

201320017A(2/2)

厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業

次世代シーケンシング・ゲノムワイド関連解析を用いた  
C型肝炎治療に伴う肝病態進展軽快、肝発癌に関わる宿主因子の解析

平成25年度 総括・分担研究報告書

研究代表者 坂本 直哉

平成26(2014)年 5月

「研究成果の刊行に関する別刷り」

## Reduced Organic Anion Transporter Expression Is a Risk Factor for Hepatocellular Carcinoma in Chronic Hepatitis C Patients: A Propensity Score Matching Study

Yutaka Yasui<sup>a,d</sup> Atsushi Kudo<sup>b</sup> Masayuki Kurosaki<sup>a</sup> Shuya Matsuda<sup>a,d</sup>  
Masaru Muraoka<sup>a,d</sup> Nobuharu Tamaki<sup>a</sup> Shoko Suzuki<sup>a</sup> Takanori Hosokawa<sup>a</sup>  
Ken Ueda<sup>a</sup> Kotaro Matsunaga<sup>e</sup> Hiroyuki Nakanishi<sup>a</sup> Kaoru Tsuchiya<sup>a</sup>  
Jun Itakura<sup>a</sup> Yuka Takahashi<sup>a</sup> Shinji Tanaka<sup>b</sup> Yasuhiro Asahina<sup>c</sup>  
Nobuyuki Enomoto<sup>d</sup> Shigeki Arij<sup>b</sup> Namiki Izumi<sup>a</sup>

<sup>a</sup>Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, and Departments of  
<sup>b</sup>Hepatobiliary Pancreatic Surgery and <sup>c</sup>Gastroenterology and Hepatology, Graduate School, Tokyo Medical and  
Dental University, Tokyo, <sup>d</sup>First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi,  
Yamanashi, and <sup>e</sup>Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna  
University School of Medicine, Kawasaki, Japan

### Key Words

Hepatocellular carcinoma · SLC22A7 · Organic anion transporter 2 · Chronic hepatitis C · Hepatocarcinogenesis

### Abstract

**Objectives:** Recent reports indicated that reduced SLC22A7 (a gene-encoding organic anion transporter 2) expression in noncancerous liver tissue predicts hepatocellular carcinoma (HCC) recurrence after curative resection. Our study aimed to elucidate the association between SLC22A7 expression and HCC development in chronic hepatitis C patients. **Methods:** HCC recurrence after local ablation therapy and SLC22A7 expression in noncancerous liver tissue were analyzed in 20 patients. Subsequently, the association between de novo HCC development and SLC22A7 expression was examined at baseline in 38 hepatitis C patients without HCC who subsequently developed HCC as well as

in 76 hepatitis C patients who did not develop HCC and were matched for age, gender and stage of fibrosis. **Results:** In the patients whose HCC had been cured, reduced SLC22A7 expression in noncancerous liver tissue was significantly associated with a high incidence of multifocal HCC recurrence. In patients without HCC at baseline, cumulative incidence of de novo HCC development was significantly higher with a reduced SLC22A7 expression than with a normal expression ( $p = 0.01$ ). This difference remained significant among patients without known risk factors for HCC like age and advanced fibrosis. **Conclusion:** Reduced SLC22A7 expression in the liver indicates a significant risk for HCC development in chronic hepatitis C, independently of other risk factors.

© 2014 S. Karger AG, Basel

## Introduction

Hepatocellular carcinoma (HCC) is the third most common cancer worldwide [1] and the most frequent primary liver cancer [2]. Chronic hepatitis C virus (HCV) infection is a major risk factor for developing HCC [3], increasing the risk by 17-fold when compared with healthy individuals [4, 5]. Among HCV-positive patients, several risk factors for HCC have been well documented, including age, obesity, sex, serum platelet count and stage of liver fibrosis [6–10]. Advanced fibrosis, in particular, is the most significant risk factor for HCC in chronic HCV patients. The response to interferon therapy is also related to HCC risk [11, 12], mainly because the treatment attenuates hepatitis in responsive individuals. However, despite the absence of known risk factors, younger patients and those with nonadvanced fibrosis also develop HCC. Thus, surveillance is insufficient and additional risk analyses are required for those chronic HCV patients without known risk factors for HCC.

As for curatively treated HCC patients, tumor differentiation or progenitor-cell feature markers of cancerous tissue have been identified as predictors of recurrence [13, 14]. In contrast, only several reports have mentioned the importance of background noncancerous liver tissue and the microenvironment; these are predictive of HCC recurrences [15, 16]. Moreover, no specific features of noncancerous liver tissue have been clarified to be associated with *de novo* HCC development.

A recent prospective study showed that reduced SLC22A7 (organic anion transporter 2, OAT2) activity in noncancerous liver tissue is associated with multifocal recurrence after curative resection, independently of age and stage of fibrosis [17]. Furthermore, this study revealed that reduced SLC22A7 expression indicates a high risk for poor prognosis [18]. This observation indicates that the function of the transporter in noncancerous liver tissue is related to hepatic carcinogenesis, which may explain HCC development in patients who have no other known risk factors.

In this study, the use of SLC22A7 as a biomarker for HCC recurrence after curative local ablation therapy was assessed in order to validate and extend previously reported observations. Subsequently, the propensity score matching method was used to match patients with and without HCC development as well as to elucidate the association between SLC22A7 expression in hepatitis tissue and the risk of HCC development in chronic HCV patients.

## Patients and Methods

### *Distant Recurrence after Radio Frequency Ablation Therapy for HCC*

#### Patients

To reveal the relationship between multifocal HCC recurrence and SLC22A7 expression in noncancerous liver tissue, we conducted a retrospective study enrolling patients who received curative local ablation therapy. Twenty of the patients who enrolled in this cohort fulfilled the following criteria: (1) their HCC was treated curatively by radio frequency ablation (RFA); (2) they were infected with HCV and (3) they underwent liver biopsy at least 6 months after curative RFA. Written informed consent was obtained from all patients. The study was approved by the Ethical Committee of the Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

#### Data Collection and Histological Evaluation

Patient characteristics, treatment details and biochemical, hematological, virological and histological data were collected at enrollment.

Liver biopsy specimens were obtained using 13-gauge needles under laparoscopy or 15-gauge needles using an ultrasound guide. Liver biopsy specimens were scored by board-certified pathologists for stage of fibrosis and grade of inflammatory activity according to the classification by Desmet et al. [19].

#### Immunohistochemical Staining of SLC22A7

All liver biopsy specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4  $\mu$ m and stained with anti-OAT2 (SLC22A) antibody (kindly provided by Dr. Anzai) at a 1:20 dilution. Immunohistochemical (IHC) staining was performed using an automated immunostainer (Ventana XT System; Ventana Medical Systems Inc., Tucson, Ariz., USA), with the same procedure as the previous study [17]. Cell staining was evaluated along the entire length of the biopsy core (>30 high-power fields). Staining was graded according to the following score:  $\leq 25\%$  = reduced staining of cells and  $>25\%$  = normal staining of cells (fig. 1). Scoring of SLC22A7 staining was performed independently by two hepatologists (K.M. and A.K.) who were blinded to the clinical outcome, and average scores were used for analysis.

#### Surveillance for HCC

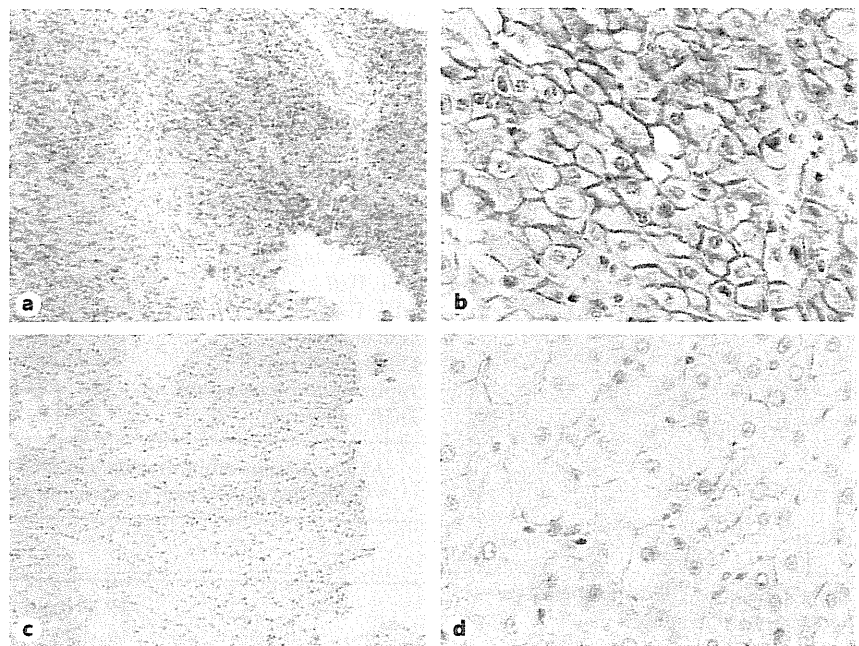
Patients were examined for HCC every 3–6 months by abdominal ultrasonography, dynamic computed tomography or magnetic resonance imaging. Serum alpha-fetoprotein levels were measured every 3 months. HCC diagnosis was confirmed from needle biopsies, surgical resection specimens or according to the typical radiological hallmarks of early enhancement and delayed washout. The start date of follow-up was the date of liver biopsy and the end date was HCC development or the latest medical attendance.

#### *Relationship between SLC22A7 and de novo HCC*

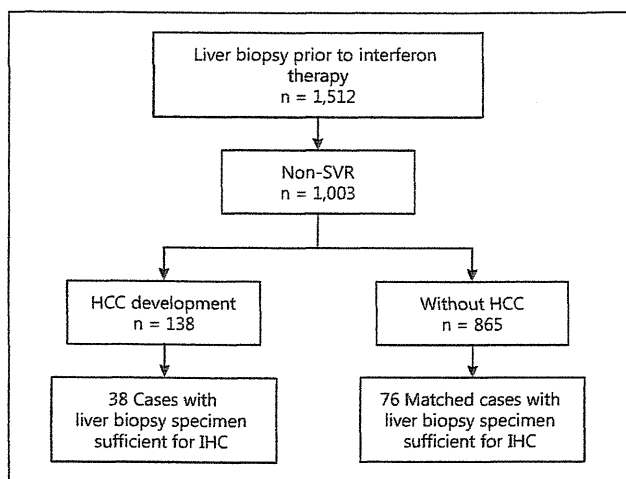
#### *Development in Chronic HCV without HCC at Baseline*

#### Patients

To elucidate the relationship between SLC22A7 and *de novo* hepatic carcinogenesis, we conducted a study in an independent cohort. A consort diagram of this study is shown in figure 2. Since 1992, 1,512 chronic HCV patients provided liver biopsies prior to interferon therapy at Musashino Red Cross Hospital. A total of 1,003 of these patients did not achieve a sustained virological re-

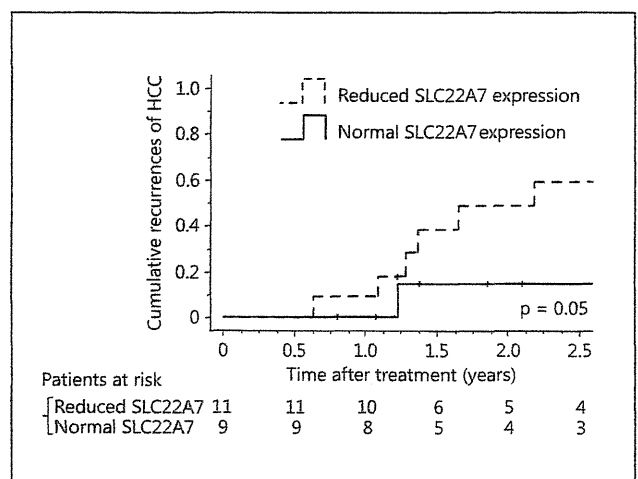


**Fig. 1.** IHC analysis of SLC22A7 in biopsy specimens. **a, b** Normal SLC22A7 expression ( $\geq 25\%$  positive cells) **a**  $\times 100$ . **b**  $\times 400$ . **c, d** Reduced SLC22A7 expression ( $< 25\%$  positive cells). **c**  $\times 100$ . **d**  $\times 400$ .



**Fig. 2.** Consort diagram of stratified analyses.

response (SVR) to therapy and among these, 132 developed HCC. We enrolled 38 non-SVR patients who developed HCC and 76 matched non-SVR patients who did not develop HCC. Ninety-four patients who developed HCC were excluded because their liver biopsy specimens were of insufficient quality for IHC analyses. Matching was performed using a propensity score matching method. Histological evaluation, IHC staining and surveillance for HCC were performed as above. The average duration of follow-up was 6.6 years for all patients and 7.9 years for patients who did not



**Fig. 3.** Cumulative incidence of HCC recurrence after curative RFA was compared between patients with normal and reduced SLC22A7 expression.

develop HCC. As above, written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

#### Propensity Score Matching

In multivariate analyses of 1,003 non-SVR patients, age, gender and stage of fibrosis were independent risk factors for HCC development. Using this multivariate logistic regression analysis, pro-

**Table 1.** Baseline characteristics of patients who underwent RFA

	Normal SLC22A7 expression (n = 9)	Reduced SLC22A7 expression (n = 11)	p value
Age, years	66.5±5.0	62.9±4.1	0.09
Gender (M/F)	4/5	3/8	0.64
Fibrosis (F0–2/F3–4)	5/4	4/7	0.65
Mean tumor size, mm	20.4±11.3	18.8±6.0	0.91
Albumin, g/dl	4.0±0.3	3.9±0.3	0.71
Bilirubin, mg/dl	0.7±0.2	0.9±0.4	0.09
AST, IU/l	82.0±47.1	74.2±30.6	0.84
ALT, IU/l	80.7±50.2	75.1±33.0	0.85
Glucose, mg/dl	100.3±11.6	123.5±38.7	0.25
Cholesterol, mg/dl	164.0±21.5	166.6±33.8	0.93
Alpha fetoprotein, ng/ml <sup>a</sup>	6.8 (3.7–106)	19.3 (5.9–87.3)	0.46
DCP, mAU/ml <sup>a</sup>	32 (14–129)	15 (14–26)	0.15

ALT = Alanine aminotransferase; DCP = des-gamma-carboxy prothrombin.

<sup>a</sup> Values are shown with median and range.

**Table 2.** Baseline characteristics of patients enrolled in study 2

	HCC cases (n = 38)	Non-HCC matching cases (n = 76)	p value
Age, years	64.6±7.1	64.6±6.4	0.98
Gender (M/F)	19/19	39/37	0.99
Fibrosis (F0–2/F3–4)	15/23	31/45	0.84
BMI	23.8±3.1	23.5±3.2	0.60
Albumin, g/dl	3.9±0.3	4.1±0.3	0.007
Bilirubin, mg/dl	0.7±0.3	0.7±0.3	0.42
AST, IU/l	83.5±39.2	66.2±37.7	0.07
ALT, IU/l	92.4±45.9	76.8±56.6	0.29
GGT, IU/l	74.6±59.0	63.2±54.0	0.42
Platelets, 10 <sup>4</sup> /μl	13.2±4.9	14.6±4.3	0.12
Glucose, mg/dl	116.8±20.9	112.4±24.1	0.16
Cholesterol, mg/dl	163.6±32.6	171.1±28.0	0.14

ALT = Alanine aminotransferase; BMI = body mass index; GGT = gamma-glutamyl transpeptidase.

propensity scores were calculated for each patient. These scores were used to match patients who developed HCC (HCC cases) with those who did not (non-HCC cases). Each HCC case was matched with 2 non-HCC cases whose propensity scores were similar to that of the HCC case (nearest-neighbor matching). Data analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, Ill., USA).

#### Statistical Analysis

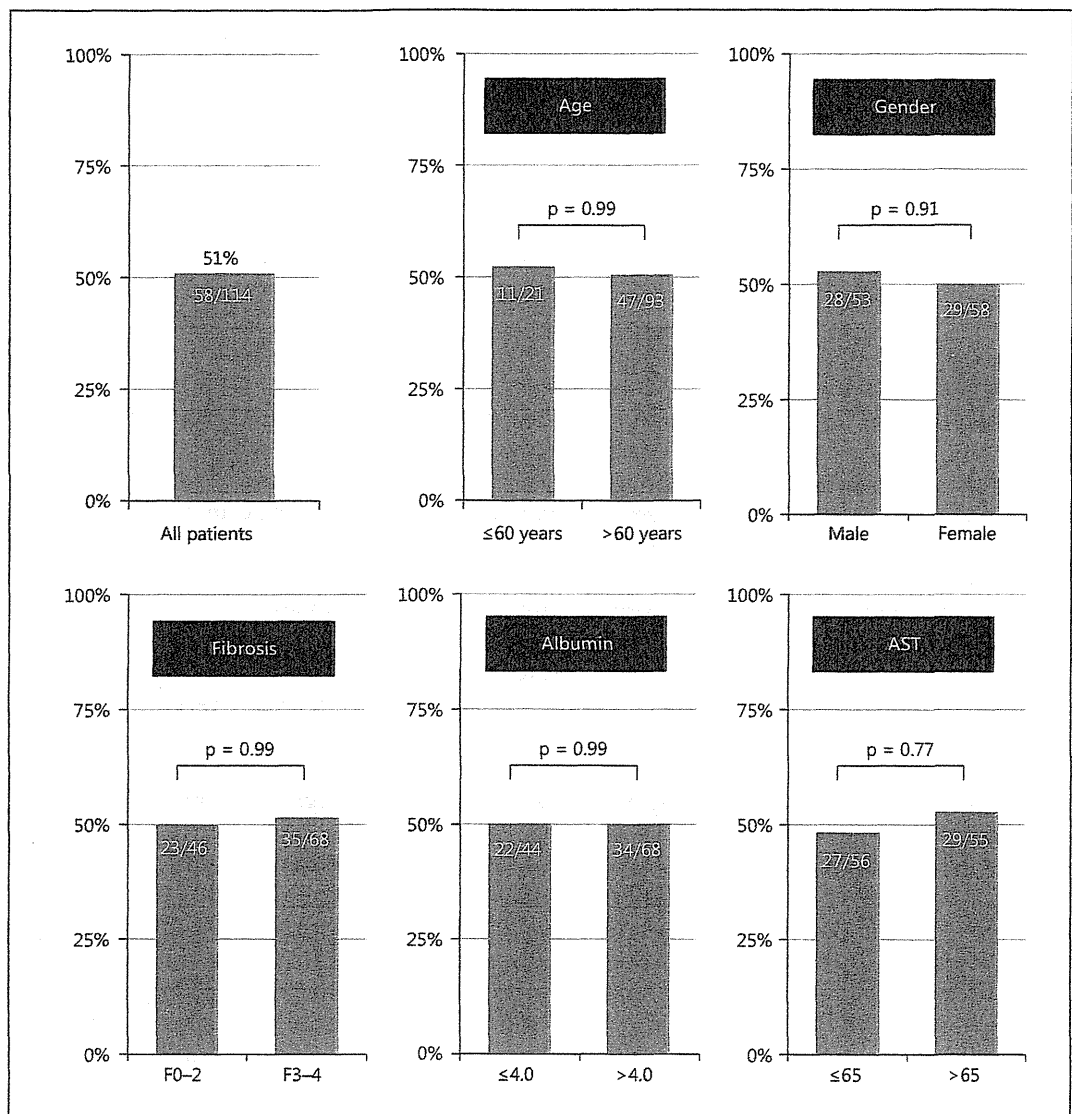
Continuous variables are reported as the mean and standard deviation (SD) or median and categorical variables are shown as counts and proportions. Statistical significance was assessed using the Student t test (mean), the Mann-Whitney U test (median) or the Fisher exact test. In all tests, 2-sided p values were calculated and differences were considered statistically significant when p < 0.05. Statistically significant differences identified in univariate analyses were further assessed in multivariate logistic regression

analysis. The stepwise and multivariate Cox proportional hazard models were used to explore independent factors that could be used to predict HCC development. Statistical analyses were performed using the SPSS software version 11.0.

## Results

### *SLC22A7 Expression and Distant Recurrence after Curative RFA*

Baseline characteristics of patients who received RFA are shown in table 1. No significant differences were observed between patients with normal SLC22A7 expression and those with reduced SLC22A7 expression. Figure 3 shows the cumulative rates of distant recurrences



**Fig. 4.** Percentage of patients with normal SLC22A7 expression according to baseline clinical findings. No significant differences in the percentage of patients with normal SLC22A7 expression were observed after stratification by age, gender, fibrosis stage, albumin and/or AST.

after curative HCC treatment. Patients with reduced SLC22A7 expression had significantly higher rates of distant recurrence than those with normal SLC22A7 expression.

#### *SLC22A7 Expression and de novo Hepatic Carcinogenesis in Chronic HCV Patients*

Patient characteristics at the time of enrollment are shown in table 2. Age, gender and stage of liver fibrosis

were matched using propensity scores. The distribution of serum albumin levels differed significantly between HCC cases and non-HCC cases. Serum aspartate aminotransferase (AST) levels were higher in patients with HCC than in those without HCC, although this was not statistically significant. Other factors, including body mass index, platelet count, serum glucose and serum cholesterol, which are known risk factors for HCC, were not significantly different between the patient groups.

**Table 3.** Factors associated with hepatic carcinogenesis according to the Cox proportional hazards model

Factors	Multivariable analysis	
	HR (95% CI)	p value
SLC22A7 (reduced expression)	3.49 (1.56–7.83)	0.002
Albumin (per 1 g/dl)	6.37 (1.56–25.6)	0.009

Normal SLC22A7 expression was found in 58 patients (51%) and reduced SLC22A7 expression was found in 56 patients. No significant differences in baseline characteristics were observed between these groups. When stratified by the matched risk factors age, gender and fibrosis stage, no significant differences were observed in the percentage of patients with normal SLC22A7 expression. Similarly, no significant differences were identified between the groups that were stratified by unmatched serum albumin and AST, which differed between HCC and non-HCC cases (fig. 4). In contrast, the percentage of patients with normal SLC22A7 expression was lower in HCC cases than in non-HCC cases (37 vs. 58%, respectively,  $p = 0.05$ ). Furthermore, among patients aged <60 years, the percentage with normal SLC22A7 expression was significantly lower in HCC cases than in non-HCC cases ( $p = 0.02$ ). This difference was observed in male patients ( $p = 0.001$ ) and in patients with nonadvanced fibrosis (i.e. stages F0–2;  $p = 0.05$ ; fig. 5). However, no significant differences were observed among patients aged >60 years, among female patients or among those with advanced fibrosis (i.e. stages F3–4).

The cumulative incidence of HCC was significantly higher in patients with reduced SLC22A7 expression than in those with normal SLC22A7 expression (33.9 vs. 13.8% after 5 years, respectively,  $p = 0.01$ ). This difference remained significant in patients without a known risk of HCC development, such as older patients and those with advanced liver fibrosis (fig. 6). Importantly, in patients aged <60 years, the cumulative incidence of HCC after 5 years was 60 and 0% in those with reduced and normal SLC22A7 expression, respectively ( $p = 0.02$ ). In patients with nonadvanced liver fibrosis, the cumulative incidence of HCC after 5 years was 31.3 and 12.0% in patients with reduced and normal SLC22A7 expression, respectively ( $p = 0.02$ ). Because serum albumin levels differed between HCC and non-HCC cases, we assessed the cumulative incidence of HCC after stratification by this variable. Receiver operating characteristic analyses re-

vealed that a level of 4.0 g/dl of serum albumin was the most appropriate cut-off for predicting HCC development. Therefore, we divided all cases into 2 groups with this cut-off. In patients with  $\geq 4.0$  g/dl of serum albumin, the cumulative incidence of HCC was significantly higher in patients with reduced SLC22A7 expression than in those with normal SLC22A7 expression (23.5 vs. 5.9% after 5 years, respectively,  $p = 0.03$ ). In contrast, among patients with <4.0 g/dl of serum albumin, the cumulative incidence of HCC after 5 years was 50.0 and 22.7% in those with reduced and normal SLC22A7 expression, respectively ( $p = 0.06$ ; fig. 6).

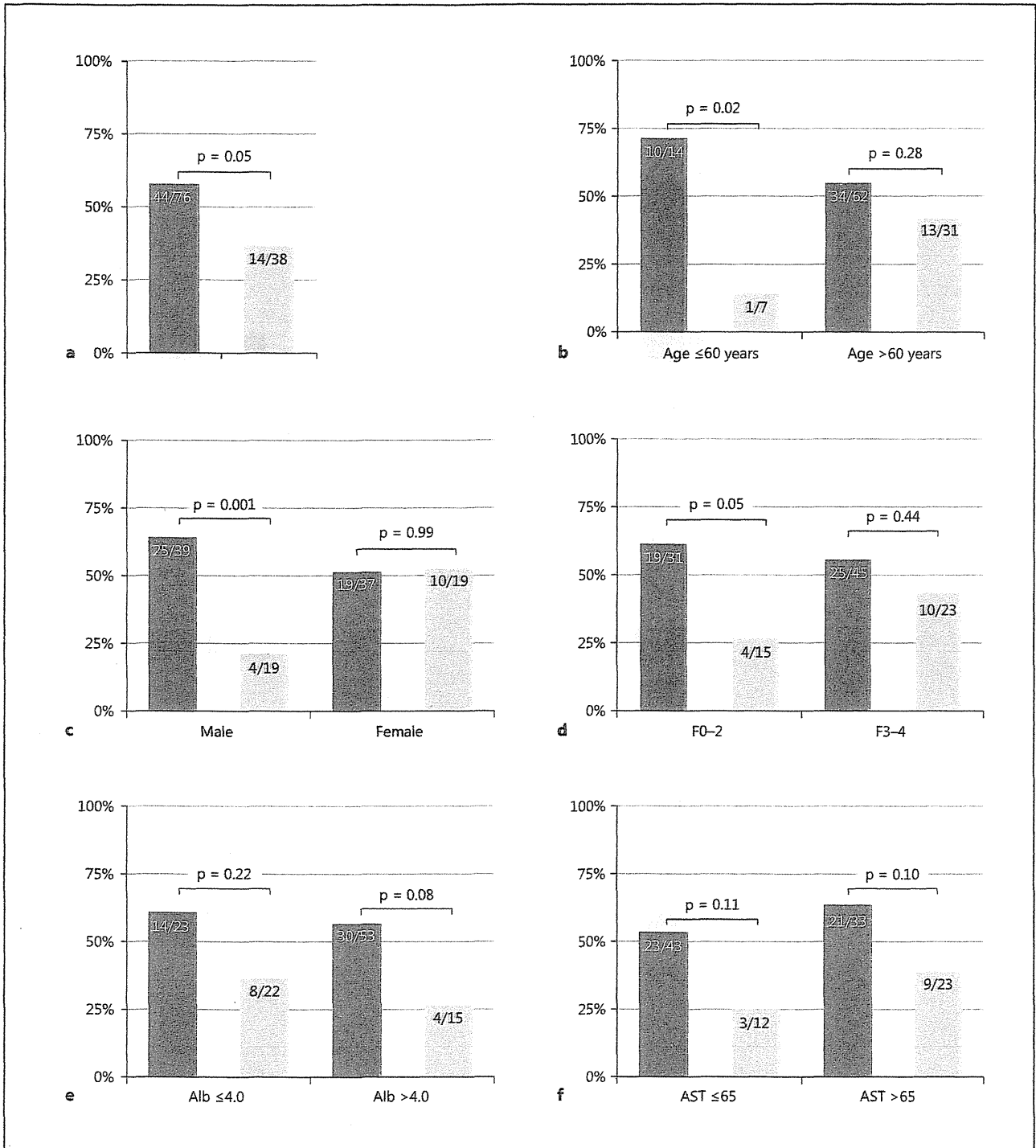
Multivariate analyses confirmed that serum albumin levels (odds ratio 3.1 and  $p = 0.003$ ) and SLC22A7 expression (odds ratio 2.6 and  $p = 0.01$ ) were independent risk factors for HCC in this cohort (table 3).

## Discussion

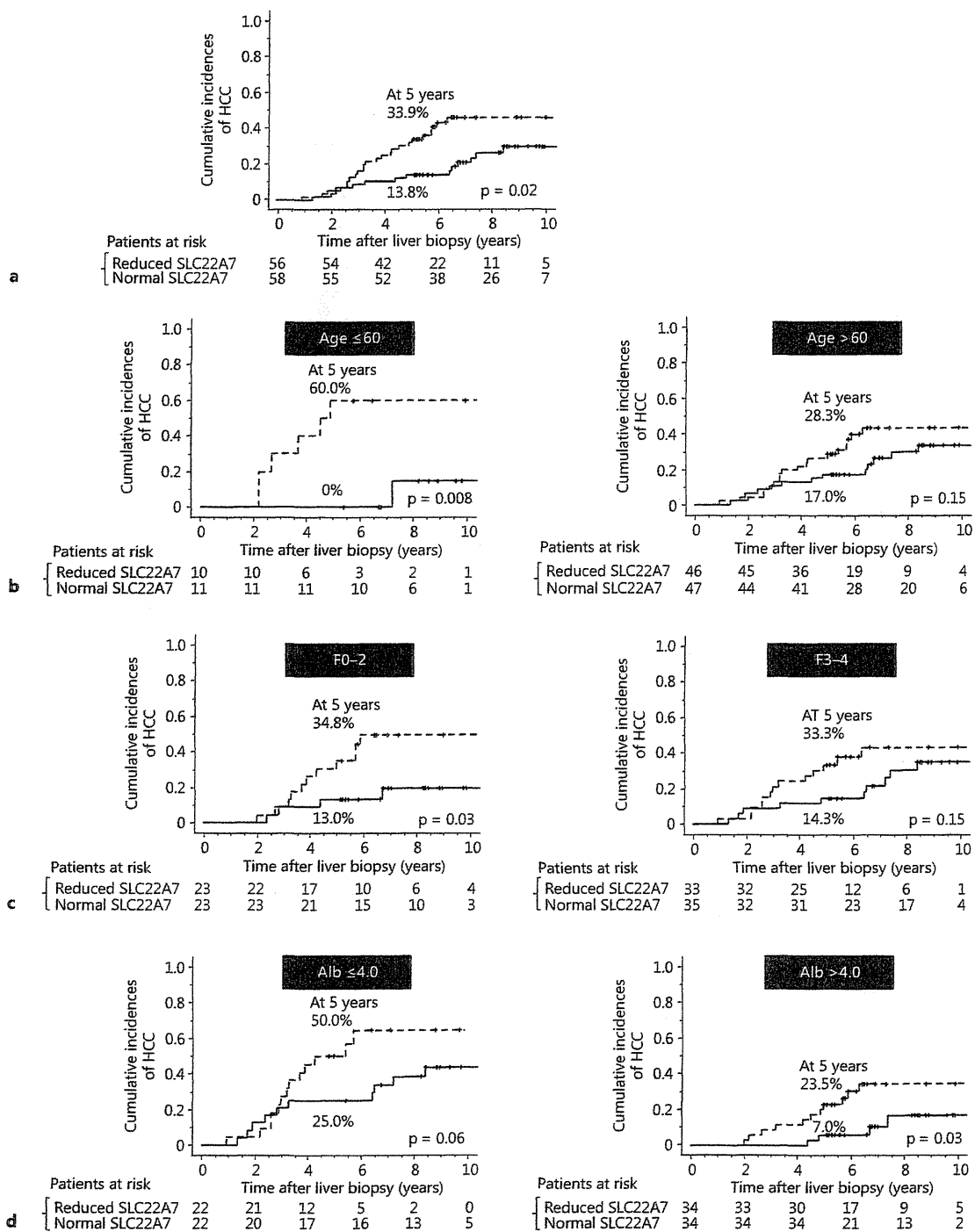
This study demonstrates higher cumulative rates of multifocal HCC recurrence after curative treatment in patients with reduced SLC22A7 expression. Moreover, SLC22A7 expression in chronic HCV tissue specimens was a significant predictor for future development of HCC in chronic HCV patients. These analyses indicate the importance of SLC22A7 expression as a predictor of multifocal HCC, de novo and after curative treatment. In particular, among patients without known risk factors for HCC, the cumulative incidence of HCC was significantly higher in those with reduced SLC22A7 expression.

A recent study showed that reduced SLC22A7 expression is an independent risk factor for recurrence after HCC resection [17]. We hypothesized that SLC22A7 might be an IHC marker for the multifocal occurrence of HCC. Initially, we validated the previously reported utility of SLC22A7 as a biomarker for HCC recurrence after curative therapy in HCC patients treated with RFA instead of resection. Subsequently, we revealed a significant association between SLC22A7 expression in hepatitis tissue and the risk of future HCC in chronic HCV patients. Indeed, previous studies show several risk factors for HCC in these patients, including failure to achieve SVR, older age, male gender, obesity and advanced fibrosis and steatosis of the liver [20–22]. According to current data, assessments of transporter function in liver biopsies contribute an additional valuable predictor. This was further emphasized in patients who lacked known risk factors, such as older age and advanced fibrosis. Given the paucity of known risk factors for HCC among younger pa-





**Fig. 5.** Percentage of patients with normal SLC22A7 expression and HCC (a). SLC22A7 staining was compared between patients who did and did not develop HCC after stratification by age (b), gender (c), fibrosis stage (d), albumin (Alb, e) and AST levels (f). Light grey and dark grey bars represent patients with and without HCC, respectively.



**Fig. 6.** Cumulative incidence of HCC according to SLC22A7 staining. **a** Comparison of the cumulative incidences of HCC in patients with normal (solid line) and reduced SLC22A7 expression (broken line). **b-d** The cumulative incidences of HCC after stratification by age (**b**), fibrosis stage (**c**) and albumin (Alb) level (**d**), respectively.

tients without advanced fibrosis, SLC22A7 expression can provide an important cost-effective screening tool. Moreover, we confirmed previous knowledge of low serum albumin levels as an independent risk factor for HCC development in patients matched for age, gender and stage of liver fibrosis. Nonetheless, in patients with higher serum albumin levels ( $\geq 4.0$  g/dl), reduced SLC22A7 expression remained a significant independent risk factor for HCC.

The *SLC22A7* gene encodes OAT2, which is distributed mainly in the liver and kidney. As a protein predominantly expressed in the liver [23], OAT2 transports several antiviral drugs as well as prostaglandins. A recent study in rats showed that OAT2 is responsible for the uptake of orotic acid [24], which reportedly promotes liver carcinogenesis [25, 26]. In the clinical setting, orotic aciduria was also observed in HCC patients without liver cirrhosis [27]. Moreover, a previous study using gene-set enrichment analysis revealed that SLC22A7 expression is significantly correlated with mitochondrial oxidoreductase activity and fatty acid metabolism. Mitochondrial dysfunction and oxidative stress are considered key mechanisms for the development of HCC. Collectively, these studies indicate that reduced SLC22A7 expression promotes hepatic carcinogenesis by increasing the concentration of orotic acid around hepatocytes and promoting oxidative stress and mitochondrial dysfunction. Our study suggests that these microenvironmental changes might occur in patients with chronic HCV in an early stage. As for HCC recurrence after surgical resection,

gene expression has been extensively investigated in tissues surrounding HCC [16, 28–30]. However, it remains unknown whether these signatures correlate with multifocal occurrence of HCC. Indeed, the precise mechanisms involved in the association between SLC22A7 expression and HCC development require further investigation.

In this study, personally gifted antibody was used for IHC. Staining performance of our antibody was similar to that of commercially available antibodies (Atlas Antibodies, Stockholm, Sweden) by a small pilot study (unpubl. data).

Our retrospective study design and low patient numbers must be acknowledged as limitations, particularly in the first study. However, this first study confirmed that our biopsy specimens were feasible for IHC analysis of SLC22A7, and we could therefore proceed to the larger matched-control study. To improve reproducibility, we conducted a propensity score matched study and only included patients who were HCV-positive and had not achieved SVR with interferon therapy, so our results may not pertain to chronic HCV patients who achieve SVR or patients with other chronic diseases of the liver. A larger prospective study will be required to confirm our results.

In conclusion, our study showed the importance of IHC staining for SLC22A7 as a predictive tool for HCC. We propose that patients with reduced SLC22A7 expression and lower serum albumin levels are candidates for intensive HCC surveillance, even if they do not exhibit other known risk factors.

## References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: Global cancer statistics. *CA Cancer J Clin* 2011;61:69–90.
- Llovet JM, Burroughs A, Bruix J: Hepatocellular carcinoma. *Lancet* 2003;362:1907–1917.
- Bartosch B, Thimme R, Blum HE, Zoulim F: Hepatitis C virus-induced hepatocarcinogenesis. *J Hepatol* 2009;51:810–820.
- El-Serag HB: Hepatocellular carcinoma: an epidemiologic view. *J Clin Gastroenterol* 2002;35:S72–S78.
- Imazeki F, Yokosuka O, Fukai K, Saisho H: Favorable prognosis of chronic hepatitis C after interferon therapy by long-term cohort study. *Hepatology* 2003;38:493–502.
- Bruix J, Sherman M: Management of hepatocellular carcinoma: an update. *Hepatology* 2011;53:1020–1022.
- Sun CA, Wu DM, Lin CC, Lu SN, You SL, Wang LY, Wu MH, Chen CJ: Incidence and cofactors of hepatitis C virus-related hepatocellular carcinoma: a prospective study of 12,008 men in Taiwan. *Am J Epidemiol* 2003;157:674–682.
- Ohki T, Tateishi R, Sato T, Masuzaki R, Imamura J, Goto T, Yamashiki N, Yoshida H, Kanai F, Kato N, Shiina S, Kawabe T, Omata M: Obesity is an independent risk factor for hepatocellular carcinoma development in chronic hepatitis C patients. *Clin Gastroenterol Hepatol* 2008;6:459–464.
- Moriyama M, Matsumura H, Aoki H, Shimizu T, Nakai K, Saito T, Yamagami H, Shiota A, Kaneko M, Goto I, Tanaka N, Arakawa Y: Long-term outcome, with monitoring of platelet counts, in patients with chronic hepatitis C and liver cirrhosis after interferon therapy. *Intervirology* 2003;46:296–307.
- Lok AS, Seeff LB, Morgan TR, di Bisceglie AM, Sterling RK, Curto TM, Everson GT, Lindsay KL, Lee WM, Bonkovsky HL, Dienstag JL, Ghany MG, Morishima C, Goodman ZD: Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. *Gastroenterology* 2009;136:138–148.
- Bruno S, Stroffolini T, Colombo M, Bollani S, Benvegna L, Mazzella G, Ascione A, Santantonio T, Piccinino F, Andreone P, Mangia A, Gaeta GB, Persico M, Fagioli S, Almasio PL: Sustained virological response to interferon-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. *Hepatology* 2007;45:579–587.
- Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Nakamura I, Murashima N, Kumada H, Kawanishi M: Effect of interferon therapy on hepatocellular carcinoma in patients with chronic hepatitis type C: A long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. *Hepatology* 1999;29:1124–1130.

- 13 Roayaie S, Obeidat K, Sposito C, Mariani L, Bhoori S, Pellegrinelli A, Labow D, Llovet JM, Schwartz M, Mazzaferro V: Resection of hepatocellular cancer  $\leq 2$  cm: results from two Western centers. *Hepatology* 2013;57:1426-1435.
- 14 Tsuchiya K, Komuta M, Yasui Y, Tamaki N, Hosokawa T, Ueda K, Kuzuya T, Itakura J, Nakanishi H, Takahashi Y, Kurosaki M, Asahina Y, Enomoto N, Sakamoto M, Izumi N: Expression of keratin 19 is related to high recurrence of hepatocellular carcinoma after radiofrequency ablation. *Oncology* 2011;80:278-288.
- 15 Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, Kammula US, Chen Y, Qin LX, Tang ZY, Wang XW: Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer Cell* 2006;10:99-111.
- 16 Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, Gupta S, Moore J, Wrobel MJ, Lerner J, Reich M, Chan JA, Glickman JN, Ikeda K, Hashimoto M, Watanabe G, Daidone MG, Roayaie S, Schwartz M, Thung S, Salvesen HB, Gabriel S, Mazzaferro V, Bruix J, Friedman SL, Kumada H, Llovet JM, Golub TR: Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med* 2008;359:1995-2004.
- 17 Kudo A, Mogushi K, Takayama T, Matsumura S, Ban D, Irie T, Ochiai T, Nakamura N, Tanaka H, Anzai N, Sakamoto M, Tanaka S, Arii S: Mitochondrial metabolism in the non-cancerous liver determine the occurrence of hepatocellular carcinoma: a prospective study. *J Gastroenterol* 2013, E-pub ahead of print.
- 18 Tanaka S, Mogushi K, Yasen M, Ban D, Noguchi N, Irie T, Kudo A, Nakamura N, Tanaka H, Yamamoto M, Kokudo N, Takayama T, Kawasaki S, Sakamoto M, Arii S: Oxidative stress pathways in noncancerous human liver tissue to predict hepatocellular carcinoma recurrence: a prospective, multicenter study. *Hepatology* 2011;54:1273-1281.
- 19 Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ: Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994;19:1513-1520.
- 20 Kurosaki M, Hosokawa T, Matsunaga K, Hirayama I, Tanaka T, Sato M, Yasui Y, Tamaki N, Ueda K, Tsuchiya K, Kuzuya T, Nakanishi H, Itakura J, Takahashi Y, Asahina Y, Enomoto N, Izumi N: Hepatic steatosis in chronic hepatitis C is a significant risk factor for developing hepatocellular carcinoma independent of age, sex, obesity, fibrosis stage and response to interferon therapy. *Hepatol Res* 2010;40:870-877.
- 21 Asahina Y, Tsuchiya K, Tamaki N, Hirayama I, Tanaka T, Sato M, Yasui Y, Hosokawa T, Ueda K, Kuzuya T, Nakanishi H, Itakura J, Takahashi Y, Kurosaki M, Enomoto N, Izumi N: Effect of aging on risk for hepatocellular carcinoma in chronic hepatitis C virus infection. *Hepatology* 2010;52:518-527.
- 22 Izumi N, Asahina Y, Kurosaki M, Yamada G, Kawai T, Kajiwara E, Okamura Y, Takeuchi T, Yokosuka O, Kariyama K, Toyoda J, Inao M, Tanaka E, Moriwaki H, Adachi H, Katsushima S, Kudo M, Takaguchi K, Hiasa Y, Chayama K, Yatsushashi H, Oketani M, Kumada H: Inhibition of hepatocellular carcinoma by PegIFN $\alpha$ -2a in patients with chronic hepatitis C: a nationwide multicenter cooperative study. *J Gastroenterol* 2013;48:382-390.
- 23 Simonson GD, Vincent AC, Roberg KJ, Huang Y, Iwanij V: Molecular cloning and characterization of a novel liver-specific transport protein. *J Cell Sci* 1994;107:1065-1072.
- 24 Fork C, Bauer T, Golz S, Geerts A, Weiland J, Del Turco D, Schomig E, Grundemann D: OAT2 catalyses efflux of glutamate and uptake of orotic acid. *Biochem J* 2011;436:305-312.
- 25 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;44:2186-2191.
- 26 Kankesan J, Yusuf A, Laconi E, Vanama R, Bradley G, Thiessen JJ, Ling V, Rao PM, Rajalakshmi S, Sarma DS: Effect of PSC 833, an inhibitor of P-glycoprotein, on 1,2-dimethylhydrazine-induced liver carcinogenesis in rats. *Carcinogenesis* 2003;24:1977-1984.
- 27 Jeffers LJ, Dubow RA, Zieve L, Reddy KR, Livingstone AS, Neimark S, Viamonte M, Schiff ER: Hepatic encephalopathy and orotic aciduria associated with hepatocellular carcinoma in a noncirrhotic liver. *Hepatology* 1988;8:78-81.
- 28 Villanueva A, Hoshida Y, Battiston C, Tovar V, Sia D, Alsinet C, Cornella H, Liberzon A, Kobayashi M, Kumada H, Thung SN, Bruix J, Newell P, April C, Fan JB, Roayaie S, Mazzaferro V, Schwartz ME, Llovet JM: Combining clinical, pathology, and gene expression data to predict recurrence of hepatocellular carcinoma. *Gastroenterology* 2011;140:1501-1512.e1502.
- 29 Teufel A, Marquardt JU, Galle PR: Novel insights in the genetics of HCC recurrence and advances in transcriptomic data integration. *J Hepatol* 2012;56:279-281.
- 30 Izumi N: Prediction and prevention of intrahepatic recurrence of hepatocellular carcinoma. *Hepatol Res* 2012;42:226-232.

**Title**

Risk factors for exceeding the Milan criteria after successful radiofrequency ablation in patients with early stage hepatocellular carcinoma

**Authors**

Kaoru Tsuchiya, M.D.<sup>1,2</sup>, Yasuhiro Asahina, M.D.<sup>3,4</sup>, Nobuharu Tamaki, M.D.<sup>1</sup>,

Yutaka Yasui, M.D.<sup>1</sup>, Takanori Hosokawa, M.D.<sup>1</sup>, Ken Ueda, M.D.<sup>1</sup>,

Hiroyuki Nakanishi, M.D.<sup>1,2</sup>, Jun Itakura, M.D.<sup>1</sup>, Masayuki Kurosaki, M.D.<sup>1</sup>,

Nobuyuki Enomoto, M.D.<sup>2</sup>, and Namiki Izumi, M.D.<sup>1</sup>

1. Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital,

Tokyo, Japan

2. First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan

3. Department of Gastroenterology and Hepatology, Tokyo Medical and Dental

University, Tokyo, Japan

4. Department of Liver Disease Control, Tokyo Medical and Dental University, Tokyo,

Japan

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/lt.23798

**Short title**

Risk factors exceeding the Milan criteria after RFA

**Keywords**

liver transplantation, locoregional therapy, recurrence,  $\alpha$ -fetoprotein, prognosis

**Tables and Figures**

Tables: 3, Total Figures: 2, Color Figures: 0

**Address for Correspondence:**

Namiki Izumi M.D., Ph.D.

Chief, Department of Gastroenterology and Hepatology

Musashino Red Cross Hospital

1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan

Tel.: +81-422-32-3111; Fax: +81-422-32-9551

E-mail: [nizumi@musashino.jrc.or.jp](mailto:nizumi@musashino.jrc.or.jp)

**Footnotes**

Designed the research:

Kaoru Tsuchiya, Yasuhiro Asahina, Nobuyuki Enomoto, Namiki Izumi

Wrote the paper: Kaoru Tsuchiya

Conducted the research:

Nobuharu Tamaki, Yutaka Yasui, Takanori Hosokawa, Ken Ueda, Hiroyuki Nakanishi,

Jun Itakura, Masayuki Kurosaki

Analyzed the data:

Kaoru Tsuchiya, Yasuhiro Asahina, Nobuyuki Enomoto

**Disclosure**

Dr. Asahina belongs to a donation-funded department funded by Chugai Pharmaceutical Co. Ltd., Toray Industries, Inc., Bristol-Myers Squibb, Dainippon Sumitomo Pharma Co.,Ltd., and MSD. The other authors of this manuscript declare no conflicts of interest in this study.

Address reprint requests to: Namiki Izumi, M.D., Ph.D.

Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital

1-26-1 Kyonan-cho, Musashino-shi, Tokyo, Japan

Tel.: 81-422-32-3111; Fax: 81-422-32-9551.

E-mail: nizumi@musashino.jrc.or.jp

#### Abbreviations

AASLD, American Association for the Study of Liver Diseases; AFP,  $\alpha$ -fetoprotein; CT, computed tomography; HCC, hepatocellular carcinoma; LDLT, living donor liver transplantation; MRI, magnetic resonance imaging; PIVKA-II, protein induced by vitamin K absence or antagonist II; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization



## ABSTRACT

### Background

Radiofrequency ablation (RFA) is an effective and safe noninvasive treatment for hepatocellular carcinoma (HCC) and may be useful as a bridging therapy in liver transplantation. Prognosis after liver transplantation in patients within the Milan criteria is excellent. The study aimed to identify risk factors associated with exceeding the Milan criteria after initial locally curative RFA therapy.

### Methods

Among 554 primary HCC patients, 323 with early stage HCC following RFA were analyzed (mean age, 66 years; HCV/HBV/others, 249/33/41; Child–Pugh A/B/C, 256/67/0). The cumulative overall survival and recurrence rate exceeding the Milan criteria were analyzed by Kaplan–Meier analysis, and factors associated with overall survival were determined by Cox proportional hazards analysis.

### Results

The overall cumulative survival rates at 1, 3, 5, and 10 years were 96%, 84%, 70%, and 41%, respectively, without liver transplantation. The cumulative recurrence rate exceeding the Milan criteria at 1, 3, and 5 years were 15%, 46%, and 61%, respectively.  $\alpha$ -Fetoprotein (AFP) >100 ng/mL and recurrence within 1 year after initial ablation

were independently associated with earlier recurrence exceeding the Milan criteria and overall survival. The 3- and 5-year survival rates of patients with both risk factors were 33.5% and 22.6%, respectively, in spite of early stage at initial ablation.

### Conclusions

Higher AFP and HCC recurrence within 1 year after RFA are risk factors for exceeding the Milan criteria and overall survival. Early liver transplantation or adjuvant therapy should be considered for patients with both risk factors.

## Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, accounting for 70%–85% of all cases, and a major cause of mortality; it is the fifth most frequently diagnosed cancer and the second most frequent cause of cancer death in men.

In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death (1, 2). At present, the major curative treatments for HCC consist

of hepatic resection, ablation therapy, and liver transplantation (3). Although hepatic

resection and ablation therapy often show excellent effects in HCC, they cannot prevent

recurrence in the remnant liver or eliminate other complications caused by concurrent

liver cirrhosis. On the other hand, liver transplantation has become a favored option for

HCC treatment because it provides not only local cure but also decreases the risks for

recurrence and progressive liver disease. Liver transplantation for cirrhotic HCC

patients who meet the Milan criteria (4) [solitary tumor  $\leq$  50mm or three or fewer

lesions (none > 30mm)] offers long-term survival similar to that observed in patients

transplanted for nonmalignant liver disease (5, 6). Some recent studies (7-9) reported

that radiofrequency ablation (RFA) is an effective and safe noninvasive treatment for

HCC, enabling complete ablation of an area up to 3 cm in diameter and is superior to

microwave coagulation and percutaneous ethanol injection therapy. In a recent study

(10), for recurrent HCC within the Milan criteria, the 1-, 3-, and 5-year tumor-free survival rates for salvage liver transplantation were all 60%; the corresponding rates were 70.2%, 48.0%, and 48.0% for hepatic resection and 41.0%, 20.3%, and 10.9% for RFA ( $P=0.004$ ). The patients in this study underwent either hepatic resection or RFA as an initial treatment for HCC within the Milan criteria. Therefore, it is very important to know when patients exceed the Milan criteria after initial RFA as a locally curative therapy for HCC. Hence, the aims of the present study were to identify the risk factors associated with recurrence exceeding the Milan criteria and clarify prognostic factors for overall survival in early stage HCC patients who received RFA as an initial therapy.

## MATERIALS and METHODS

### Patients

Between July 1999 and July 2005, 554 primary HCC patients were admitted to the Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital (Tokyo, Japan). The patients received the following appropriate therapies according to the appropriate guidelines released during study period by the Liver Cancer Study Group of Japan and BCLC staging system (11): 323 were treated by RFA, 35 by surgical resection,