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ORIGINAL ARTICLE

Up-regulation of dbpA mRNA in hepatocellular carcinoma associated with metabolic syndrome

Gulanbar Obulhasim · Mahmut Yasen · Kazunori Kajino · Kaoru Mogushi · Shinji Tanaka · Hiroshi Mizushima · Hiroshi Tanaka · Shigeki Arii · Okio Hino

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Abstract
Purpose Metabolic syndrome (MS) is a group of recognized risk factors for the development of hepatocellular carcinoma (HCC) in patients with chronic liver disease. The aim of this study was to analyze the clinicopathological characteristics of HCC patients with MS and the risk factors for recurrence. Also, the aim was to investigate the cold shock protein: DNA-binding protein A (dbpA) expression in HCC patients with MS.
Methods A total of 243 patients who underwent curative resections for HCC were classified into two groups. dbpA expression was investigated in 66 HCC patients with MS and in 30 patients without MS by using real-time RT-PCR. Promoter methylation status was examined by using MS-PCR.
Results The incidence of metabolic factors affect the HCC significantly higher in non-B non-C patients than in hepatitis B virus (HBV) or hepatitis C virus (HCV) patients ($P < 0.001$). Univariate analysis of HCC patients with MS recurrence revealed aspartate amino transferase (AST), multiple tumors, liver damage, hepatic vein invasion, advanced cancer stages ($P < 0.01$), alpha-fetoprotein (AFP) and diabetes mellitus type II ($P < 0.05$) as risk factors. Multivariate analysis, AST, multiple tumors, and hepatic vein invasion ($P < 0.01$) were identified as independent factors for the recurrence. dbpA mRNA was higher in patients with MS than in those without MS ($P = 0.016$), and it was mostly upregulated in non-B non-C HCC patients with MS than in non-B non-C HCC patients without HBV or HCV. Especially, in HCC patients with diabetes mellitus type II, the mRNA and protein levels were highly upregulated. The dbpA expression was regulated by promoter methylation status ($P < 0.05$).
Conclusions This study identifies that dbpA may accelerate the hepatocarcinogenesis in HCC patients with MS via inflammation-induced and oxidative stress pathways. The demethylation-related epigenetic activation may be one of the regulating factors for HCC patients with MS.

Keywords Hepatocellular carcinoma · Metabolic syndrome · dbpA expression · Oxidative stress

G. Obulhasim · K. Kajino · O. Hino
Department of Pathology and Oncology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

G. Obulhasim · M. Yasen
Department of Surgery, Xinjiang Uyghur Tumor Hospital of Xinjiang Medical University, 30 Beijing Rood, Urumqi 830011, Xinjiang, China

M. Yasen (✉) · S. Tanaka · S. Arii
Department of Hepato-Biliary-Pancreatic Surgery, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan
e-mail: mahmut@bioinfo.tmd.ac.jp

K. Mogushi · H. Mizushima · H. Tanaka
Department of Computational Biology and Bioinformatics, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common tumor worldwide with an increasing incidence and the third leading cause of cancer-related death [1, 2]. Although many risk factors of HCC are well defined, including hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol, and nonalcoholic fatty liver disease (NAFLD), a hepatic manifestation of the metabolic syndrome (MS) is also a newly identified risk factor in chronic liver disease (CLD)

and HCC [3, 4]. Indeed, obesity and diabetes are two conditions clearly associated with the development of NAFLD, which is currently recognized as one of the leading causes of CLD.

Recent studies have shown that patients with cryptogenic CLD frequently have risk factors for NAFLD and metabolic steatosis [5–8]. Metabolic steatosis or NAFLD is the most common cause of chronic liver injury in worldwide countries. Histological signs of necroinflammation, indicating the presence of nonalcoholic steatohepatitis (NASH), are present in 20–30% of cases, while steatosis on its own has a benign course. Recent reports have shown that while ~40% were related to overweight or obesity, 20% were related to diabetes mellitus (DM) and another 20% to hyperlipidemia [9, 10]. NASH could also progress to hepatic fibrosis or even cirrhosis; also, it had been reported to develop into HCC [11–13].

Hepatocellular carcinoma is one of the most important diseases in patients with CLD. Also, some epidemiological studies have reported that obesity and DM are risk factors for HCC. Furthermore, hepatic steatosis is a risk factor for HCC in patients with chronic virus infection as well. The association of metabolic syndrome, which is a series of conditions including insulin resistance obesity, hypertension, and hyperlipidemia, with malignance attracted increasing attention. As an inevitable consequence of insulin resistance, hyperinsulinemia plays an important role in the occurrence and prognosis of cancer. However, the molecular mechanisms of hepatocarcinogenesis remain unknown.

Previously, we reported DNA-binding protein A (dbpA) as a candidate molecule that can accelerate the process of the inflammation-induced hepatocarcinogenesis [14–17]. dbpA is a member of Y-box binding protein family and the DNA-binding domain, called the cold shock domain, about 70 amino acid residues, which is highly conserved from bacteria to humans [18]. dbpA was identified as the protein binding to the epidermal growth factor receptor enhancer or c-erbB-2 promoter [19]. dbpA expression was associated with advanced stages of human HCC [20]. However, no study was conducted to investigate the relationship between dbpA expression and MS-associated HCC. In the present study, we analyze the clinicopathological characteristics of HCC patients with MS and the risk factor for recurrence. We also investigate the dbpA expression and methylation status in HCC patients with or without MS high-risk factors.

Methods

Study population and samples

We analyzed 243 patients with primary HCC. All patients who had undergone surgical resection in the Department of Hepato-Biliary-Pancreatic Surgery at the Tokyo Medical and Dental University Hospital between April 2000 and March 2008 were analyzed. Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. A total of 334 liver specimens including 167 pairs of tumor and corresponding nontumor tissues were snap frozen in liquid nitrogen and then stored at −80 °C for DNA/RNA analysis. A part of the tissue sample was fixed in formalin and embedded in paraffin for histological diagnosis. Histological diagnosis was made when two pathologists specialised in liver disease reached the same consensus. The hepatitis virus status of patients with HCC was as follows: 39 patients were positive for HBV surface antigen, 129 were positive for HCV antibody, and 75 were negative for both. In addition, we collected the main metabolic risk factors, including DM (plasma glucose ≥126 mg/dl), obesity [body mass index (BMI) ≥25 kg/m^2], dyslipidemia (triglycerides ≥150 mg/dl or high-density lipoprotein cholesterol <40 mg/dl in males or <50 mg/dl in females), and hypertension (blood pressure >140/90 mmHg). Metabolic aggregate in this study was defined as the presence of two or more of these components: high glucose, high blood pressure, high triglycerides, low high-density lipoprotein cholesterol, and overweight, or the presence of any of the following factors—DM, hypertension, and dyslipidemia, in addition to being overweight. Among them, we defined two groups of 243 HCC patients, presence of any of MS factors (MS group, $n = 147$) and no identified risk factors (CG group, $n = 96$). The details of the clinicopathological data from the patients, classified by MS status, are listed in Table 1.

Expression analysis

Total RNA was extracted from 192 liver specimens using RNeasy Mini kit (Qiagen, Valencia, CA, USA; Hilden, Germany) and treated with RNase-free DNase I according to the manufacturer's instructions. Integrity of the obtained RNA was assessed using Agilent Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). All samples had an RNA integrity number (RIN) >5.0. Gene expression was performed by using quantitative real-time PCR (qRT-PCR). In brief, 2 μg of total RNA from tissues was reverse transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit's (Applied Biosytems) random primer according to the manufacturer's directions. Quantitative PCR was performed using the SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on the AB 7500 Fast Real-Time PCR System (Applied Biosystems). The primer and PCR product sizes are listed in Table 2. Quantitative analysis of specific

Table 1 Clinicopathological features of 243 patients with primary HCC according to with or without MS risk factors

Clinicopathologic factors	MS group ($n = 147$)	CG group ($n = 96$)	P value
Age in years (mean ± SD)	67.5 ± 7.99	64.8 ± 9.34	0.017*
Gender (male:female)	116:31	62:34	0.018*
Viral infection (HBV:HCV:NBNC)	14:75:58	25:54:17	<0.001*
Liver damage (A:B:C)	112:31:4	60:35:1	0.025*
AST (IU/L, mean ± SD)	52.9 ± 36.3	58.5 ± 33.5	0.235
ALT (IU/L, mean ± SD)	50.2 ± 39.1	52.9 ± 37.1	0.588
Plt ($\times 10^9$/L, mean ± SD)	15.7 ± 9.26	14.4 ± 7.74	0.266
ICG-R15 (%, mean ± SD)	18.6 ± 11.8	19.9 ± 11.9	0.414
PT% (mean ± SD)	85.2 ± 11.4	80.4 ± 13.2	0.003*
T.bil (mg/dl, mean ± SD)	0.88 ± 0.43	0.98 ± 0.76	0.207
Alb (g/dl, mean ± SD)	3.80 ± 0.47	3.71 ± 0.51	0.127
AFP (ng/ml, log10)	1.51 ± 0.99	1.96 ± 1.20	0.002*
PIVKA-II (mAU/ml, log10)	2.19 ± 1.03	2.26 ± 1.04	0.636
Tumor max. size (mean ± SD)	4.55 ± 3.03	4.71 ± 4.26	0.733
Solitary or multiple	102:45	68:28	0.887
Capsular formation (pfc)(−:+)	37:110	23:73	0.880
Capsular invasion (pfc-inf) (−:+)	78:69	41:55	0.118
Portal vein invasion (pvp) (−:+)	100:47	60:36	0.408
Hepatic vein invasion (pvv) (−:+)	129:18	86:10	0.838
Vascular invasion (pvp/pvv) (−:+)	93:54	57:39	0.590
Recurrence (absence:presence)	82:65	53:43	1.000
Stages (I:II:III:IV)	16:59:55:17	15:41:22:18	0.125
Background of liver (CH:LC:NL)	74:67:6	38:52:6	0.236
Alcohol consumption (%)	59 (40.1)	33 (34.4)	0.418
Body mass index (kg/m^2)	23.7 ± 3.74	22.6 ± 3.28	0.029*
Diabetes mellitus type II (%)	67 (45.5)	0 (0.0)	<0.001*
Hypertension (%)	108 (73.5)	0 (0.0)	<0.001*
Dyslipidemia (%)	44 (29.9)	0 (0.0)	<0.001*

AST aspartate amino transferase, *ALT* alanine amino transferase, *PLT* platelet, *ICG-R15* indocyanine green retention rate at 15 min, *PT%* prothrombin time, *T.bil* total bilirubin, *Alb* albumin, *AFP* α-fetoprotein, *PIVKAII* protein induced by vitamin K absence or antagonists II, + positive, − negative

* Statistical significance

mRNA expression was performed. The C_T values were calculated using the 7500 SDS software. The level of mRNA for each sample was calculated. The expression of *dbpA* gene was normalized with the expression of control gene and the fold difference between tumor and corresponding nontumor tissue was calculated by using the ΔΔCT method (Applied Biosystems User Bulletin #2, 1997).

Methylation-specific PCR

Total genomic DNA was extracted from 122 frozen liver tissues by using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA; Hilden, Germany). Bisulfite modification of the extracted DNA (2 μg) was performed by using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The methylation status of the dbpA promoter was detected in 61 pairs of HCC and corresponding nontumor liver tissues by using the methylation-specific PCR methods according to Herman et al. [21]

Immunohistochemistry staining

Four-micrometer-thick tissue sections were prepared from 61 HCC patients' formalin-fixed paraffin-embedded specimens. The tissue sections were treated with the primary antibody and antirabbit polyclonal dbpA (1 μg/ml) in phosphate-buffered saline containing 1% bovine serum albumin. Tissue sections were then stained with an automated immunostainer (BenchMark® XT; Ventana Medical Systems, Tucson, AZ, USA) by using heat-induced epitope

Table 2 Primer sets for quantitative real-time reverse transcription PCR and methylation-specific PCR

Gene symbol	Primer sequence	Product size (bp)
dbpA	qRT: ATGGAGTTCCTGTGGAAGGGAGTCG (S)	171
	qRT: CAGAGAACTGCCTATCAGTGGCAGG (AS)	
	M1: GTTTTGTAAGCGATTCGC (S)	174
	M1: AAATTTTTCTAAACGACGCA (AS)	
	UM1: TTGGTTTTGTAAGTGATTTGT (S)	174
	UM1: AAATTTTTCTAAACAACACACCA (AS)	
	M2: AGCGAGGAGTTTAAGGAGC (S)	170
	M2: TCGATAACGATTAATCGACG (AS)	
	UM2: GAGTGAGGAGTTTAAGGAGT (S)	170
	UM2: CTCAATAACAATTAATCAACA (AS)	
GAPDH	qRT: AGCCACATCGCTCAGACA (S)	120
	qRT: GCCCAATACGACCAAATCC (AS)	

GAPDH glyceraldehyde-3-phosphate dehydrogenase, *S* sense primer, *AS* antisense primer, *qRT-PCR* quantitative real-time reverse transcription PCR, *M* methylation, *U* unmethylation

retrieval and a standard diaminobenzidine detection kit (Ventana). Staining was evaluated by two independent observers and interpreted to be positive when >10% of tumor cells showed the positive signal.

Statistical analysis

Statistical comparisons of the clinicopathological characteristics for significance were performed by the χ^2 test or Fisher's exact test. To investigate those factors that predicted recurrence-free survival, univariate and multivariate analyses were performed by Cox proportional hazard models. Differences in dbpA mRNA levels between groups and the association between methylation states were analyzed by using the Student t test and Kruskal–Wallis test. Data were expressed as the mean ± standard deviation. Fisher's exact test was performed to estimate the association between gene expression and methylation status in each group. A P value of <0.05 was considered statistically significant. Statistical analysis was performed with PASW Statistics 18 software for Windows (IBM).

Results

Clinicopathological characteristics and risk factors of recurrence in HCC patients with MS

A total of 243 patients were included in the study. Two groups of subjects were compared. All patients with HCC were analyzed, including 45.5% with DM type II, 73.5% with hypertension, and 29.9% with dyslipidemia. The MS group was composed of 147 patients (116 males and 31 females), with a mean age of 67.5 ± 7.99 years. In the group of patients with HCC related to MS risk factors, the etiology was related to HBV in 14 patients (9.5%), HCV infection in 75 patients (51%), and non-B non-C infection in 58 patients (39.5%). Similarly, the CG group was 26% with HBV, 56% with HCV, and 17.7% with non-B non-C ($P < 0.001$). Serum AFP was lower in the MS group than in the CG group ($P = 0.002$). There was no statistical difference between the MS and CG groups in other biological and pathological factors (Table 1).

We were able to follow the postoperative course for all patients. The follow-up period until death or the end point of this study was 4–2,623 days (median 687 days). In the MS group, 82 patients survived without recurrence (median 439 days) and 65 patients survived with recurrence (median 273 days) of HCC within the follow-up period ($P = 0.0099$). In CG group, 53 patients survived without recurrence (median 211 days) and 43 patients with recurrence (median 286 days) of HCC within the follow-up period ($P = 0.363$). We examined the association between clinicopathological factors and recurrence-free survivals of patients with MS. Univariate Cox regression analysis demonstrated that the risk factors of recurrence were revealed as virus infection, liver damage, aspartate amino transferase (AST), alanine amino transferase (ALT), prothrombin time (PT%), albumin (Alb), alpha-fetoprotein (AFP), multiple tumors, hepatic vein invasion, advanced cancer stages, and DM type II. According to Cox hazard multivariate analysis, AST ($P = 0.005$), multiple tumors ($P < 0.001$), hepatic vein invasion ($P = 0.006$), and liver damage ($P = 0.048$) were identified as independent factors for recurrence of HCC with MS (Table 3).

dbpA mRNA levels upregulated in non-B non-C associated HCC patients with MS

We investigated the mRNA expression levels of dbpA on 66 pairs of HCC patients with MS and 30 pairs of HCC patients without MS. With the hepatic background, there were 15 HBV hepatitis, 41 HCV hepatitis, and 40 non-B and non-C patients. In the MS group patients, 6% were

Table 3 Univariate and multivariate analysis of the recurrence-free survival of 147 HCC patients with MS

Clinicopathologic factors	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age in years (mean ± SD)	1.020 (0.990–1.052)	0.196		
Gender (male:female)	0.920 (0.515–1.643)	0.778		
Viral infection (NBNV:HBV + HCV)	0.534 (0.314–0.909)	0.021*	0.782 (0.424–1.442)	0.431
Background of liver		0.686		
Normal liver	NA			
Chronic hepatitis	1.371 (0.328–5.728)			
Liver cirrhosis	1.626 (0.388–6.811)			
Liver damage		<0.001*		0.048*
A	0.441 (0.136–1.436)		0.406 (0.073–2.255)	
B	1.234 (0.365–4.167)		1.173 (0.282–4.890)	
AST (IU/L, mean ± SD)	1.015 (1.009–1.020)	<0.001*	1.013 (1.004–1.021)	0.005*
ALT (IU/L, mean ± SD)	1.009 (1.003–1.015)	0.001*	0.997 (0.988–1.006)	0.553
Plt ($\times 10^9$/L, mean ± SD)	0.998 (0.972–1.025)	0.880		
ICG-R15 (%, mean ± SD)	1.011 (0.994–1.029)	0.213		
PT% (mean ± SD)	0.976 (0.954–0.999)	0.038*	0.997 (0.963–1.032)	0.857
T.bil (mg/dl, mean ± SD)	1.465 (0.865–2.482)	0.155		
Alb (g/dl, mean ± SD)	0.541 (0.327–0.897)	0.017*	1.211 (0.554–2.647)	0.631
AFP, ng/ml (≥20 vs. <20)	0.531 (0.324–0.870)	0.012*	0.841 (0.457–1.546)	0.577
AFP, ng/ml (≥100 vs. <100)	0.694 (0.404–1.194)	0.187		
PIVKA-II, mAU/ml (≥100 vs. <100)	0.827 (0.502–1.361)	0.454		
Tumor max size (cm, mean ± SD)	1.060 (0.981–1.146)	0.141		
No. of tumors (solitary vs. multiple)	3.540 (2.152–5.824)	<0.001*	3.648 (2.006–6.632)	<0.001*
Capsular formation (pfc) (−: +)	0.978 (0.557–1.716)	0.937		
Capsular invasion (pfc-inf) (−: +)	0.821 (0.501–1.344)	0.432		
Severe portal vein invasion (pvp)	0.549 (0.211–1.428)	0.219		
Hepatic vein invasion (pvv) (−: +)	0.388 (0.200–0.750)	0.005*	0.297 (0.126–0.700)	0.006*
Vascular invasion (pvp/pvv) (−: +)	0.710 (0.431–1.169)	0.178		
TNM stages (I/II vs. III/IV)	0.493 (0.298–0.815)	0.006*	1.077 (0.568–2.042)	0.82
Alcohol consumption	1.136 (0.689–1.874)	0.617		
Body mass index (kg/m^2)	0.988 (0.919–1.063)	0.752		
Diabetes mellitus type II	0.585 (0.354–0.965)	0.036*	0.798 (0.451–1.412)	0.438
Hypertension	0.838 (0.475–1.476)	0.540		
Dyslipidemia	1.110 (0.650–1.897)	0.702		

AST aspartate amino transferase, *ALT* alanine amino transferase, *PLT* platelet, *PT%* prothrombin time, *ICG-R15* indocyanine green retention rate at 15 min, *T.bil* total bilirubin, *Alb* albumin, *AFP* α-fetoprotein, *PIVKAII* protein induced by vitamin K absence or antagonists II, + positive, − negative

* Statistical significance

with HBV, 41% with HCV, and 53% with non-B and non-C. In the CG group patients, 36.7% were with HBV, 46.6% with HCV, and 16.7% with non-B and non-C. First, the dbpA mRNA expression levels were compared between tumor tissues and nontumor tissues in the MS group and CG group patients, respectively. The dbpA transcript levels in the tumor tissues were significantly higher than that in the nontumor tissues both in the MS group ($P < 0.001$; Fig. 1a) and the CG group patients (Fig. 1b; $P < 0.001$). Second, the dbpA mRNA expression levels were compared between the MS group and the CG group patients. The dbpA transcript levels in the MS group patients were higher than that in the CG group patients (Fig. 1c). Third, the dbpA mRNA expression levels were compared between non-B and non-C patients and HBV or HCV patients' tumor and nontumor tissues. In the MS group, the dbpA transcript levels in non-B non-C patients were higher than that in the HBV or HCV patients, both in the tumor and nontumor tissues (Fig. 2a, b). However, there was no significant correlation in CG group.

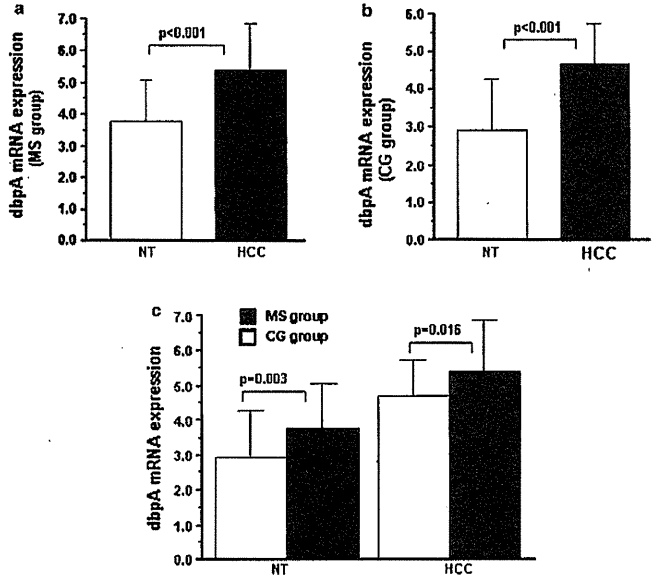

Fig. 1 dbpA mRNA expression level in HCC patients a mRNA expression level of dbpA were compared between tumor and nontumor tissues in MS group, b between tumor and nontumor tissues in CG group, and c between CG group and MS group. *HCC* hepatocellular carcinoma, *MS* metabolic syndrome, *NT* adjacent nontumor tissues

dbpA expression in HCC patients associated with MS

We comparatively analyzed the mRNA expression levels of dbpA according to the presence or absence of MS factors in cancer and corresponding noncancerous tissues. There was a significant correlation when the absence and presence of MS factors associated with HCC of cancerous and noncancerous specimens, respectively, were compared ($P < 0.01$; Fig. 3a, b). In all MS factors, the expression of dbpA in patients with DM type II was significantly higher than that in patients without DM both in the cancerous ($P < 0.001$) and the corresponding noncancerous tissues ($P < 0.05$). Dyslipidemia was present in patients with the presence of MS factors, only in the cancer tissues ($P < 0.05$). However, there was a tendency, but no statistical significance between high or low BMI index patients ($P = 0.077$) and between present or absent hypertension patients ($P = 0.089$). Immunohistochemical analysis was performed for the evaluation of the clinical significance of dbpA protein expression by using formalin-fixed paraffin-embedded tissue samples from 61 patients. As shown in Fig. 4, the cytoplasm and nuclear membrane staining of dbpA was clearly detected in the cancer tissues. Consequently, the specific overexpression of dbpA was recognized in 19 out of 27 cases (70.4%) of DM (+) patients and there was a statistical significance compared between patients with DM (+) and those without DM (−) ($P = 0.038$; Table 4).

Methylation analysis of dbpA in HCC patients with MS

Methylation-specific PCR (MS-PCR) was done for 122 liver tissues. The relationship of dbpA mRNA expression levels in patients with absence or presence of MS factors according to the promoter methylation and unmethylation status, both in the cancer and noncancerous tissues, was analyzed. The statistical correlation was obtained only in cancerous tissues but not in noncancerous tissues. The results are shown in Fig. 4. There was a statistical significance between methylated and unmethylated patients whose dbpA transcript levels were detected when MS factors were present ($P = 0.003$), but not when MS factors were absent. In DM type II patients, there was a statistical correlation between methylated and unmethylated patients' dbpA transcript levels ($P = 0.013$). In patients without DM, there was a similar tendency, but the difference was not statistically significant ($P = 0.063$). The dbpA mRNA expression level was also compared between methylated or unmethylated subjects according to the presence or absence of the MS factors. In demethylation status, the dbpA

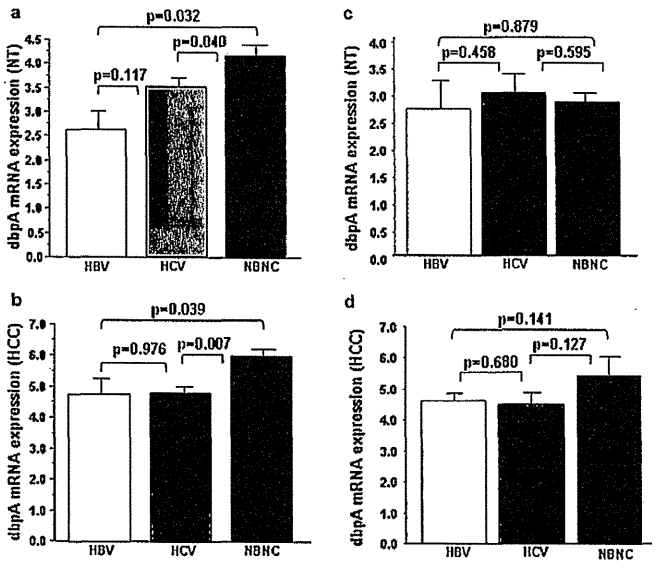

Fig. 2 dbpA mRNA expression in HCC patients with or without MS according to hepatitis background. **a**, **b** mRNA expression level of dbpA was compared between NBNC and HBV or HCV associated HCC patients with MS (MS group). **c**, **d** between NBNC and HBV or HCV associated HCCs patients without MS (CG group). *HBV* hepatitis B virus, *HCV* hepatitis C virus, *HCC* hepatocellular carcinoma, *MS* metabolic syndrome, *NBNC* non-B non-C hepatitis, *NT* adjacent nontumor tissues

mRNA levels were higher in DM type II patients than in non-DM type II patients ($P = 0.046$) and in patients with the presence of than in patients with the absence of MS factors ($P = 0.048$). We also analyzed the relationship of dbpA protein expression and the promoter methylation status in patients with the presence or absence of MS factors. The results are shown in Table 5. There was a statistical correlation between dbpA protein expression in methylated or unmethylated DM type II patients ($P < 0.01$) and between the presence and absence of MS risk factors in cancerous tissues ($P < 0.01$). However, the prognosis analysis showed that there was no statistic correlation between overall survival and recurrence-free survival with methylated or unmethylated HCC patients with MS.

Discussion

The risk of HCC patients with MS and the carcinoma is poorly understood. Regarding pathogenesis, it has been proposed that steatosis and several factors associated with MS, such as obesity, diabetes, and insulin resistance, may predispose patients with cirrhosis to HCC. In addition, an increased risk of HCC has been found in patients with diabetes, mostly type II DM [22–24].

In the present study, we did a comparative analysis of HCC patients with and without MS based on clinicopathological viewpoint and the risk hazard of recurrence. Also, we examined the expression and regulatory mechanisms of dbpA in patients with HCC. Based on this study, we speculate that metabolic factors may affect the risk of HCC not only in those with hepatic virus infection but also in those without hepatic virus infection, via a common or a different pathway. More specifically, metabolic factors may play a role in those without hepatitis virus infection through NAFLD and related conditions and in promoting carcinogenesis after infection. In our study, data showed that only the serum AFP is lower in the MS group than in the CG group. These differences of tumor markers between MS-HCC and non-MS-HCC are interesting and should be studied further. In most of the MS-associated HCC patients, there is an absence of hepatitis virus infection. Also, the serum AFP levels in patients with the absence of hepatitis virus infection are lower than in patients with hepatitis virus infection. It seems that AFP is not a sensitive marker in the diagnosis of MS-associated HCC. The presence of hepatic inflammation and/or fibrosis based on virus infection may be the underlying cause of increased serum AFP levels in patients with HCC.

The principal interest in this study was that on multivariate analysis, the only variables markedly associated with high

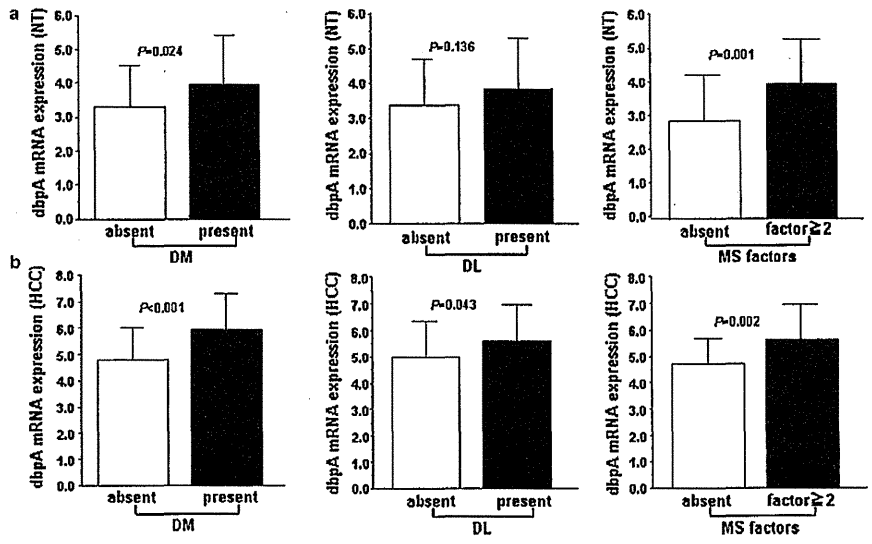

Fig. 3 dbpA mRNA expression in HCC patients according to absence or presence of MS factors. **a** mRNA expression levels of dbpA were compared between absence or presence of DM, absence or presence of DL between absence or presence of any two MS factor patients with associated NTs and **b** associated HCCs. *DM* diabetes mellitus type II, *DL* dyslipidemia, *HCC* hepatocellular carcinoma, *MS* metabolic syndrome, *NT* adjacent nontumor

incidence of tumor recurrence were the high preoperative AST levels. Recurrence of HCC is thought to be derived from intrahepatic metastases through the hepatic vascular and, more frequently, from metachronous multicentric carcinogenesis in the remnant liver. Chronic active hepatitis and cirrhosis are also significant risk factors for intrahepatic recurrence through multicentric carcinogenesis. In cirrhotic patients, the aminotransferase serum level is usually considered an index of the activated inflammatory activity that reflects the etiopathogenetic mechanism of hepatocyte necrosis [25]. Hypertransaminasemia is the clinical expression of a biologic activity at the basis of the multicentric carcinogenesis. In an experimental model, Marks et al. [26] demonstrated that vigorous necrosis is the cause of intensive proliferation of tissue cells, which correlate with the development of multicentric carcinogenesis by an increased rate of mutations and promotion. In our present study, the preoperative AST levels were higher in hepatic cirrhosis or chronic hepatitis associated MS-HCC patients than in nonchronic hepatitis or cirrhosis patients (data not shown). We suggested that the preoperative serum aminotransferase levels, as an index of hepatic inflammation, correlate with the recurrence rate among resected patients for HCC with cirrhosis.

The biological mechanism by which metabolic factors lead to HCC has not been fully clarified. Studies suggested that metabolic factors such as obesity lead to insulin resistance and steatosis, which are associated with the release of inflammatory mediators and cytokines. Based on this, obesity and diabetes cause hepatic inflammation, leading to oxidative stress and lipid peroxidation, subsequently resulting in hepatic injury, progress to hepatic fibrosis or cirrhosis and HCC [27–29].

Previously, our study group reported that the dbpA was a candidate molecule to accelerate the process of the inflammation-induced hepatocarcinogenesis [14, 15]. However, this opens a question: what are the roles of dbpA in MS-associated hepatocarcinogenesis? To confirm this question, the dbpA mRNA expression levels were comparatively investigated in the MS group or CG group patients. The dbpA transcript levels in the MS group patients were higher than in the CG group patients, both in the tumor and nontumor tissues (Fig. 1c). In the MS group, the dbpA transcript levels in non-B non-C patients were higher than in the HBV or HCV patients, both in the tumor and nontumor tissues (Fig. 2a, b). It is suggested that the aberrant expression of dbpA can accelerate not only the

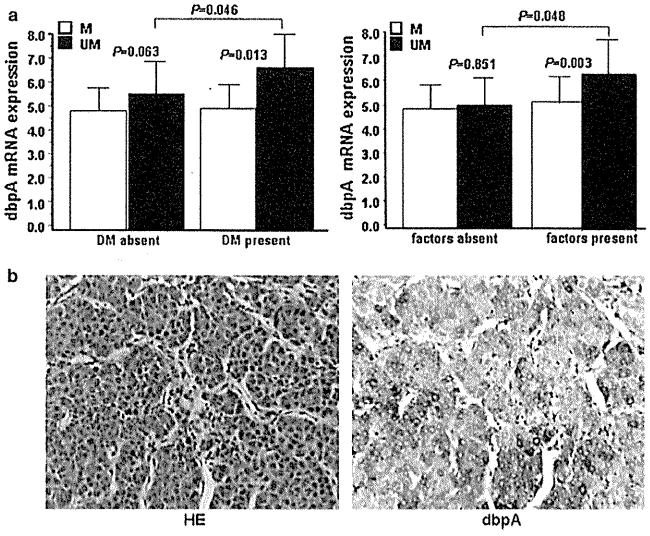

Fig. 4 Relationship of dbpA mRNA expression and the methylation/unmethylation status in HCC according to absence or presence of MS factors. a Between absence or presence of DM and between absence or presence of any of MS factors associated with tumor tissues. b dbpA positive staining with HCC patients with DM (+), shown are the representative cases that dbpA was demethylated. *DM* diabetes mellitus type II, *M* methylation, *UM* unmethylation

Table 4 Association between dbpA protein expression in HCC patients according to with or without MS factors

Factors	dbpA expression					
	Noncancerous			Cancer		
	Negative (n = 37)	Positive (n = 24)	P value	Negative (n = 28)	Positive (n = 33)	P value
Alcohol (−)	22	13	0.793	17	18	0.795
Alcohol (+)	15	11		11	15	
BMI <25	23	16	0.790	17	22	0.790
BMI ≥25	14	8		11	11	
DM (−)	24	10	0.113	20	14	0.038*
DM (+)	13	14		8	19	
DL (−)	27	16	0.774	23	20	0.093
DL (+)	10	8		5	13	
HP (−)	15	11	0.793	11	15	0.795
HP (+)	22	13		17	18	
Factor absent	9	4	0.539	7	6	0.547
≥1 factor present	28	20		21	27	
≥2 factors present	20	13	0.739	14	19	0.527
≥3 factors present	8	6	0.694	4	10	0.252

DM diabetes mellitus, *BMI* body mass index (kg/m^2), *DL* dyslipidemia, *HP* hypertension

* Statistical significance, Fisher's exact probability test

viral inflammation but also the nonviral inflammation-induced HCC. In nonviral-related HCC, most patients were associated with MS factors. In MS factors, especially, in DM type II patients, the dbpA was upregulated in tumor tissues and nontumor tissues, both in the mRNA transcript levels and also in the protein level in tumor tissues. The result suggested that dbpA transcripts is increased insulin resistance hepatocytes or liver cells that may be activated

Table 5 Association between dbpA protein expression and methylation or unmethylation status of patients according to with or without MS factors

dbpA expression	Factors	Noncancerous			HCC		
		M	UM	P value	M	UM	P value
Negative	Alcohol (−)	15	7	0.730	10	7	0.137
	Alcohol (+)	9	6		3	8	
Positive	Alcohol (−)	2	11	0.357	7	11	0.283
	Alcohol (+)	4	7		3	12	
Negative	BMI <25	16	7	0.495	10	7	0.137
	BMI ≥25	8	6		3	8	
Positive	BMI <25	4	12	1.000	8	14	0.430
	BMI ≥25	2	6		2	9	
Negative	DM (−)	13	11	0.083	7	13	0.096
	DM (+)	11	2		6	2	
Positive	DM (−)	2	8	1.000	10	4	<0.001*
	DM (+)	4	10		0	19	
Negative	DL (−)	15	12	0.065	12	11	0.333
	DL (+)	9	1		1	4	
Positive	DL (−)	4	12	1.000	7	13	0.701
	DL (+)	2	6		3	10	
Negative	HP (−)	9	6	0.730	5	6	1.000
	HP (+)	15	7		8	9	
Positive	HP (−)	2	9	0.649	6	9	0.448
	HP (+)	4	9		4	14	
Negative	MS absent	5	4	0.691	4	3	0.670
	MS present	19	9		9	12	
Positive	MS absent	1	3	1.000	5	1	0.005*
	MS present	5	15		5	22	

M methylation, *UM* unmethylation, *N* negative stain, *P* positive stain, *DM* diabetes mellitus, *BMI* body mass index (kg/m^2), *DL* dyslipidemia, *HP* hypertension, *MS* metabolic syndrome factors

* Statistical significance, Fisher exact probability

by oxidative stress. Previously, our study groups demonstrated that dbpA is aberrantly upregulated by several stressors such as UV irradiation, hypoxia, and partial hepatectomy. Several genes such as insulin-like growth factor binding protein 1 and insulin-like growth factor binding protein 2 were upregulated in gene expression profile of dbpA transgenic mice [30]. Insulin-like growth factor binding protein 1 is induced during the regeneration of the liver and is implicated in the maintenance of hepatocyte differentiation and metabolism. There is a complex relationship between IGF-I, IGF-binding proteins, growth hormone, and insulin. IGF-I directly inhibits insulin secretion and increases insulin sensitivity. An increased ratio of total IGF-I to IGFBP-1 has been proposed as a surrogate marker of increased IGF-I bioactivity. IGF-I bioactivity progressively increases with increasing severity of insulin resistance and hyperinsulinemia [31].

In this study, we also investigated the association between dbpA expression and methylation status according to patients with or without MS background. Results showed that the dbpA mRNA expression was regulated with promoter methylation status in HCC patients with MS part of cancerous specimens. Our results suggested that the demethylation-related epigenetic activation may accelerate the hepatocarcinogenesis in the liver of HCC patients with DM type II. In a previous study, we described for the first time that dbpA is frequently activated by hypomethylated in HCC. Our study showed that the specific Sp1-binding sites are located in the CpG region of dbpA promoter. Perhaps in HCC, this hypomethylation of dbpA gene may result in increasing Sp1 binding and subsequent transactivation [32]. Currently, we are unable to clearly explain how DM type II can cause demethylation of dbpA promoter and increase the transcription and translation of the molecule yet. Further study is required to confirm the mechanistic aspect.

In conclusion, we quantitatively evaluated dbpA mRNA expression levels in HCC patients with MS. DM type II

was a main risk factor for HCC patients with MS, and the dbpA was aberrantly expressed. The expression was partially regulated by epigenetic mechanisms such as promoter methylation status, and the demethylation-related epigenetic activation may be one of the regulated factors for HCC patients with MS.

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References

1. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745–750
2. Siegel AB, Zhu AX. Metabolic syndrome and hepatocellular carcinoma. Cancer 2009;115:5651–5661
3. Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. Gastroenterology 2004;127:S5–S16
4. Regimbeau JM, Colombat M, Mognol P et al. Obesity and diabetes as a risk factor for hepatocellular carcinioma. Liver Transpl 2004;10:S69–S73
5. El-Serag HB, Tran T, Elerhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology 2004;126:460–468
6. Wong GL, Wong VW, Choi PC et al. Metabolic syndrome increases the risk of liver cirrhosis in chronic hepatitis B. Gut 2009;58:111–117
7. Paradis V, Zalinski S, Chelbi E et al. Hepatocellular carcinomas in patients with metabolic syndrome often develop without significant liver fibrosis: A pathological analysis. Hepatology 2009; 49:851–859
8. Zen Y, Katayanagi K, T Tsuneyama, Harada K, Araki I, Nakanuma Y. Hapatocellular carcinoma arising in non-alcoholic steatohepatitis. Pathology Int 2001;51:127–131
9. Powell EE, Cooksley WG, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. Hepatology 1990;11:74–80
10. Hui JM, Kench JG, Chitturi S et al. Long-term outcomes of cirrhosis in nonalcoholic steatohepatitis compared with hepatitis C. Hepatology 2003;38:420–427
11. Marrero JA, Fontana RJ, Su GL, Conjeevaram HS, Emick DM, Lok AS. NAFLD may be a common underlying liver disease in patients with hepatocellularcarcinoma in the United States. Hepatology 2002;36:1349–1354
12. Bugianesi E, Leone N, Vanni E et al. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology 2002;123:134–140
13. Ratziu V, Bonyhay L, Di Martino V et al. Survival, liver failure, and hepatocellular carcinoma in obesity-related cryptogenic cirrhosis. Hepatology 2002;35:1485–1493
14. Hino O, Kajino K, Umeda T, Arakawa Y. Understanding the hypercarcinogenic state in chronic hepatitis: a clue to the prevention of human hepatocellular carcinoma. J Gastroenterol 2002;37:883–887
15. Umeda T, Hino O. Molecular aspects of human hepatocarcinogenesis mediated by inflammation: from hypercarcinogenic state to normo-or hypocarcinogenic state. Oncology 2002;62:38–42
16. Kajino K, Yamamoto T, Hayashi J, Umeda T, Takahara T, Hino O. Recombination hot spot of Hepatitis B virus genome bind to members of the HMG domain protein family and the Y box protein binding protein family, implication of these proteins in genomic instability. Intervirology 2001;44:311–316
17. Arakawa Y, Kajino K, Kano S et al. Transcription of dbpA, a Y box binding protein, is positively regulated by E2F1: implications in hepatocarcinogenesis. Biochem Biophys Res Commun 2004; 322:297–302
18. Wolffe AP, Tafuri S, Ranjan M, Familari M. The Y-box factors: a family of nucleic acid binding proteins conserved from *Escherichia coli* to man. New Biol 1992;4:290–298
19. Sakura H, Maekawa T, Imamoto F, Yasuda K, Ishii S. Two human genes isolated by a novel method encode DNA-binding proteins containing a common region of homology. Gene 1998; 73:499–507
20. Yasen M, Kajino K, Kano S et al. The up-regulation of Y box binding proteins (DNA binding protein A and Y-box binding protein-1) as prognostic marker of hepatocellular carcinoma. Clin Cancer Res 2005;11:7354–7361
21. Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation specific PCR. A novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996;93:9821–9826
22. Lagiou P, Kuper H, Stuver SO, Tzonou A, Trichopoulos D, Adami HO. Role of diabetes mellitus in the etiology of hepatocellular carcinoma. J Natl Cancer Inst 2000; 92:1096–1099.
23. Caldwell SH, Crespo DM, Kang HS, Al-Osaimi AM. Obesity and hepatocellular carcinoma. Gastroenterology 2004;127:S97–S103
24. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology 2004;126:460–468
25. Tarao K, Takemiya S, Tamai S et al. Relationship between the recurrence of hepatocellular carcinoma (HCC) and serum alanine aminotransferase levels in hepatectomized patients with hepatitis C virus-associated cirrhosis and HCC. Cancer 1997;79:688–694
26. Marks F, Bertsch S, Grimm W et al. Hyperplastic transformation and tumor promotion in mouse epidermis: possible consequences of disturbances of endogenous mechanism controlling proliferation and differentiation. Carcinogenesis 1987;2:97–116
27. Yuan JM, Govindarajan S, Aeakawa K, Yu MC. Synergism of alcohol, diabetes and viral hepatitis on the risk of hepatocellular carcinoma in blacks and whites in the U.S. Cancer 2004; 101:1009–1017
28. Yu MC, Yuan MJ. Environmental factors and risk for hepatocellular carcinoma. Gastroenterology 2004;127:S72–S78
29. Tellez-Avila FI, Sanchez-Avila F, García-Saenz-de-Sicilia M et al. Prevalence of metabolic syndrome, obesity and diabetes type 2 in cryptogenic cirrhosis. World J Gastroenterology 2008; 14:4771–4775
30. Tobita H, Kajino K, Inami K et al. Gene expression profile of DNA binding protein A transgenic mice. Int J Oncology 2006; 29:373–679
31. Brugts MP, van Duijn CM, Hofland LJ et al. IGF-1 bioactivity in an elderly population: relation to insulin sensitivity, insulin levels, and the metabolic syndrome. Diabetes 2010;59:505–508
32. Yasen M, Obulhasim G, Kajino K et al. DNA binding protein A expression and methylation status in hepatocellular carcinoma and the adjacent tissue. Int J Oncol. 2012;40:789–797

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Mitochondrial metabolism in the noncancerous liver determine the occurrence of hepatocellular carcinoma: a prospective study

Atsushi Kudo · Kaoru Mogushi · Tadatoshi Takayama · Satoshi Matsumura · Daisuke Ban · Takumi Irie · Takanori Ochiai · Noriaki Nakamura · Hiroshi Tanaka · Naohiko Anzai · Michiie Sakamoto · Shinji Tanaka · Shigeki Arii

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Abstract
Background Recurrence determines the postoperative prognosis with hepatocellular carcinoma (HCC). It is unknown how the liver dysfunction involving organic anion transporter failure causes the occurrence of HCCs. This study was designed to elucidate the link between liver dysfunction and multicentric occurrence (MO) after radical hepatectomy.
Methods Forty-nine samples of noncancerous liver tissue from HCC patients within the Milan criteria who were treated at our institution between January 2004 and August 2008 were examined as a training set by using genome-wide gene expression analysis. Using the independent 2-institutional cohort of 134 patients between September 2008 and December 2009, we performed a validation study using tissue microarray analysis. Cox proportional hazard regression analyses for MFS were performed to estimate the risk factors.
Results In the Gene Ontology database (GO:0015711), SLC22A7 expression was the best predictor of MO-free survival [MFS] (Fold, 0.726; $P = 0.001$). High SLC22A7 gene expression prevented the occurrence of HCC after hepatectomy (odds ratio [OR], 0.2; $P = 0.004$). Multivariate analyses identified SLC22A7 expression as an independent risk factor (OR, 0.3; $P = 0.043$). In the validation study, multivariate analyses of MFS identified SLC22A7 expression as an independent risk factor (OR, 0.5; $P = 0.012$). As judged by gene set enrichment analysis, SLC22A7 down regulation was associated with mitochondrion ($P = 0.008$) and oxidoreductase activity ($P = 0.006$). Sirtuin 3 as a regulator of mitochondrial metabolism also determined MFS ($P = 0.018$).
Conclusions The mitochondrial pathways may affect SLC 22A7 function to promote the occurrence of HCC. (Word count: 246).

Keywords Hepatocellular carcinoma · Mitochondria · Sirtuin 3 · SLC22A7 · Organic anion transporter

Abbreviations

CI	Confidence interval
FDR	False discovery rate
GSEA	Gene set enrichment analysis
HCC	Hepatocellular carcinoma
HR	Hazard ratio
MO	Multicentric occurrence
NES	Normalized enrichment score
OR	Odds ratio

Accession number of repository for expression microarray data: GSE40873.

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A. Kudo (✉) · S. Matsumura · D. Ban · T. Irie · T. Ochiai · N. Nakamura · S. Tanaka · S. Arii
Department of Hepatobiliary-Pancreatic Surgery, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
e-mail: kudomsrg@tmd.ac.jp

K. Mogushi · H. Tanaka
Department of Bioinformatics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

T. Takayama
Department of Digestive Surgery, Nihon University School of Medicine, Tokyo, Japan

N. Anzai
Department of Pharmacology and Toxicology, Dokkyo Medical University School of Medicine, Mibu, Tochigi 321-0293, Japan

M. Sakamoto
Department of Pathology, Graduate School of Medicine, Keio University, Tokyo, Japan

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Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide because of its high fatality (overall ratio of mortality to incidence of 0.93) [1]. In 2008, an estimated 748,000 new cases of HCC and 696,000 deaths associated with HCC occurred [1]. Underlying liver diseases increase the risk of HCC occurrence, although the mechanism by which this occurs is unclear [2]. Multicentric occurrence (MO) is a crucial problem irrespective of anatomic resection to prevent local recurrences. The clinical courses and biological features of MO definitely differ from those of local recurrence and intrahepatic metastases [3, 4].

Various treatments are selected for MO of HCC [5]. Anatomic resection proposed by Makuuchi et al., was implemented to overcome the local recurrence involving micro-dissemination into the portal vein and intrahepatic metastasis [6]. Resection is regarded as a first-line therapy when HCC occurrence is within the Milan criteria. However, even after curative treatments involving anatomic resection, considerable risk of MO has been reported [7]. There is no benefit of anatomic resection in HCC with MO [8]. Noncancerous liver tissue with oncogenic potential may explain the risk of MO after hepatectomy [3]. The criteria for MO are defined in the classification of the Liver Cancer Study Group of Japan [4].

This study was designed to elucidate whether noncancerous liver function involving transporter activity influences the MO of early-stage HCC. This study excluded patients beyond the Milan criteria to reduce malignant factors of the primary tumor. Genome-wide gene expression analysis was used to elucidate the link between this hepatocellular function and MO of HCC. Hepatocellular organic anion transporters exchange materials that are indispensable for mitochondrial metabolism, and they detoxify the sinusoidal microcirculation. Xenobiotics transported through organic anion transporters, are detoxified in hepatocytes and excreted into bile [9]. In the organic anion transporter genes according to the Gene Ontology database (GO:0015711) as a hepatocellular function, the best predictor for MO of HCC was SLC22A7. According to recent reports, SLC22A7 expressed on the hepatocellular sinusoidal membrane takes up orotic acid [10, 11]. An experimental study reported that exposure to dietary orotic acid with hepatectomy promotes liver carcinogenesis [12]. In this study, we present evidence indicating that decreased SLC22A7 expression associated with mitochondrial disability might play a causative role in liver carcinogenesis and that it will be a biomarker for predicting MO even after curative hepatic resection.

Methods

Training set

Between January 2004 and August 2008, 231 curative hepatectomies for HCC were performed at Tokyo Medical and Dental University Hospital (Tokyo, Japan). In total, 69 of 115 patients within the Milan criteria were ethically informed according to the guidelines of our institutional review board. This study excluded "beyond Milan", a contraindication for anatomic resection and liver transplantation. Trans-arterial embolization, radiofrequency ablation, and systemic chemotherapy are available options when tumor recurrence is evident after the first hepatectomy. Serum alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) levels were measured monthly, and ultrasonography, computed tomography, and magnetic resonance imaging were performed every 3 months. The criteria for MO of HCC were defined according to the classification of the Liver Cancer Study Group of Japan (the recurrent tumor consists of early HCC occurring in a different hepatic segment with or without dysplastic nodules in peripheral areas, or well differentiated HCCs with peripheral moderately or poorly differentiated HCC) [4]. Any tumor, regardless of the time to recurrence, arising in the same segment as the initial tumor (or within 2 cm from the surgical stump when performing segmentectomy) was considered a "local" recurrence [13].

Genome-wide gene expression analysis

All samples of noncancerous liver tissue obtained from the resected specimens were separately frozen immediately and stored at −80 °C. Total RNA was extracted using an RNeasy kit (Qiagen, Hilden, Germany). Contaminating DNA was removed by digestion with RNase-free DNase (Qiagen). Upon checking the RNA integrity of the samples using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), 49 samples were given an RNA integrity number exceeding 5.0. After preparing complementary RNA by 1-cycle target labeling and with a control reagents kit (Affymetrix, Santa Clara, CA, USA), hybridization and signal detection of HG-U133 Plus 2.0 arrays (Affymetrix) were performed according to the manufacturer's instructions. The 49 microarray datasets were normalized using the robust multiarray average method of R

statistical software (version 2.12.1) together with the Bio-Conductor package. Estimated gene expression levels were obtained as log2-transformed values, and 62 control probe sets were selected for further analysis.

Selection of organic anion transporter genes for HCC occurrence

First, probe sets corresponding to known genes were selected on the basis of the NetAffx annotation file, version 32 (available at: http://www.affymetrix.com/analysis/index.affx). Next, probe sets of organic anion transporter genes were selected according to GO:0015711 (52 probes). The 35 probes were matched to the criteria. The univariate Cox proportional hazards regression model was used to estimate the relationship between the gene expression pattern and MO for each probe set. Probe sets that had a $P < 0.005$ by the likelihood ratio test were selected.

Validation study on immunohistochemical analysis using tissue microarrays

To validate the clinical significance of SLC22A7 expression, 134 patients who visited Tokyo Medical and Dental University Hospital and Nihon University Hospital between September 2008 and December 2009 were enrolled in the prospective multicenter cohort. The candidate gene was assessed by immunohistochemical staining on tissue microarrays using resected liver samples from patients with HCC within the Milan criteria with an anti-SLC22A7 antibody (provided by Dr. Anzai) at a 1:20 dilution [14] by the use of an automated immunostainer (Ventana XT System; Ventana Medical Systems, Inc., Tucson, AZ, USA) as described previously. The SLC22A7 staining was judged by 2 investigations, and staining of less than 25 % of cells was judged as negative (Fig. 1c, d).

Gene set enrichment analysis (GSEA)

To investigate the biological backgrounds correlated with a particular gene expression pattern, we used GSEA version 2.0.7 with MSigDB gene sets version 3.0. Gene set category C5, which is based on the GO database, was used. Gene sets satisfying both $P < 0.05$ and a false discovery rate (FDR) <0.25 were considered significant. The customized sirtuin 3-related gene set involving 147 genes was constructed to examine the relationship with SLC22A7 according to the supplemental Fig. 1.

Statistical analysis

Univariate and multivariate Cox regression analyses were performed using SPSS 20.0 (IBM, Armonk, NY, USA). The median value was selected for the cut-offs of clinical variables in the training set and the validation set. The MO-free survival (MFS) was evaluated by the Kaplan–Meier method and the log-rank test. Two-sided $P < 0.05$ were considered significant. Values are given as the mean ± SD unless otherwise stated.

Results

Baseline characteristics

All possible curative resections within the Milan criteria (R0) were attempted for the 49 patients in the training set and the 134 patients in a multicenter validation study (Table 1). The mean observation time in the training set and the validation set were 21.1 and 16.4 months, respectively. The mean MFS in the training set and the validation set were 12.3 and 10.6 months. There was no difference in the mean MFS between the training and validation sets ($P = 0.602$), though the mean observation time was longer in the training set ($P = 0.010$). There was no difference between the two studies in age, gender, viral infection (HBV and HCV), serum albumin, total bilirubin, AFP, DCP, platelet count, tumor number, pathological invasion into the portal vein, liver cirrhosis, and MO occurrence rate. There were differences in Child class B ($P = 0.03$), ICG-R15 value ($P = 0.02$), tumor size ($P < 0.0001$), and anatomic resection ($P < 0.0001$), respectively.

The predictive factors for MFS in the training set

As shown in Table 2, low SLC22A7 expression was the best predictor of MFS ($P = 0.001$; fold difference between the mean expression levels of patients with and without MO = 0.726). Table 3 presents the link between MFS and SLC22A7 gene expression in the 49 HCC patients within the Milan criteria. The MO was observed in seventeen patients (35 %). Univariate analyses identified HCV infection, serum albumin levels, serum platelet counts, and SLC22A7 gene expression as risk factors for HCC occurrence. These results led us to determine which risk factors were independently predictive of the prognosis of HCC patients. According to multivariate analysis, SLC22A7 gene expression determined prognosis independently. Figure 1a indicates that the cumulative recurrence-free survivals were significantly associated with SLC22A7 expression. (Log-rank test, $P = 0.001$).

Validation study for SLC22A7 expression among patients within the Milan criteria

Table 4 illustrates the link between MFS and SLC22A7 protein expression as judged by a tissue microarray in 134

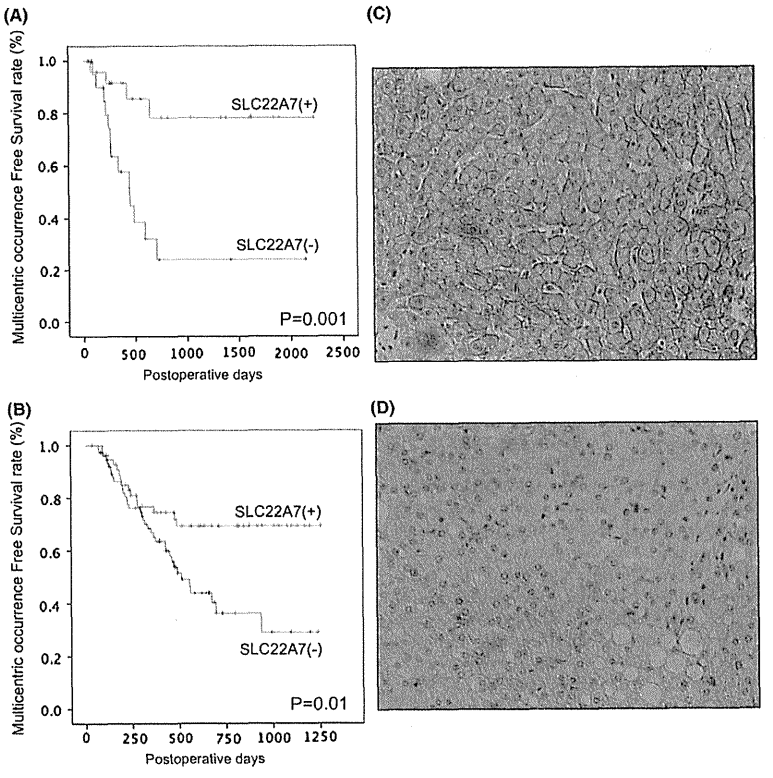

Fig. 1 **a** Training study. MFS of postoperative HCC patients within the Milan criteria with high (SLC22A7 (+) group) and low (SLC22A7 (−) group) SLC22A7 gene expression in noncancerous liver tissue. The median expression level for each gene was used as a cutoff value. The green lines denote the Kaplan–Meier curves for the SLC22A7 (+) group. *Blue line* denotes low SLC22A7 expression in the SLC22A7 (−) group. The prognosis of the SLC22A7 (−) group was significantly worse ($P = 0.001$). **b** Validation study using a prospective multicenter cohort. The *green lines* denote the Kaplan–Meier curves for SLC22A7 (+) group. The *blue line* denotes the MFS in the SLC22A7 (−) group. Note the poor survival of the SLC22A7 (−) group ($P = 0.01$). **c** Immunohistochemical analysis (magnification, ×20) with high expression of SLC22A7. **d** Immunohistochemical analysis (magnification, ×20) with low expression of SLC22A7 protein

HCC patients within the Milan criteria. The SLC22A7 protein was expressed at the hepatocellular sinusoidal membrane in noncancerous tissues (Fig. 1c). The MO was observed in 52 patients (38 %). Low SLC22A7 expression was confirmed when the immunohistochemical staining of the tissue microarray was less than 25 % (Fig. 1d). Univariate analyses identified anatomic resection and low SLC22A7 expression as risk factors for HCC occurrence (Table 4). Other clinicopathological factors did not determine MO. These results led us to determine which factors were independently predictive of prognosis. As shown in Table 4, SLC22A7 expression determined prognosis (OR, 0.5; 95 % CI, 0.3–0.8; $P = 0.012$). In Fig. 1b, the cumulative MFS was significantly associated with SLC22A7 expression ($P = 0.010$). The 1-year MFS in patients with low SLC22A7 expression was 65.2 %, compared with 76.7 % in those with high SLC22A7 expression.

GSEA evaluation of SLC22A7 expression in HCC

The dataset had 54,675 native features. After collapsing the features into gene symbols, 20,606 genes were identified.

Gene set size filters (min = 15, max = 500) resulted in the filtering out of 446/1454 gene sets. The remaining 998 gene sets containing 7,605 genes were used in the analysis. The p value of SLC22A7 was ranked at 261st out of 21,050 genes included in the HG-U133 Plus 2.0 array. In total, 552 of 998 gene sets were positively correlated with SLC22A7 expression. Seventy-seven gene sets were significant at FDR of <25 %. Thirty-six gene sets were significantly enriched at a nominal $P < 1$ %. Conversely, 446 of 998 gene sets were negatively correlated with SLC22A7 expression. No gene sets were significantly enriched at FDR < 25 %. Two gene sets were significantly enriched at a nominal $P < 1$ %. As shown in Fig. 2, mitochondrion ($P = 0.008$; FDR = 0.199; NES = 1.804), oxidoreductase activity ($P = 0.006$; FDR = 0.157; NES = 1.854), and fatty acid metabolic process ($P = 0.021$; FDR = 0.177; NES = 1.723) were significantly correlated with SLC22A7 expression. By analyzing the gene expression profiles of 49 samples of noncancerous tissue, GSEA showed that the 27 of the 62 gene sets (44 %) were closely related with mitochondrial genes involving oxidoreductase activity and fatty acid metabolic process at FDR of 20 % with a nominal $P < 0.05$ (Supplementary Table 1). Mitochondrial sirtuin 3, reported as the regulator of fatty acid oxidation, oxidative damage and orotic acid concentrations, prevents deacetylates and stimulates ornithine transcarbamylase (OTC) and modulates amino acid catabolism and β-oxidation [15, 16]. The correlation between SLC22A7 and sirtuin3 expression levels was 0.300 ($P = 0.034$). As shown in Fig. 2d, decreased sirtuin 3 gene expression also determined patient MFS ($P = 0.018$). These results led us to examine whether the customized sirtuin 3-related gene set correlates with SLC22A7. The GSEA revealed a remarkable correlation with SLC22A7 ($P = 0.008$; FDR = .008; NES = 1.786), as shown in Supplemental Figs. 2 and 3. Mitochondrial factor may be confounding factor of the two factors, though the detailed mechanism remains unknown.

Table 1 Baseline characteristics

Variables	Training set Mean (SD)	Validation set Mean (SD)	P
Age	66.8 ± 10.3	67.3 ± 9	0.941
Male/female	34/15	97/37	0.411
Viral infection			
HBV	11	17	0.136
HCV	31	85	0.363
Laboratory data			
Prothrombin time (%)	83.7 ± 17.9	92.3 ± 12.8	<0.0001*
Albumin (g/dL)	3.9 ± 0.5	4 ± 0.5	0.067
Total bilirubin (mg/dL)	0.8 ± 0.4	0.8 ± 0.4	0.401
Platelet (×10⁴/mL)	14.1 ± 6.8	14.4 ± 5.6	0.509
Child-Pugh A/B	42/7	128/6	0.03*
ICG-R15 (%)	20.2 ± 11.9	16.3 ± 10.2	0.015*
Tumor factor			
Diameter (cm)	3.2 ± 1	2.5 ± 1	<0.0001*
Number	1.4 ± 0.9	1.2 ± 0.5	0.159
AFP (ng/mL)	350 ± 1305	175 ± 750	0.272
DCP (mAU/mL)	1053 ± 3389	1345 ± 7086	0.958
Pathological vp (+)	9	22	0.790
Anatomic resection			
Yes	24	28	<0.0001*
Liver background			
NL/CH/LC	3/17/29	6/71/57	0.081
Multicentric occurrence			
+	17	52	0.371

DCP des-gamma-carboxy prothrombin

* $P < 0.05$ was considered significant

Discussion

The retrospective training study and validated prospective multicenter study provided evidence that low SLC22A7 expression promoted the occurrence of HCC after hepatectomy in patients within the Milan criteria. This is the first study to elucidate the link between the occurrence of HCC and SLC22A7 expression in noncancerous liver tissue. Moreover, this gene expression is closely related with the gene sets of mitochondrion in noncancerous liver, as judged by GSEA evaluation in the training set (Fig. 2a).

Table 2 The univariate analyses to estimate the relationship between a gene expression pattern and MO of HCC for each probe set of organic anion transporter genes according to the Gene Ontology database (GO:0015711)

Probe set	Symbol	Title	Fold	P
221661_at	SLC22A7	Solute carrier family 22 (organic anion transporter), member 7	0.726	0.001*
1557918_s_at	SLC16A1	Solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	0.831	0.005
210366_at	SLC10A1	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	0.936	0.017
207185_at	SLC16A1	Solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	0.907	0.071
202236_s_at	SLCO1A2	Solute carrier organic anion transporter family, member 1A2	0.912	0.074

The best organic anion transporter genes in GO-0015711. † Fold values were calculated by the ratio of mean expression levels in the patients with MO to that in patients without MO

* $P < .005$ was considered significant (Cox's proportional hazard ratio)

Table 3 The risk factors determining MO in 49 HCC patients within the Milan criteria (training set)

Variables	Univariate		P	Multivariate		P
	OR	95 %CI		OR	95 %CI	
Age (years)						
>68	1.7	(0.6–4.4)	0.288			
Gender						
Female	1.2	(0.5–3.3)	0.694			
HCV						
(+)	0.2	(0.1–0.9)	0.027*	0.4	(0.1–1.7)	0.206
HBV						
(+)	2.7	(0.6–11.9)	0.190			
Total bilirubin (×mg/dL)						
≧0.8	1.9	(0.7–5.5)	0.241			
Albumin (g/dL)						
≧4.0	0.2	(0.1–0.6)	0.002*	0.3	(0.1–1.0)	0.056
Prothrombin time (%)						
≧84.4	0.7	(0.3–1.8)	0.426			
Platelet (×10⁴/μL)						
≧11.9	0.3	(0.1–0.8)	0.017*	0.8	(0.2–2.8)	0.700
Child-Pugh A vs. B	0.4	(0.1–1.4)	0.140			
ICG-R15 (%)						
≧20	2.3	(0.9–6.1)	0.101			
Tumor diameter (cm)						
>3	0.9	(0.4–2.4)	0.871			
Multiple	1.6	(0.5–5.7)	0.444			
AFP (ng/mL)						
≧12	1.1	(0.9–1.3)	0.337			
DCP (mAU/mL)						
≧38	0.6	(0.2–1.6)	0.284			
Capsule						
(+)	0.4	(0.0–20)	0.309			
Capsule invasion						
(+)	0.5	(0.2–1.3)	0.151			
Pathological vp						
(+)	0.9	(0.2–4.0)	0.909			
CM type vs. SNEG	1.0	(0.3–3.5)	0.972			
SN type vs. SNEG	0.3	(0.1–1.1)	0.061			
Moderately differentiated						
Vs. well	0.8	(0.2–3.5)	0.775			
Poorly differentiated						
Vs. well	0.2	(0.1–2.0)	0.233			
Liver cirrhosis						
(+)	1.7	(0.6–4.6)	0.298			
SLC22A7 expression						
High	0.2	(0.1–0.6)	0.004*	0.3	(0.1–1.0)	0.043*
Anatomic resection						
(+)	0.7	(0.3–1.8)	0.426			

DCP des-gamma-carboxy prothrombin *SN* simple nodular type, *SNEG* simple nodular type with extranodular growth, *MC type* confluent multinodular type

* $P < .05$ was considered significant

Table 4 The risk factors that determine MO in 134 HCC patients within the Milan criteria (validation set)

Variables	Univariate		P	Multivariate		P
	OR	95 %CI		OR	95 %CI	
Age						
≧67	1.08	(0.62–1.89)	0.792			
Gender						
Female	0.88	(0.46–1.68)	0.701			
HCV						
(+)	1.13	(0.64–1.98)	0.681			
Body weight						
≧58.3	1.00	(0.58–1.74)	0.989			
Total bilirubin (mg/dL)						
≧0.7	0.79	(0.45–1.39)	0.418			
Albumin (g/dL)						
≧4.1	0.70	(0.40–1.21)	0.200			
Prothrombin time (%)						
≧97 %	0.79	(0.46–1.37)	0.406			
Platelet (×10⁴/μL)						
≧13.8	0.80	(0.46–1.37)	0.413			
Child-Pugh score						
≧6	0.81	(0.41–1.62)	0.550			
ICG-R15 (%)						
≧14.3	1.17	(0.68–2.03)	0.568			
Tumor diameter (cm)						
≧2.4	0.88	(0.51–1.52)	0.644			
Multiple tumor						
(+)	1.28	(0.57–2.84)	0.550			
Pathological vp						
(+)	0.99	(0.48–2.04)	0.985			
AFP (ng/mL)						
≧8.7	1.41	(0.82–2.50)	0.216			
DCP (mAU/mL)						
>36	0.75	(0.43–1.30)	0.302			
Liver cirrhosis						
(+)	0.70	(0.21–2.33)	0.558			
Anatomic resection						
(+)	0.52	(0.24–1.16)	0.110			
SLC22A7 expression						
High	0.46	(0.25–0.84)	0.012*	0.46	(0.25–0.84)	0.012*

DCP des-gamma-carboxy prothrombin

* $P < 0.05$ was considered significant

The GSEA showed that the 44 % of SLC22A7-related gene sets were closely related with mitochondrial genes involving oxidoreductase activity and fatty acid metabolic process. Mitochondrial metabolism may also involved fatty acid synthase and oxidoreductase activity.

A previous study demonstrated that the gene expression profiles of the surrounding nontumoral liver tissue, but not the tumor tissues, were highly correlated with survival in the training set of Japanese patients and in validation sets in the United States and Europe ($P = 0.04$) [17]. Anatomic resection was not identified as a prognostic factor in the two studies. The HCV infection, platelet counts, and serum

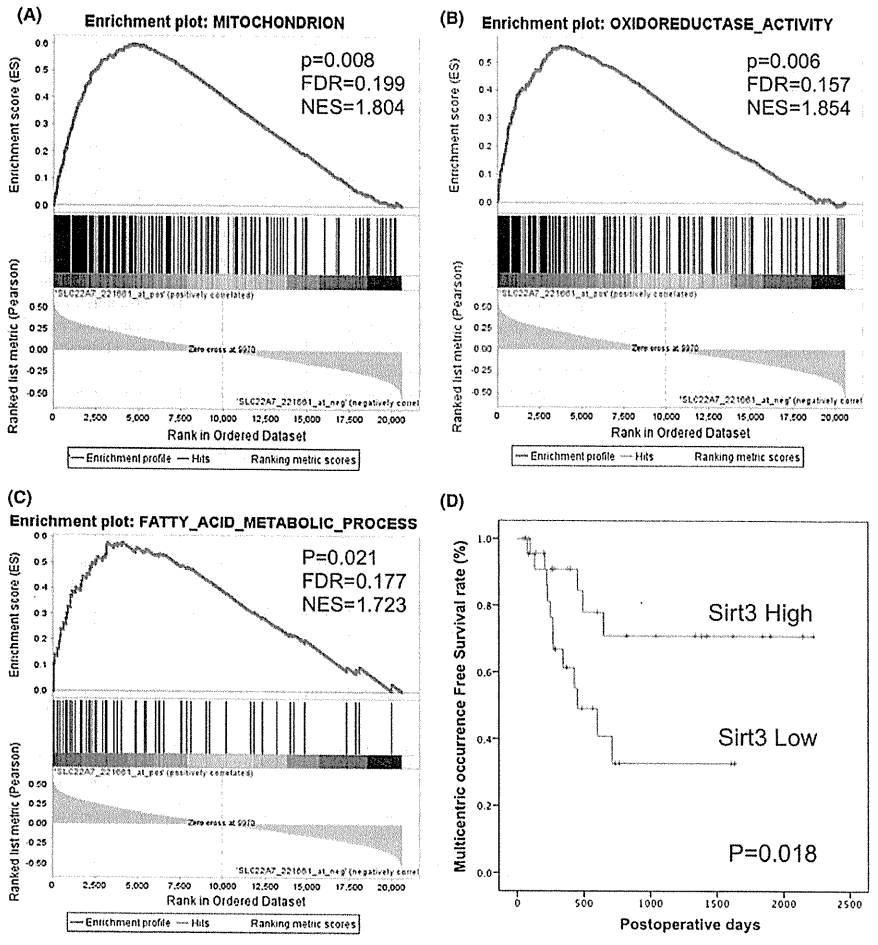

Fig. 2 GSEA evaluation associated with SLC22A7: **a** mitochondrion ($P = 0.008$; FDR = 0.199; NES = 1.804), **b** oxidoreductase activity ($P = 0.006$; FDR = 0.157; NES = 1.854), and **c** fatty acid metabolic process ($P = 0.021$; FDR = 0.177; NES = 1.723). **d** Low expression of sirtuin 3 was associated with a poor prognosis ($P = 0.018$)

albumin levels, which were recognized as risk factors for the occurrence of HCC in univariate analyses of the training set (Table 3), did not decide the clinical outcome in the multicenter validation study (Table 4). Multivariate analysis of the training set and the validation study revealed the low SLC22A7 expression to be the only reliable factor for predicting MO of HCC. The correlation between SLC22A7 gene expression and platelet counts were 0.167 ($P = 0.246$), and 0.134 ($P = 0.355$), respectively (data not shown). There was no difference in SLC22A7 expression between virus negative patients, HBV positive patients and HCV positive patients

($P = 0.439$). The SLC22A7 expression may be independent of platelet count and serum albumin decrease, predicting another aspect of liver functional reserve.

The precise indicator derived from noncancerous liver tissue determined the prognosis, which was not governed by tumor progression. According to the genome-wide gene expression analysis in the training set (Table 1), SLC22A7 best determined the clinical outcome in the organic anion transporter genes (GO:0015711), as judged by Cox regression analysis in ($P = 0.001$). Figure 1a shows the significant difference in tumor-free survival after hepatectomy according to SLC22A7 expression ($P = 0.001$).

The information obtained in the training set was validated in a prospective multicenter study using tissue microarrays (Fig. 1b). To this end, low SLC22A7 expression certainly determined MFS (Table 4). Regarding occurrence-free survival in the present study, de novo HCC may occur at 1 year after hepatectomy in patients with low SLC22A7 expression (Fig. 1). In this context, the prognostic curves appeared to be compatible with clinical observations. Anatomic resection did not decrease the risk for MFS in the multicenter study. It is reasonable that anatomic resection is not available in noncancerous liver with low SLC22A7 expression promoting de novo HCC. The aforementioned criteria were enough to determine the MO of HCC, since the anatomic resection prevents local recurrence within Milan criteria.

Whether oxidative stress resulting from reactive oxygen species in noncancerous tissue or cellular mitochondrial dysfunction promotes hepatocarcinogenesis has been discussed [18, 19]. Antioxidants such as glutathione play an important role and serve as essential components of the detoxification mechanism [9]. Our previous study identified CYP1A2 as an index for HCC recurrence [18]. The CYP1A2 expression is significantly decreased in the steatotic liver induced with orotic acid [20, 21]. The CYP1A2 and SLC22A7 are regulated by interferon-alpha 2b in human primary hepatocytes [22]. Interferon-alpha 2b induced partial remission of hepatoma [23]. These reports imply the possibility that CYP1A2 and SLC22A7 down regulation, are an early alert symptom of MO. In this aspect, decreased SLC22A7 expression may serve as a reliable surrogate biomarker for the prognosis and treatment of HCC.

Organic anion transporters are responsible for the uptake and exclusion of xenobiotics and organic anions [24, 25]. Serum organic anions and xenobiotics are emptied into the Disse's space, taken up by transporters at the hepatocellular sinusoidal membrane, and detoxified in the cytoplasm [26, 27]. The SLC22A7 expressed on the hepatocellular sinusoidal membrane takes up orotic acid [11]. Orotic acid has been regarded as a promoter of liver carcinogenesis, although the detailed mechanisms are unknown [28–30]. Moreover, mitochondrial sirtuin 3 may be involved in the metabolic cycle of orotic acid. Sirtuin 3, by regulating OTC activity to decrease orotic acid, inhibits hepatocellular carcinoma cell growth [31]. Previous research reported that exposure to orotic acid after hepatectomy promotes liver carcinogenesis [12]. Orotic aciduria and encephalopathy were observed in HCC patients without liver cirrhosis [32]. We focused on Sirtuin 3 regulating orotic acid production, because SLC22A7 transports orotic acids. Sirtuin 3 was reported as the regulator of mitochondrial metabolism and the inhibitor of hepatocellular carcinoma cell growth. The present findings indicate that the decreased sirtuin3 expression coinciding with decreased SLC22A7 may regulate hepatocellular orotic acid concentrations to promote MO of HCC (Fig. 2d). The gene expression levels of SLC22A7 correlated with that of sirtuin3. Furthermore, the customized sirtuin 3-related gene set revealed significant correlation with SLC 22A7 expression (Supplementary Figs. 2, 3).

In conclusion, the down regulation of SLC22A7 in noncancerous liver tissue may have promoted the MO of early-stage HCC in the training and multicenter validation studies. Evaluating SLC22A7 expression may be useful for selecting treatment strategies. Further research is required to determine whether the hepatocellular mechanisms involving mitochondrial metabolism increase hepatocellular liver carcinogenesis. Such studies could address whether antioxidant therapy or another therapy to prevent hepatocarcinogenesis would become available in patients with low SLC22A7 expression.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Yang JD, Roberts LR. Hepatocellular carcinoma: a global view. Nat Rev Gastroenterol Hepatol. 2010;7:448–58.
2. Schlitt HJ, Schnitzbauer AA. Hepatocellular carcinoma: agents and concepts for preventing recurrence after curative treatment. Liver Transpl. 2011;17(Suppl 3):S10–2.
3. Utsunomiya T, Shimada M, Imura S, Morine Y, Ikemoto T, Mori M. Molecular signatures of noncancerous liver tissue can predict the risk for late recurrence of hepatocellular carcinoma. J Gastroenterol. 2010;45:146–52.
4. Japan LCSGo: The general rules for the clinical and pathological study of primary liver cancer (in japanese). 5th ed. Tokyo: Kanehara; 2009. p. 43.

5. Arii S, Yamaoka Y, Futagawa S, Inoue K, Kobayashi K, Kojiro M, et al. Results of surgical and nonsurgical treatment for small-sized hepatocellular carcinomas: a retrospective and nationwide survey in japan. The liver cancer study group of japan. Hepatology. 2000;32:1224–9.
6. Hasegawa K, Kokudo N, Imamura H, Matsuyama Y, Aoki T, Minagawa M, et al. Prognostic impact of anatomic resection for hepatocellular carcinoma. Ann Surg. 2005;242:252–9.
7. Imamura H, Matsuyama Y, Tanaka E, Ohkubo T, Hasegawa K, Miyagawa S, et al. Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. J Hepatol. 2003;38:200–7.
8. Kobayashi A, Miyagawa S, Miwa S, Nakata T. Prognostic impact of anatomical resection on early and late intrahepatic recurrence in patients with hepatocellular carcinoma. J Hepatobiliary Pancreat Surg. 2008;15:515–21.
9. Kudo A, Kashiwagi S, Kajimura M, Yoshimura Y, Uchida K, Arii S, et al. Kupffer cells alter organic anion transport through multidrug resistance protein 2 in the post-cold ischemic rat liver. Hepatology. 2004;39:1099–109.
10. Sekine T, Cha SH, Tsuda M, Apiwattanakul N, Nakajima N, Kanai Y, Endou H. Identification of multispecific organic anion transporter 2 expressed predominantly in the liver. FEBS Lett. 1998;12:179–82.
11. Fork C, Bauer T, Golz S, Geerts A, Weiland J, Del Turco D, et al. Oat2 catalyses efflux of glutamate and uptake of orotic acid. Biochem J. 2011;436:305–12.
12. Laconi E, Vasudevan S, Rao PM, Rajalakshmi S, Pani P, Sarma DS. The development of hepatocellular carcinoma in initiated rat liver after a brief exposure to orotic acid coupled with partial hepatectomy. Carcinogenesis. 1993;14:2527–30.
13. Takayama T, Makuuchi M, Hirohashi S, Sakamoto M, Yamamoto J, Shimada K, et al. Early hepatocellular carcinoma as an entity with a high rate of surgical cure. Hepatology. 1998;28:1241–6.
14. Enomoto A, Takeda M, Shimoda M, Narikawa S, Kobayashi Y, Yamamoto T, et al. Interaction of human organic anion transporters 2 and 4 with organic anion transport inhibitors. J Pharmacol Exp Ther. 2002;301:797–802.
15. Hallows WC, Yu W, Smith BC, Devries MK, Ellinger JJ, Someya S, et al. Sirt3 promotes the urea cycle and fatty acid oxidation during dietary restriction. Mol Cell. 2011;41:139–49.
16. Hirschey MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, et al. Sirt3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature. 2010;464:121–5.
17. Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, et al. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med. 2008;359:1995–2004.
18. Tanaka S, Mogushi K, Yasen M, Ban D, Kudo A, Arii S, et al. Oxidative stress pathways in noncancerous human liver tissue to predict hepatocellular carcinoma recurrence: a prospective, multicenter study. Hepatology. 2011;54:1273–81.
19. Marra M, Sordelli IM, Lombardi A, Lamberti M, Tarantino L, Giudice A, et al. Molecular targets and oxidative stress biomarkers in hepatocellular carcinoma: an overview. J Transl Med. 2011;9:171.
20. Su GM, Sefton RM, Murray M. Down-regulation of rat hepatic microsomal cytochromes p-450 in microvesicular steatosis induced by orotic acid. J Pharmacol Exp Ther. 1999;291:953–9.
21. Zhang WV, Ramzan I, Murray M. Impaired microsomal oxidation of the atypical antipsychotic agent clozapine in hepatic steatosis. J Pharmacol Exp Ther. 2007;322:770–7.
22. Chen C, Han YH, Yang Z, Rodrigues AD. Effect of interferon-alpha2b on the expression of various drug-metabolizing enzymes and transporters in co-cultures of freshly prepared human primary hepatocytes. Xenobiotica. 2011;41:476–85.
23. Locker GJ, Mader RM, Steiner B, Wenzl E, Zielinski CC, Steger GG. Benefit of interferon-alpha2b in a patient with unresectable hepatoma and chronic infection with hepatitis c virus. Eur J Gastroenterol Hepatol. 2000;12:251–3.
24. Kudo A, Ban D, Aihara A, Irie T, Ochiai T, Nakamura N, Tanaka S, Arii S. Decreased Mrp2 transport in severe macrovesicular fatty liver grafts. J Surg Res. 2012;178(2):915–21.
25. Ban D, Kudo A, Sui S, Tanaka S, Nakamura N, Ito K, et al. Decreased mrp2-dependent bile flow in the post-warm ischemic rat liver. J Surg Res. 2009;153:310–6.
26. Norimizu S, Kudo A, Kajimura M, Ishikawa K, Taniai H, Suematsu M, et al. Carbon monoxide stimulates mrp2-dependent excretion of bilirubin-ixalpha into bile in the perfused rat liver. Antioxid Redox Signal. 2003;5:449–56.
27. Sui S, Kudo A, Suematsu M, Tanaka S, Ito K, Arii S, et al. Preservation solutions alter mrp2-dependent bile flow in cold ischemic rat livers. J Surg Res. 2010;159:572–81.
28. Rao PM, Nagamine Y, Roomi MW, Rajalakshmi S, Sarma DS. Orotic acid, a new promoter for experimental liver carcinogenesis. Toxicol Pathol. 1984;12:173–8.
29. Laurier C, Tatematsu M, Rao PM, Rajalakshmi S, Sarma DS. Promotion by orotic acid of liver carcinogenesis in rats initiated by 1,2-dimethylhydrazine. Cancer Res. 1984;44:2186–91.
30. Denda A, Laconi E, Rao PM, Rajalakshmi S, Sarma DS. Sequential histopathological analysis of hepatocarcinogenesis in rats during promotion with orotic acid. Cancer Lett. 1994;82:55–64.
31. Zhang YY, Zhou LM. Sirt3 inhibits hepatocellular carcinoma cell growth through reducing mdm2-mediated p53 degradation. Biochem Biophys Res Commun. 2012;423:26–31.
32. Jeffers LJ, Dubow RA, Zieve L, Reddy KR, Livingstone AS, Neimark S, et al. Hepatic encephalopathy and orotic aciduria associated with hepatocellular carcinoma in a noncirrhotic liver. Hepatology. 1988;8:78–81.