis (PCA) on whole-genome expression profiling was employed to study the correlation between the 10 normal liver controls and 24 pairs of tumorous and non-tumorous liver tissues from HCC patients. The PCA scatter plot confirmed that the group of normal liver controls could be easily discriminated from the other two groups of liver tissues studied (Fig. 1A). Furthermore, the normal liver controls were more consistent in overall gene expression pattern as demonstrated by a smaller ellipse, as compared with the bigger ellipse observed in the other two groups showing a heterogeneous gene expression pattern among the samples (Fig. 1A). Using normal liver controls as a baseline for gene expression studies, we observed that among the 42 iron-regulatory genes studied, Tf and albumin were the only two with significantly reduced expression in non-tumorous liver

Table 1. Modulation of Iron-Regulatory Gene Expression in HCC

Affymetrix probe set ID[a]	Gene name	Iron-related diseases[b]	Patient no.[c]			Median[d]	
			I	D	NC	N	T
Regulation of iron absorption							
220491_at	Hepcidin (HAMP)	HH Type 2b[e]	0	26	1	2863	75
235754_at	Hemochromatosis (HFE)	HH Type 1[e]	2	0	25	127	146
228621_at	Hemojuvelin (HJV)	HH Type 2a[e]	11	8	8	1051	1320
201891_s_at	β2-microglobulin (B2M)	B2−/− mice[e]	1	11	15	5104	4068
Iron uptake							
210215_at	Transferrin receptor 2 (TfR2)	HH Type 3[e]	1	20	6	2464	1266
208691_at	Transferrin receptor 1 (TfR1)	TfR1 +/− mice	12	6	9	422	474
203124_s_at	Divalent metal ion transporter 1 (DMT1)	DMT1 defects[e]	6	4	17	201	201
222453_at	Cytochrome b reductase 1 (CYBRD1)		3	11	13	173	106
212110_at	Solute carrier family 39 (zinc transporter), member 14 (SLC39A14)		2	20	5	1933	1194
231667_at	Solute carrier family 39 (metal ion transporter), member 5 (SLC39A5)		0	17	10	250	25
218424_s_at	STEAP family member 3 (STEAP3)	Steap3 −/− mice	0	24	3	463	188
Iron export							
227253_at	Ceruloplasmin (CP)	Aceruloplasminemia[e]	1	17	9	4200	2723
223044_at	Ferroportin 1 (FPN)	HH Type 4[e]	4	9	14	3968	3469
Heme export							
222906_at	Feline leukemia virus subgroup C cellular receptor (FLVCR1)		14	0	13	68	115
Iron transport							
214063_s_at	Transferrin (Tf)	Atransferrinemia[e]	0	23	4	7860	5752
212531_at	Lipocalin 2 (LCN2)		18	1	8	44	118
Iron storage							
214211_at	H-ferritin (FTH1)	Fth +/− mice	15	2	10	354	439
213187_x_at	L-ferritin (FTL)	Hyperferritinemia-cataract syndrome	3	2	22	5789	4854
Gene regulation							
207071_s_at	Iron regulatory protein 1 (IRP1)		0	19	8	578	319
225892_at	Iron regulatory protein 2 (IRP2)	IRP2 −/− mice	7	1	19	267	337
204194_at	BTB and CNC homology 1 (BACH1)		16	0	11	69	116
Iron salvage							
39763_at	Hemopexin (HPX)		0	24	3	5376	2637
206697_s_at	Haptoglobin (HP)	Hp −/− mice	0	22	5	7725	4582
211298_s_at	Albumin (ALB)		0	19	8	7495	5784
208581_x_at	Metallothionein 1X (MT1X)		1	23	3	7748	3089
212185_x_at	Metallothionein 2A (MT2A)		1	24	2	10674	4290
203665_at	Heme oxygenase 1 (HMOX1)	Hmox 1 deficiency[e]	4	8	15	121	96
218121_at	Heme oxygenase 2 (HMOX2)		1	3	23	78	72
Mitochondrial iron metabolism							
205565_s_at	Frataxin (FXN)	Friedreich ataxia[e]	0	17	9	90	56
203646_at	Ferredoxin 1 (FDX1)		1	10	16	103	64
230069_at	Sideroflexin 1 (SFXN1)	f (flexed tail) mice	3	11	13	380	276
200793_s_at	Aconitase 2 (ACO2)		5	4	18	239	245
Heme synthesis							
205633_s_at	Aminolevulinate, delta-, synthase 1 (ALAS1)		4	22	1	1196	535
204788_s_at	Protoporphyrinogen oxidase (PPOX)	Variegate porphyria	11	0	16	77	97
DNA synthesis (iron containing)							
201477_s_at	Ribonucleotide reductase M1 polypeptide (RRM1)		11	0	16	120	160
201890_at	Ribonucleotide reductase M2 polypeptide (RRM2)		21	0	6	10	104

Table 1. Continued.

Affymetrix probe set ID[a]	Gene name	Iron-related diseases[b]	Patient no.[c]			Median[d]	
			I	D	NC	N	T
Oxygen transport (heme containing)							
211745_x_at	Hemoglobin, alpha 1 (HBA1)	Thalassemia[e]	1	24	2	1356	389
214414_x_at	Hemoglobin, alpha 2 (HBA2)	Thalassemia[e]	1	25	1	1342	347
211696_x_at	Hemoglobin, beta (HBB)	Thalassemia[e]	1	23	3	1165	383
204419_x_at	Hemoglobin, gamma G (HBG2)		0	7	20	70	53
Regulation of hepcidin expression							
214955_at	Transmembrane protease, serine 6 (TMPRSS6)	Iron-refractory iron deficiency anemia	1	15	11	392	198
221577_x_at	Growth differentiation factor 15 (GDF15)		8	13	6	151	118

[a] Affymetrix probe set ID with '_at' suffixes (unique probe sets) or the highest signal intensity are chosen for the representative 42 iron-regulatory genes.
[b] Iron-related diseases are those observed in human being or mouse model when loss of gene functions.
[c] Patient no. represents patient numbers in each category of gene changes: 'I' denotes 'Increase', 'D' denotes 'Decrease' and 'NC' denotes 'No Change' when comparing tumorous (T) to paired adjacent non-tumorous liver tissues (N) in 27 patients with HCC.
[d] The median of signal intensity levels of each gene are defined in non-tumorous liver tissues (N) and tumorous tissues (T).
[e] Diseases that associated with iron overload.

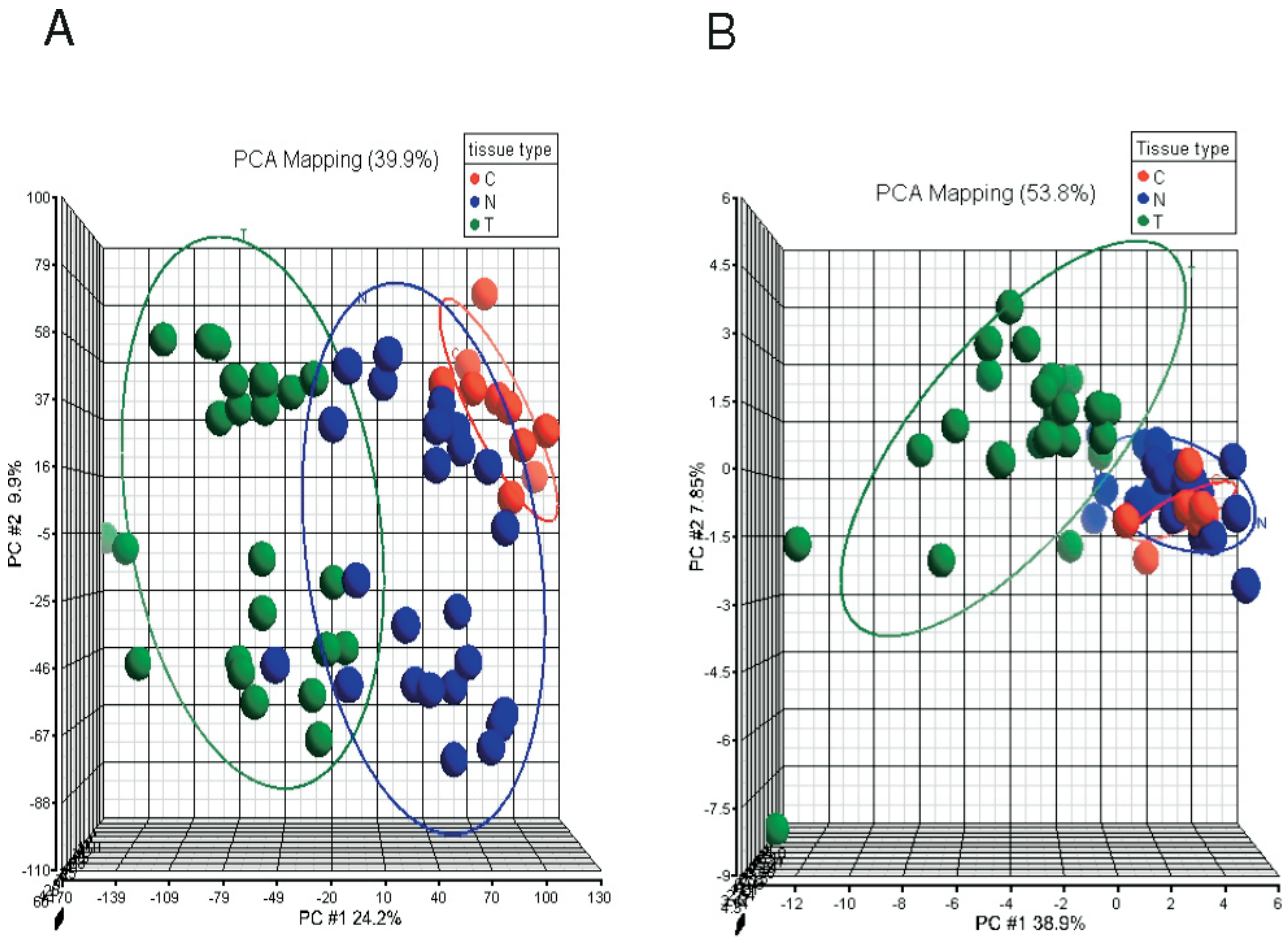

Figure 1. PCA scatter plot demonstrates the segregation of tumorous tissues from non-tumorous liver tissues and normal liver controls using (A) genome-wide gene expression profiles and (B) 42 iron-regulatory genes alone. Each object represents a sample which is colored by green for tumorous tissue (T, $n=24$), blue for paired non-tumorous tissues (N, $n=24$) and red for normal liver controls (C, $n=10$). Ellipsoids are drawn to look at the distribution in each tissue group. A color version of this figure is available in the online journal.

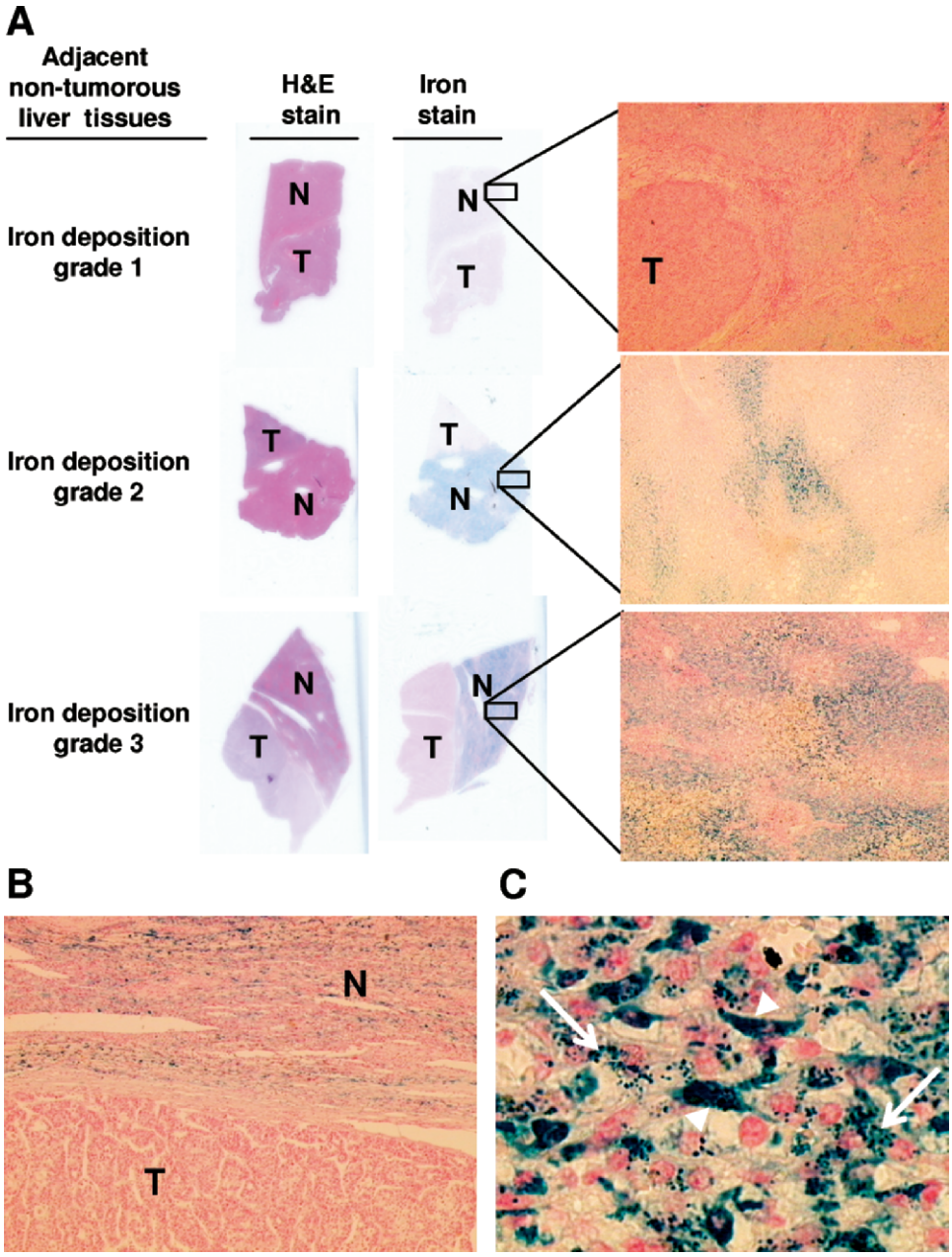

Figure 2. Perls' iron staining reveals iron-free in tumorous tissues and excessive iron deposits in the non-tumorous liver tissues in patients with HCC. (A) Slides contained consecutive liver sections of tumorous lesions (T) and adjacent non-tumorous liver tissues (N) are stained with Perls' and H&E stain as observed with the naked eye. Representative adjacent non-tumorous liver tissues with iron deposition of grade 1, 2 and 3, respectively, are shown (×25). Blue staining shows iron deposits. (B) The interphase between iron-free tumorous lesion (T) and adjacent non-tumorous liver tissues with iron deposits (N) (×100). (C) Grade 3 iron depositions in non-tumorous liver tissues. Arrows indicate the accumulation of iron in hepatocytes; arrowheads indicate the persistence of iron deposits in Kupffer cells (×400). A color version of this figure is available in the online journal.

tissues of HCC patients, most likely associated with the underlying chronic HBV infection. We also confirmed that the expression of the majority of these iron-regulatory genes was significantly reduced in the tumorous tissues of HCC patients as compared with normal liver controls.

For the 42 iron-regulatory genes, we were able to discriminate tumorous tissues from adjacent non-tumorous liver tissues and the normal liver controls by PCA (Fig. 1B). The three dimensional PCA scatter plot shows that the green ellipse representing the distribution of tumorous tissues is well segregated from the other two ellipses (Fig. 1B), indicating that the expression of these iron-regulatory genes is significantly different in the HCC tumorous tissues. The more scattered distribution of the tumorous liver tissues could reflect the heterogeneity of expression of the iron-regulatory genes in these samples. The distribution of non-tumorous tissues of HCC patients (blue ellipse) and normal liver controls (red ellipse) is indistinguishable (Fig. 1B),

Table 2. Univariate Analysis of Clinical Characteristics of 24 HCC Patients Related with Iron Deposition Grading of Their Non-Tumorous Liver Tissues

Patient characteristics	Iron deposition in non-tumorous liver			
	Grade 0–1	Grade 2	Grade 3	*P* value[a]
Number of patients	7	10	7	NS
Age (yrs) (mean [SD])	62 [11]	54 [15]	53 [15]	NS
Sex (F/M)	2/5	1/9	1/6	NS
Histology				0.0185*
Fibrosis stage 1–2	4	2	6	
Fibrosis stage 3–4	0	0	1	
Fibrosis stage 5–6	3	8	0	
Tumor grade				NS
Well differentiated (G1)	1	0	2	
Moderate differentiated (G2)	4	7	3	
Poorly differentiated (G3)	2	3	2	
Tumor diameter[b]				NS
<2 cm	0	3	0	
2–5 cm	4	4	2	
>5 cm	3	3	5	

[a] χ^2 test is applied with *P* value of 0.05 as significant. NS, not significant.
[b] Tumor size is defined as the largest diameter of the tumor.
* $P < 0.05$.

suggesting that the expression of the iron-regulatory genes in the non-tumorous liver tissues is comparable to the normal liver controls. However, the presence of a longer axis found in the blue ellipse indicates the likely modulation of the expression of some of the iron-regulatory genes in the non-tumorous liver tissues.

Iron Staining Revealed a Lack of Iron Accumulation in Tumorous Tissues and Excessive Iron Deposits in the Non-Tumorous Liver Tissues. To ascertain whether the modulation of the expression of iron-regulatory genes in HCC bears potential physiological relevance, we performed iron staining in the corresponding liver sections containing tumorous and surrounding non-tumorous tissues. Semi-quantitation of hepatic iron content was assessed by a pathologist without prior knowledge of the molecular data. All tumorous tissues (T) were stained negative for iron (Fig. 2A and B), whereas different degrees of iron deposition (Iron Grade 0–1 to 3) were detected at the adjacent non-tumorous liver tissues (Fig. 2A). Iron deposits in the non-cirrhotic, non-tumorous liver tissues, which appeared as blue granules, were found predominantly in hepatocytes and to a lesser extent in Kupffer cells (Fig. 2C). Further examination revealed that 60% to 95% of the iron deposits detected were found within the hepatocytes. Additionally, histological examination revealed that iron deposition is distributed around the periportal regions of the liver with a decreasing intensity towards the centrilobular regions.

In summary, non-tumorous liver tissues of 7 patients with grade 0–1 of iron deposition were considered as physiologically normal, without iron loading (Table 2). For the remaining 17 patients with excess iron deposition at the non-tumorous liver tissues, 10 were mild (Iron Grade 2) and 7 were moderate (Iron Grade 3) iron deposition (Table 2). There was no significant association between the grade of iron deposition in the non-tumorous liver tissue and age, gender, grading of tumor, and size of tumor of HCC patients studied. Interestingly, we observed that all non-tumorous liver tissues with grade 3 iron deposition (7/7) were scored with fibrosis stage 1–4, showing non-cirrhosis. In comparison, 80% (8/10) of non-tumorous tissues examined with grade 2 of iron deposition were scored with either fibrosis stage 5 (incomplete cirrhosis) or stage 6 (cirrhosis) (Table 2). These observations indicate that liver cirrhosis could be a confounding factor in hepatic iron deposition.

Significantly Increased Expression of Hepcidin, TfR2, Ferroportin 1 and DMT1 in Iron-Loaded Adjacent Non-Cirrhotic, Non-Tumorous Liver Tissues when Compared with Normal Liver Controls. To investigate whether the non-tumorous liver tissues of HCC patients could actively respond to the iron burden, we took cirrhosis as a possible confounding factor. However, after grouping the non-tumorous liver tissues into cirrhosis and non-cirrhosis, the sample size of those HCC patients without iron deposition was too small to perform a statistically meaningful comparison (3 for cirrhosis and 4 for non-cirrhosis, Table 2). Hence, we employed normal liver tissues as a baseline to monitor the modulation of iron-regulatory genes for the iron-loaded, non-cirrhotic, non-tumorous liver tissues. Out of the 42 iron-regulatory genes studied, the expression of hepcidin, TfR2, ferroportin 1 and DMT1 was significantly increased, whereas Tf and albumin were significantly reduced in the iron-loaded non-cirrhotic liver tissues as compared with normal liver controls (Fig. 3).

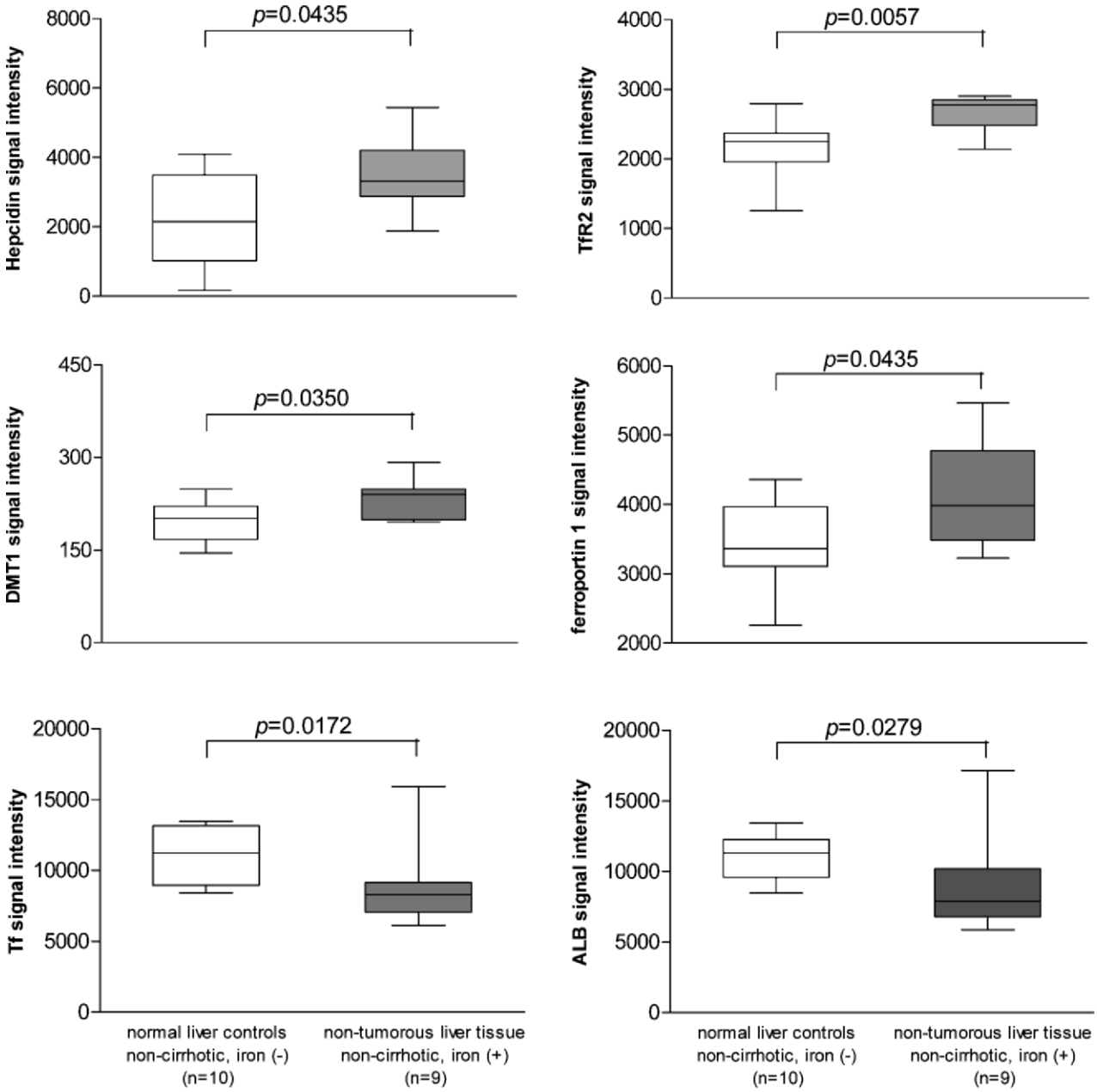

Figure 3. Box plots show the comparison of hepcidin, TfR2, ferroportin 1, DMT1, Tf and albumin gene expression between iron-loaded adjacent non-cirrhotic, non-tumorous liver tissues ($n = 9$) and normal liver controls ($n = 10$). The horizontal line located in the box shows the median value. The top and bottom lines of the box are 25th and 75th percentiles, respectively; approximately 50% of the values fall within this range. The whiskers show the highest and lowest values not considered outliers (extreme values). $P < 0.05$ was considered to be statistically significant as determined by Mann-Whitney *U* test.

Discussion

We have studied a group of HCC patients with chronic HBV infection, a significant risk factor for hepatocarcinogenesis in Singapore (20). In this study, we employed histological normal liver tissues resected from patients with hepatic metastasis of colorectal cancer as additional normal liver controls for our molecular analysis. Using a genome-wide gene expression profiling approach, non-tumorous liver tissues from HCC patients could be discriminated from the normal liver controls (Fig. 1A). The differential expression was likely to be associated with the presence of underlying chronic liver diseases in the HCC patients that was absent in the control group. In support of this suggestion, we were able to demonstrate the significant down-regulation of Tf and albumin gene expression in the non-tumorous liver tissues when compared to normal liver controls. Our data are consistent with the report of low serum Tf detected in HBV carriers and HCC patients with chronic HBV infection when compared with healthy controls (21).

HCC often arises in individuals with underlying

chronic liver disease. Hence, the prevalence of iron loading in the non-tumorous liver tissues of HCC patients is also believed to be associated with the underlying chronic liver disease. Several studies have indeed shown that chronic HBV infection is associated with mild iron deposition in the liver (22, 23). Patients with chronic HCV infection were also reported to have more severe hepatic iron deposition than those with chronic HBV infection (24).

In this study, we observed the marked reduction of many important iron-regulatory genes within the tumorous liver tissues of HCC patients. This could partially reflect the loss of differentiated hepatocyte function since hepatocytes play major roles in iron metabolism. Dysfunction of key regulators of iron homeostasis, including hepcidin, TfR2, Tf and CP, has also been linked to HH and/or hepatic iron overload (25, 26). It is therefore likely that the presence of tumor in patients with chronic HBV infection could further deteriorate the overall liver functions, particularly relating to iron homeostasis, and could progressively result in iron accumulation in the non-tumorous liver tissues. Chapoutot *et al*. demonstrated that hepatic iron deposits are more severe and common in HCC patients with chronic HCV infection (14) and further suggested that progression of iron loading could be associated with HCC. This report is similar to our present observation when HCC developed in patients with chronic HBV infection. We observed a comparatively more frequent and higher grade of iron deposition in the non-tumorous liver tissues. Additionally, we demonstrated that iron deposits are more prevalent within the periportal hepatocytes of the non-tumorous liver tissues, indicating that intestinal absorption of dietary iron was excessive in these HCC patients. However, the importance of this observation is unclear, given that hepatic iron content does not significantly correlate with tumor mass and the impact of the reduction in expression of iron regulators in a focal lesion may not be pronounced enough to have a physiological impact.

The lack of iron accumulation is an early feature of hepatocarcinogenesis (15). Through gene profiling expression studies, we observed the induction of TfR1 protein and suppression of ferritin-L protein in the HCC tumorous lesions (27). We suggest that neoplastic cells need to secure an adequate supply of iron for proliferation and metabolism but demand may outpace supply. We observed increased expression of ribonucleotide reductase (RRM2), which is an essential enzyme for cell proliferation in tumorous tissues (Table 1). The up-regulation of ferritin-H may reflect an increased demand for ferroxidase activity to ensure adequate supply of bioavailable iron. Interestingly, the expression of TFR1 was significantly and negatively correlated with TFR2 expression in the tumorous tissues (Pearson correlation, $r = 0.4877$, $P = 0.0156$), suggesting that the uptake of iron in cancer cells might have shifted to a more efficient pathway involving TFR1, a receptor with 25-fold higher affinity for transferrin than TFR2 (28). Therefore, TFR1 might be able to compensate for the down-regulation of TFR2 in HCC and provide sufficient iron for proliferation. Consistent with reports by Holmström *et al*. (29) and Kijima *et al*. (30), we observed a drastic reduction in hepcidin expression within the iron-depleted tumorous lesions and this could have physiological consequences resulting from the high proliferation rate of the tumor cells outpacing the availability of iron. The transporters responsible for NTBI uptake, namely Slc39A14 and Slc39A5, were also significantly down-regulated in tumorous tissue compared to the non-tumorous liver tissues and normal liver controls (Table 1). This may also help to explain the lack of iron accumulation in tumor lesions since NTBI could facilitate hepatic iron deposition.

Although the reason for the presence of excess iron in the non-tumorous liver tissues of HCC patients remains uncertain, we observed that hepcidin, TfR2, ferroportin 1 and DMT1 expression were up-regulated in iron-loaded non-cirrhotic, non-tumorous liver tissues when compared with normal liver controls (Fig. 3). This is consistent with the upregulation of hepatic hepcidin, TfR2 and ferroportin 1 mRNA in patients with chronic HCV infection and positively correlates with the hepatic iron concentration (31, 32). This is also supported by the observation of increased hepatic hepcidin expression in a mouse model with experimental iron overload (33). Contrary to several reports showing the lack of adaptive up-regulation of hepcidin in response to the significant primary hepatic iron overload in HH patients and murine models that mimic hemochromatosis (8, 34–36), we find that the liver tissues of HCC patients can still respond appropriately to the hepatic iron burden as demonstrated by the up-regulation of hepcidin in the non-tumorous liver tissues in response to increased iron storage.

In conclusion, the reduction of hepcidin expression within the iron-depleted tumorous lesions possibly reflects the physiological consequences of the obligate demand for iron in the rapidly proliferating neoplastic cells, while the up-regulation of hepcidin expression in the adjacent non-tumorous liver tissues is likely a physiological response to iron loading. However, free iron, being a catalyst for the formation of highly toxic radicals, could induce lipid peroxidation (25) and lead to tissue damage and liver failure. Hence, we propose that therapeutic strategies based on chelation of iron may potentially inhibit the deterioration of liver function in patients with HCC and that the assessment of hepatic iron deposition should be included as a routine test for patients with HCC, regardless of the etiologic factors.

We acknowledge the contribution of patient tissues for this study from the National Cancer Centre Tissue Repository.

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