hypothesis is supported by the demonstration that plasma VEGF concentrations increased shortly after treatment with TACE.[24-26] It is believed that these increases in plasma VEGF concentration are related to the induction of tissue hypoxia.[27] However, the peak time point of VEGF elevation during sorafenib administration was different from that previously reported in TACE, in which a transient elevation of VEGF was observed within 7 days after TACE.[24-26] This observed difference may be related to the continuous induction of hypoxia by sorafenib administration.

It is noteworthy that, in our study, decreases in plasma VEGF observed within 8 weeks of sorafenib administration were associated with better OS. One possible reason for this association may be that the decrease in VEGF concentrations reflects a decrease in the number of tumor cells secreting VEGF. An association between changes in VEGF concentrations and disease progression was observed in a previous study of an anti-VEGF antibody, bevacizumab, in patients with advanced HCC.[23] In that study, plasma VEGF-A concentrations decreased from baseline in all patients after 8 weeks of bevacizumab therapy and increased to near baseline levels in 5 of 6 patients at the time of disease progression. Unfortunately, plasma VEGF-A levels after 8 weeks of bevacizumab in that study were available for only 8 of 46 patients who were enrolled the study, and plasma VEGF-A levels after 4 weeks were not evaluated. In our study, all patients were evaluated before and every 4 weeks after starting sorafenib. Moreover, we demonstrated the usefulness of plasma VEGF concentrations at 8 weeks and not at 4 weeks. Zhu et al[28] reported that plasma levels of VEGF and placental growth factor increased after cediranib, a pan-VEGFR tyrosine kinase inhibitor monotherapy for advanced HCC. In that study, progression-free survival was correlated inversely with baseline levels of VEGF, soluble VEGFR2 (sVEGFR2), and basic fibroblast growth factor and with on-treatment levels of basic fibroblast growth factor and insulin-like growth factor-1; and progression-free survival was directly associated with on-treatment levels of interferon-γ. Because changes of VEGF concentrations during therapy were not identified as a prognostic factor in the study by Zhu et al, biomarkers that predict prognosis may be different among different types of tyrosine kinase inhibitors. Jayson et al[29] reported that plasma VEGF-A in patients who received bevacizumab was potentially predictive and prognostic in metastatic breast, gastric, and pancreatic cancers; however, it was only prognostic (and not predictive) in metastatic colorectal cancer, nonsmall cell lung cancer, and renal cell carcinoma. In our study, we measured plasma VEGF concentrations and not plasma VEGF-A concentrations. Sorafenib is a multikinase inhibitor, whereas bevacizumab is a humanized monoclonal antibody that recognizes and blocks VEGF-A expression. Further studies to evaluate the clinical usefulness of determining VEGF and VEGF-A concentrations during sorafenib therapy are necessary in various cancers. Although the precise mechanism underlying the association between serial changes in VEGF and disease progression is unclear, the findings of the current study are extremely valuable for clinical practice in predicting the prognosis of patients who receive treatment with sorafenib.

Llovet et al[5] studied plasma biomarkers as predictors of outcome in patients with advanced HCC. They measured plasma biomarkers in 491 patients at baseline and in 305 patients after 12 weeks in a phase 3, randomized, controlled trial (the SHARP trial). Those authors concluded that angiopoietin-2 and VEGF were independent predictors of survival in patients with advanced HCC and that none of the tested biomarkers significantly predicted response to sorafenib. In our study, by measuring plasma VEGF monthly, we demonstrated that the changes 8 weeks after starting sorafenib were important for predicting OS.

It has been reported that modified RECIST guidelines are useful for predicting efficacy and prognosis after patients with advanced HCC receive treatment with sorafenib.[30] However, modified RECIST can only be used for typical hypervascular HCC, and not for atypical HCC, including poorly differentiated HCC and diffuse-type HCC. Moreover, the percentage of patients in our study who had PD was only 11.1% (9 of 63 patients), and the objective response rate (CR + PR vs SD) could not predict OS, suggesting that using only modified RECIST guidelines was insufficient for predicting OS in most patients who received sorafenib (non-PD patients). Therefore, it is important to identify a predictive biomarker for those patients who can expect long survival during sorafenib therapy, although their radiologic findings may not be categorized as objective responses.

From this point of view, decreases in VEGF observed in non-PD patients at week 8 may identify patients who have a favorable prognosis. According to our results, the median survival of patients who had a VEGF decrease was extremely good at 31.0 months, and we demonstrated that a VEGF decrease, but not modified RECIST or AFP, was the only significant post-therapeutic factor associated with favorable survival after sorafenib administration (Table 3). In our study, all

patients who had both a VEGF decrease and an AFP response survived during the observation period (median, 19.7 months). Taken together, the combination of a plasma VEGF decrease, an AFP response, and modified RECIST is useful for predicting an extremely favorable prognosis.

This study had a few limitations. The first was our subanalysis of consecutive patients. However, the median survival for the 23 excluded patients who were available for estimation was equivalent to that of the included patients (16.8 months); therefore, it is unlikely that selection bias affected our results. The second limitation is that we measured only plasma VEGF concentrations. In previous studies, many factors, including VEGF-A, short VEGF-A isoform, sVEGFR1, sVEGFR2, sVEGFR3, angiopoietin-2, and insulin-like growth factor-2, were evaluated as biomarkers. However, to our knowledge, this is the first clinical study to demonstrate the early dynamic changes in plasma VEGF concentrations in patients who received sorafenib. Finally, the number of patients in this study was relatively small to make recommendations to physicians. Our results indicated that patients who have decreased VEGF concentrations at 8 weeks have a favorable prognosis, regardless of their radiologic findings. However, further studies with a larger number of patients will be necessary to propose new recommendations.

In conclusion, changes in plasma VEGF concentrations during sorafenib treatment are dynamic in patients with advanced HCC, and an observed decrease in the plasma VEGF concentration 8 weeks after starting sorafenib is associated significantly with favorable OS. Today, because many clinical trials of new molecular-targeted agents for HCC are being conducted, it is necessary for hepatologists and oncologists to determine the time when alternative agents should be started as a second or third line of treatment. Our results have potentially important clinical implications for physicians and may influence their decisions regarding a treatment strategy for advanced HCC in individual patients.

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CONFLICT OF INTEREST DISCLOSURES

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ORIGINAL ARTICLE

Risk Factors for Exceeding the Milan Criteria After Successful Radiofrequency Ablation in Patients With Early-Stage Hepatocellular Carcinoma

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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. The prognosis after liver transplantation for patients within the Milan criteria is excellent. This study was aimed at identifying risk factors associated with exceeding the Milan criteria after initial locally curative RFA therapy. Among 554 primary HCC patients, 323 with early-stage HCC after RFA were analyzed (mean age = 66 years). Two hundred forty-eight patients had hepatitis C virus, 33 patients had hepatitis B virus, and 41 patients had neither hepatitis B nor hepatitis C; 256, 67, and 0 patients were classified as Child-Pugh A, B, and C, respectively. The rates of cumulative overall survival and recurrence exceeding the Milan criteria were analyzed with Kaplan-Meier analysis, and factors associated with overall survival were determined with Cox proportional hazards analysis. The cumulative overall survival rates at 1, 3, 5, and 10 years were 96.2%, 84.4%, 69.9%, and 40.6% respectively, without liver transplantation. The cumulative rates of recurrence exceeding the Milan criteria at 1, 3, and 5 years were 15.1%, 46.0%, and 61.1% respectively. An alpha-fetoprotein (AFP) level > 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 for patients with both risk factors were 33.5% and 22.6%, respectively, despite an early stage at initial ablation. In conclusion, a higher AFP level and HCC recurrence within 1 year of RFA are risk factors for exceeding the Milan criteria and for overall survival. Early liver transplantation or adjuvant therapy should be considered for patients with both risk factors. *Liver Transpl 20:291-297, 2014.* © 2013 AASLD.

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Abbreviations: AFP, alpha-fetoprotein; CI, confidence interval; CLIP, Cancer of the Liver Italian Program; CT, computed tomography; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; PIVKA-II, protein induced by vitamin K absence or antagonist II; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization.

Kaoru Tsuchiya, Yasuhiro Asahina, Nobuyuki Enomoto, and Namiki Izumi designed the research. Kaoru Tsuchiya wrote the article. Nobuharu Tamaki, Yutaka Yasui, Takanori Hosokawa, Ken Ueda, Hiroyuki Nakanishi, Jun Itakura, and Masayuki Kurosaki conducted the research. Kaoru Tsuchiya, Yasuhiro Asahina, and Nobuyuki Enomoto analyzed the data.

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See Editorial on Page 257

Accounting for 70% to 85% of all cases, hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver 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 on 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 not only provides a local cure but also decreases the risks for recurrence and progressive liver disease. Liver transplantation for HCC patients with cirrhosis who meet the Milan criteria[4] (a solitary tumor $\leq$ 50 mm or 3 or fewer lesions, none > 30 mm) offers long-term survival similar to that observed for patients undergoing transplantation for nonmalignant liver disease.[5,6] Some recent studies[7-9] have reported that radiofrequency ablation (RFA) is an effective and safe noninvasive treatment for HCC, enables 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] the 1-, 3-, and 5-year tumor-free survival rates were all 60% for salvage liver transplantation for recurrent HCC within the Milan criteria; 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 that 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 to clarify prognostic factors for overall survival for early-stage HCC patients receiving RFA as an initial therapy.

PATIENTS AND METHODS

Patients

Between July 1999 and July 2005, 554 primary HCC patients were admitted to the Department of Gastroenterology and Hepatology at Musashino Red Cross Hospital (Tokyo, Japan). The patients received the following appropriate therapies according to the appropriate guidelines released during the study period by the Liver Cancer Study Group of Japan and according to the Barcelona Clinic Liver Cancer staging system[11]: 323 were treated with RFA, 35 were treated with surgical resection, 158 were treated with transcatheter arterial chemoembolization (TACE), 10 were treated with systemic cytotoxic chemotherapy, 2 were treated with percutaneous microwave coagulation, 4 were treated with percutaneous ethanol injection therapy, 2 were treated with radiation therapy, and 20 were treated with the best supportive care. There were no patients who underwent liver transplantation. Among these 554 patients, 323 were treated with RFA as an initial curative therapy for primary HCC, and they were included in the following analyses. The inclusion criteria for RFA were as follows: a solitary HCC tumor-$\leq$ 50 mm or 3 or fewer lesions, none > 30 mm; 3 or fewer lesions without major vascular or biliary invasion; a total bilirubin concentration < 2.5 mg/dL; a platelet count > $3 \times 10^4/mm^3$; and prothrombin activity > 50%. Some patients refused hepatic resection and chose RFA voluntarily because of concerns about complications or physician recommendations, which took into account impairment of liver function, HCC location, and cardiopulmonary dysfunction. Patients who had ascites uncontrolled by diuretics and/or had extrahepatic metastases were excluded. The reasons that the patients were selected for RFA instead of liver transplantation were as follows: a Child-Pugh classification of A (n = 256 or 79.3%), an age > 65 years (n = 198 or 61.3%), and heart or lung disease complications (n = 6 or 1.9%). The number of patients who were classified as Child-Pugh B and were younger than 66 years of age was 28 (8.7%). Among these patients, there was 1 patient who had severe heart disease; the remaining 27 patients did not have living donors. Written informed consent was obtained from all patients, and this study was approved by the ethics committee of Musashino Red Cross Hospital and was conducted in accordance with the Declaration of Helsinki.

HCC Diagnosis

The HCC diagnosis was confirmed by typical radiographic findings on dynamic computed tomography (CT) with or without hepatic arterial and portal angiography and magnetic resonance imaging or by needle biopsy. For triple-phase dynamic CT scans, arterial, portal, and equivalent phases were set at 35, 70, and 150 seconds, respectively, after the injection of the contrast agent. Spiral CT scans were obtained from 5-mm-thick sections. Board-certified radiologists diagnosed HCC on the basis of typical patterns, such as an early-phase hyperattenuation area or late-phase hypoattenuation on dynamic CT or magnetic resonance imaging. Liver biopsy was performed when a definitive diagnosis was not provided by imaging techniques, and the final diagnosis was confirmed by certified pathologists who were unaware of the patient's clinical data.

RFA Procedure

RFA was performed under local anesthesia with the percutaneous approach (n = 279) or under general anesthesia with the laparoscopic approach (n = 44);

both were used under real-time ultrasound guidance. The laparoscopic approach was selected for patients with HCC located on or near the liver surface.[12] We used an internally water-cooled 17-gauge cooled-tip electrode with an impedance-controlled generator (Cosman generator, Cool-Tip system, Radionics, Burlington, MA). Ultrasonography was performed with a 3.0- to 6.0-MHz convex probe and the Aloka SSD-5500 (Aloka, Tokyo, Japan), Sonoline Elegra (Siemens, Erlangen, Germany), and Aplio XV systems (Toshiba Medical Systems, Tokyo, Japan). When the target nodule was >20 mm in diameter, we performed multiple needle insertions and multiple ablations of 1 nodule.

Assessment of Treatment Efficacy and Follow-Up

A dynamic CT scan with a section thickness of 5 mm was performed to evaluate the efficacy of ablation 1 to 3 days after RFA. Complete HCC ablation was defined as hypo-attenuation of the entire tumor. Patients whose ablation was judged to be incomplete received additional therapy 1 week after the first ablation, which was continued until the treatment was judged to be completely effective. Blood was sampled every 2 to 3 months and tested for indicators of liver function and the markers alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II). A dynamic CT scan was scheduled every 3 to 4 months, and chest CT or bone scintigraphy was performed if extrahepatic recurrence was suspected. HCC recurrence was defined as the detection of an early enhanced lesion by dynamic CT scanning concomitant with late washout. Local tumor progression was defined as the appearance of viable cancer tissue touching the initially treated tumor and distant recurrence separated from the primary site. When intrahepatic HCC recurrence was detected, RFA was performed if the recurrence met the initial inclusion criteria. If there was no indication for RFA, we chose TACE, percutaneous ethanol injection therapy, surgical resection, systemic chemotherapy, or symptomatic therapy according to the guidelines established by the Liver Cancer Study Group of Japan[11] and the American Association for the Study of Liver Diseases.[3] The end of follow-up was tumor progression beyond the Milan criteria, death, or latest medical attendance up to March 31, 2012.

Statistical Analysis

The primary endpoint of the present analysis was tumor progression beyond the Milan criteria, and the secondary endpoint was death. The cumulative incidences of recurrence exceeding the Milan criteria and survival after successful initial RFA were determined with the Kaplan-Meier method, and the risk factors associated with recurrence exceeding the Milan criteria and death were identified with a Cox proportional hazards regression model independently for tumor progression and death. The survival analysis was performed on a per-patient basis. The starting date for follow-up was defined as the completion date of the initial RFA session. Multivariate analysis was performed with a Cox proportional hazards model and included variables with a marginal P value < 0.05 according to univariate analysis. All statistical analyses were performed with StatView 5.0 (SAS, Inc., Cary, NC).

TABLE 1. Patient Characteristics (n = 323)

Characteristic	Value
Age (years)*	66 ± 9
Follow-up (years)†	4.0 (0.6-12.2)
Sex [n (%)]	
Male	191 (59.1)
Female	132 (40.9)
Clinical and laboratory data	
AFP (ng/mL)†	25.6 (1.2-76,600)
PIVKA-II level (mAU/mL)†	25.0 (7.0-10,600)
Child-Pugh score [n (%)]	
A	256 (79.3)
B	67 (20.7)
Pathology	
Maximum HCC diameter [n (%)]	
≤20 mm	117 (36.2)
21-30 mm	158 (48.9)
31-50 mm	48 (14.9)
Number of HCC nodules [n (%)]	
Single	226 (70.0)
Multiple	97 (30.0)
CLIP score [n (%)]	
0	174 (53.6)
1	114 (35.2)
2	32 (9.9)
3	3 (0.9)
Lymph node involvement (%)	0
Metastasis (%)	0
Major associated liver diseases [n (%)]	
HCV	248 (76.8)
HBV	31 (9.6)
HCV + HBV	3 (0.9)
Other	41 (12.7)

*The data are presented as the mean and standard deviation.
†The data are presented as the median and range.

RESULTS

The patient characteristics are shown in Table 1. The minimum follow-up period was 7 months, and the median follow-up period was 4.0 years (0.6–12.2 years). During follow-up, HCC recurred in 270 of the 323 patients (83.6%), and local tumor progression was observed in 47 patients (14.6%). Tumor progression beyond the Milan criteria was observed in 193 patients; 174 of these patients (90.2%) died because of tumor progression, and 19 (9.8%) died without tumor progression. The cumulative survival rates at 1, 3, 5, 7, and 10 years were 96.2%, 84.4%, 69.9%, 52.7%, and 40.6%, respectively. The cumulative rates of recurrence exceeding the Milan criteria at 1, 3, and

TABLE 2. Cox Proportional Hazards Analysis for Recurrence Exceeding the Milan Criteria (n = 323)

Factor	Univariate Analysis: *P* Value	Multivariate Analysis: *P* Value	Multivariate Analysis: HR (95% CI)
Age > 65 years	0.64		
Child-Pugh score: B versus A	0.10		
AFP level > 100 ng/mL	<0.001	0.006	1.59 (1.14-2.23)
PIVKA-II level > 100 mAU/mL	<0.001	0.21	1.26 (0.87-1.84)
Tumor size > 20 mm	0.003	0.01	1.54 (1.09-2.16)
Tumor number > 2	0.29		
Early recurrence*	<0.001	<0.001	2.76 (2.05-3.71)

*Within the first year after RFA.

TABLE 3. Cox Proportional Hazards Analysis for Overall Survival (n = 323)

Factor	Univariate Analysis: *P* Value	Multivariate Analysis: *P* Value	Multivariate Analysis: HR (95% CI)
Age > 65 years	0.64		
Child-Pugh score: B versus A	<0.001	<0.001	2.42 (1.61-3.64)
AFP level > 100 ng/mL	<0.001	<0.001	2.03 (1.37-3.00)
PIVKA-II level > 100 mAU/mL	0.14		
Tumor size > 20 mm	0.94		
Tumor number > 2	0.004	0.06	1.45 (0.99-2.13)
Early recurrence*	<0.001	<0.001	2.09 (1.43-3.03)

*Within the first year after RFA.

5 years were 15.1%, 46.0%, and 61.1%, respectively. Major complications were observed in only 2 cases (0.6%): one was gastric penetration after the ablation of segment 2, and the other was hemothorax after the ablation of segment 7. Both patients recovered without surgery.

Risk Factors for Exceeding the Milan Criteria and Overall Survival

A univariate analysis showed that a higher AFP level (>100 ng/mL), a higher PIVKA-II level (>100 mAU/mL), a larger tumor size (diameter > 20 mm), and an earlier recurrence of intrahepatic lesions (within 1 year of initial RFA) were significantly associated with the risk for recurrence exceeding the Milan criteria (Table 2). A multivariate analysis with a Cox proportional hazards model indicated that a higher AFP level [hazard ratio (HR) = 1.59, $P = 0.006$], a larger tumor size (HR = 1.54, $P = 0.012$), and early recurrence within 1 year of initial RFA (HR = 2.76, $P < 0.001$) were independent risk factors associated with recurrence exceeding the Milan criteria (Table 2). No association was observed between recurrence exceeding the Milan criteria and the Child-Pugh score. Risk factors associated with overall survival are shown in Table 3. A multivariate analysis with a Cox proportional hazards model indicated that a higher initial AFP level (HR = 2.03, $P < 0.001$), Child-Pugh class B (HR = 2.42, $P < 0.001$), and early recurrence within 1 year of initial RFA (HR = 2.09, $P < 0.001$) were independent risk factors associated with overall survival. There was no significant difference in overall survival or recurrence exceeding the Milan criteria between the patients whose imaging findings according to the modified Response Evaluation Criteria in Solid Tumors 3 months after RFA indicated an noncomplete response (n = 11) and the patients with a complete response (n = 312).

Predictability of the Long-Term Survival Rate and Recurrence Exceeding the Milan Criteria by Risk Group

To predict long-term survival and recurrence exceeding the Milan criteria, we formed risk groups on the basis of 2 relevant clinical predictors: the initial tumor marker (AFP level > 100 ng/mL) and the presence of earlier recurrence. The probability within the Milan criteria according to these predictors are shown in Fig. 1, and the cumulative survival rates are shown in Fig. 2. The 3- and 5-year survival rates for patients with both risk factors were 33.5% and 22.6%, respectively, although the patients were initially treated with

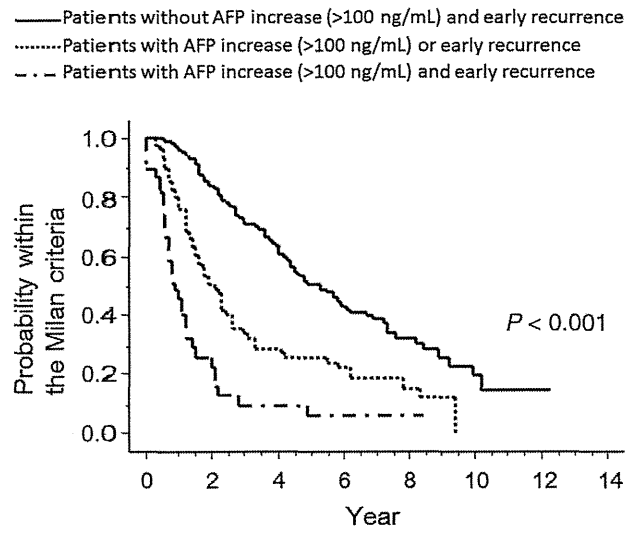

Figure 1. Probability within the Milan criteria stratified by the number of risk factors. Probability within the Milan criteria for patients with more risk factors was significantly lower than the rate for patients with fewer risk factors ($P < 0.001$).

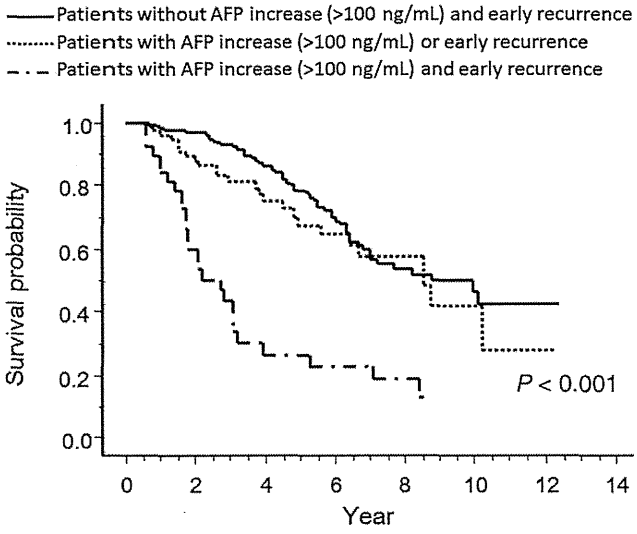

Figure 2. Cumulative survival rate stratified by the number of risk factors. The cumulative survival rate for patients with more risk factors was significantly lower than the rate for patients with fewer risk factors ($P < 0.01$).

RFA for early-stage HCC. Probability within the Milan criteria for the low-risk patients (n = 203) who had none of the risk factors (initial AFP and early recurrence) at 1, 3, and 5 years were 95.9%, 70.7%, and 51.1%, respectively, and the cumulative survival rates for the low-risk group at 1, 3, 5, 7, and 10 years were 98.5%, 93.1%, 78.0%, 56.5%, and 46.6%, respectively.

DISCUSSION

In the present study, long-term survival after RFA was similar to that for patients undergoing hepatic resection[13-17] and especially for patients with early-stage HCC. Moreover, major complications were observed in only 0.6% of the patients, and this indicates that RFA has considerable merit with respect to both effectiveness and safety. The overall outcomes were similar to those in a study by Tateishi et al.,[7] who reported a 5-year survival rate of 54.3% and a major complication rate of 1.9% per session. Ogihara et al.[16] reported that RFA was less invasive and was associated with a lower complication rate and lower costs in comparison with resection. Their data also indicated that RFA was effective in ensuring local control of stage T1 HCC and was associated with survival rates similar to those obtained with surgical resection. Cucchetti et al.[18] verified that RFA was more cost-effective than resection for patients with very early HCC and in the presence of 2 or 3 nodules ≤ 30 mm, and for patients with single larger early-stage HCCs, surgical resection remained the best strategy to adopt as a result of better survival rates at an acceptable increase in cost.

Llovet et al.[19] reported that RFA was a useful bridging therapy for liver transplantation because a higher dropout rate (38%/year) was reported for patients without adequate adjuvant therapy for HCC. In a recent study of recurrent HCC within the Milan criteria,[10] the 1-, 3-, and 5-year tumor-free survival rates for salvage liver transplantation were all 60%, and excellent 10-year survival would be expected for these patients. Therefore, it is very important to clarify the risk factors associated with exceeding the Milan criteria after locally curative RFA. We determined the probability and risk factors for tumor progression beyond the Milan criteria after successful locally curative RFA for primary HCC. Our results showed a rate of recurrence exceeding the Milan criteria of 15.1% at 1 year and a rate of 46.0% at 3 years, and patients who had a larger tumor size (diameter > 20 mm) and/or a higher AFP level (>100 ng/mL) at their initial presentation and early recurrence after initial RFA were at a high risk for recurrence exceeding the Milan criteria. Therefore, in such high-risk patients, RFA should be carefully considered as a bridging therapy for liver transplantation, and the physician should follow these patients carefully for tumor progression even after successful initial RFA.

We reported that keratin 19 expression was related to a high rate of recurrence of HCC after RFA in 249 patients,[20] and Ziol et al.[21] reported that endothelial cell–specific molecule 1 in stromal cells was predictive of recurrence after RFA for early HCC in 150 patients. However, there is no HCC-specific biomarker that can be measured to link the post-RFA biology to recurrence and outcomes and that is better than serum AFP. Tateishi et al.[22] reported on the prediction of the recurrence of HCC after RFA in 416 patients. Tumor marker levels were determined immediately before and 2 months after the treatment. The timing and frequency of measuring AFP would be 2 months after RFA and then every 2 to 3 months.

There were no significant differences in the rates of overall survival or recurrence exceeding the Milan

criteria among patients with hepatitis C virus (HCV), hepatitis B virus (HBV), and patients who had neither HBV nor HCV (NBNC). Among patients with HCV (n = 248), a larger tumor size (diameter > 20 mm), an AFP level > 100 ng/mL, and recurrence within 1 year after the initial ablation were independently associated with earlier recurrence exceeding the Milan criteria. An AFP level > 100 ng/mL and recurrence within 1 year of the initial ablation were independently associated with overall survival. In patients with HBV (n = 31), an AFP level > 100 ng/mL was the only independent factor that was associated with overall survival. In patients with NBNC (n = 41), recurrence within 1 year after the initial ablation was the only independent factor that was associated with earlier recurrence exceeding the Milan criteria. The patients who were positive for both hepatitis B surface antigen and HCV antibodies (n = 3) were excluded from this analysis. However, the number of patients positive for hepatitis B surface antigen or negative for both hepatitis B surface antigen and HCV antibodies were too small to clarify the differences due to the underlying cause of liver disease.

In the initial study population of 554 primary HCC patients, the 35 patients who underwent surgical resection were Child-Pugh A patients or patients without cirrhosis, so they could not undergo liver transplantation. The 158 patients who received TACE, the 10 patients who received systemic cytotoxic chemotherapy, the 20 patients who received the best supportive care, and the 2 patients who received radiation therapy exceeded the Milan criteria. The remaining 6 patients were more than 65 years old and could not undergo liver transplantation. We did not include the patients who received TACE as an initial therapy in this study because they already exceeded the Milan criteria. The number of patients who received other therapies (resection, microwave coagulation therapy, and percutaneous ethanol injection) was too small for an analysis of recurrence and prognosis.

In our study, the incidence rate for exceeding the Milan criteria was similar to the data reported by Yamashiki et al.,[23] whose overall rates of recurrence exceeding the Milan criteria were 9.0% and 32.8% at 1 and 3 years, respectively. Similarly to us, they found that a high serum level of AFP or PIVKA-II and a tumor diameter > 30 mm affected recurrence exceeding the Milan criteria as a result of tumor progression. An elevated AFP level may be related to the histological grading. Parfitt et al.[24] reported that the histological grade of tumor differentiation and macroscopic vascular invasion were independent predictors of long-term survival after liver transplantation. However, the most significant risk factor in our cohort was early recurrence after initial RFA. This suggests that careful surveillance for recurrence is necessary even after complete local ablation, and if early recurrence occurs within 1 year, liver transplantation should be considered as soon as possible to prevent the loss of the indication, even in patients whose initial tumor size and number are small. Importantly, liver function tests, such as albumin levels and prothrombin activity, were not identified as risk factors for recurrence exceeding the Milan criteria in our cohort, and this suggests that preserved liver function itself does not necessarily indicate that there has been adequate waiting time.

Here we calculated the risk score from 2 simple factors: the initial tumor marker and early recurrence after initial complete RFA. The 3- and 5-year survival rates for patients with both risk factors were 33.5% and 22.6%, respectively, despite an early stage at initial ablation. Conversely, the 3- and 5-year survival rates for patients with neither risk factor were 93.1% and 78.0%, respectively. The number of patients with both risk factors was small (12.1%); however, new therapeutic strategies (early transplantation or repeated adjuvant therapy) were necessary to achieve long-term survival.

Takada et al.[25] reported that repeated nontransplant treatments for recurrent HCC such as RFA and transluminal arterial embolization before living donor liver transplantation might increase the risk of recurrence and impair the survival advantage conferred by living donor liver transplantation. Because our study was focused mainly on recurrence exceeding the Milan criteria, we did not assess whether RFA performed before liver transplantation affected the final outcomes of patients who actually underwent liver transplantation. Therefore, further controlled studies are warranted to confirm whether bridging therapy with RFA actually leads to better survival after transplantation. Nevertheless, liver transplantation should be considered before the patient exceeds the Milan criteria in order to achieve excellent survival after liver transplantation.

In conclusion, RFA presents a promising bridging therapy for liver transplantation in patients who are at low risk of tumor progression. However, patients with a higher AFP level at the time of initial RFA and with earlier recurrence even after successful RFA should be considered for timely liver transplantation or new adjuvant therapy. For these patients, the 3- and 5-year survival rates were less than 50%, although they were classified as early-stage at the time of initial therapy.

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

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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.

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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 μm 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: ≤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-

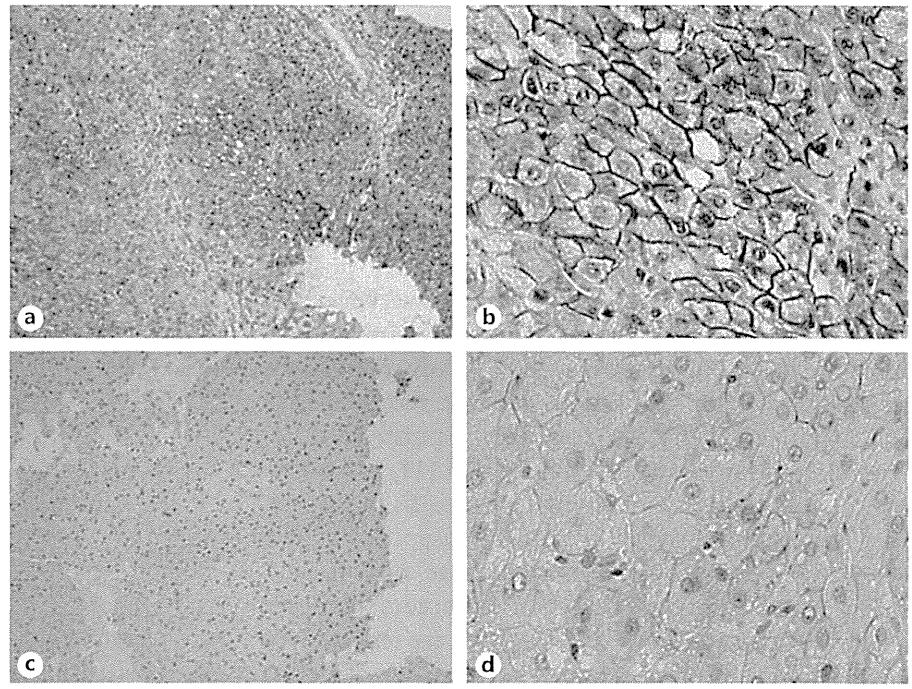

Fig. 1. IHC analysis of SLC22A7 in biopsy specimens. **a**, **b** Normal SLC22A7 expression (≥25% positive cells) **a** ×100. **b** ×400. **c**, **d** Reduced SLC22A7 expression (<25% positive cells). **c** ×100. **d** ×400.

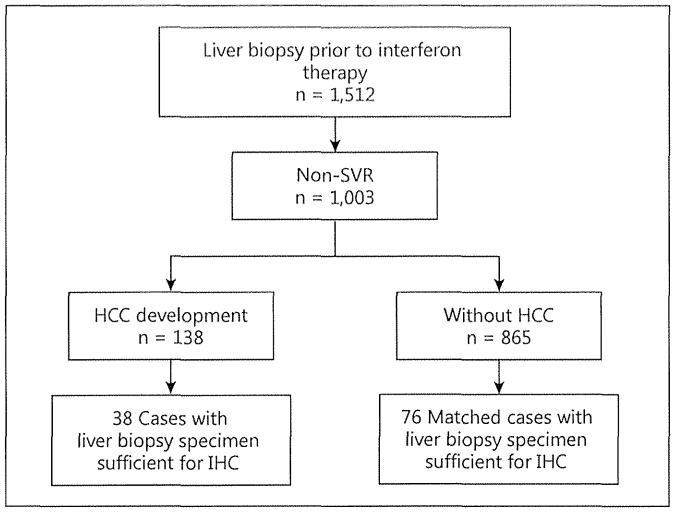

Fig. 2. Consort diagram of stratified analyses.

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

sponse (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 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[a]	6.8 (3.7–106)	19.3 (5.9–87.3)	0.46
DCP, mAU/ml[a]	32 (14–129)	15 (14–26)	0.15

ALT = Alanine aminotransferase; DCP = des-gamma-carboxy prothrombin.
[a] 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, $10^4/\mu l$	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.

pensity 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

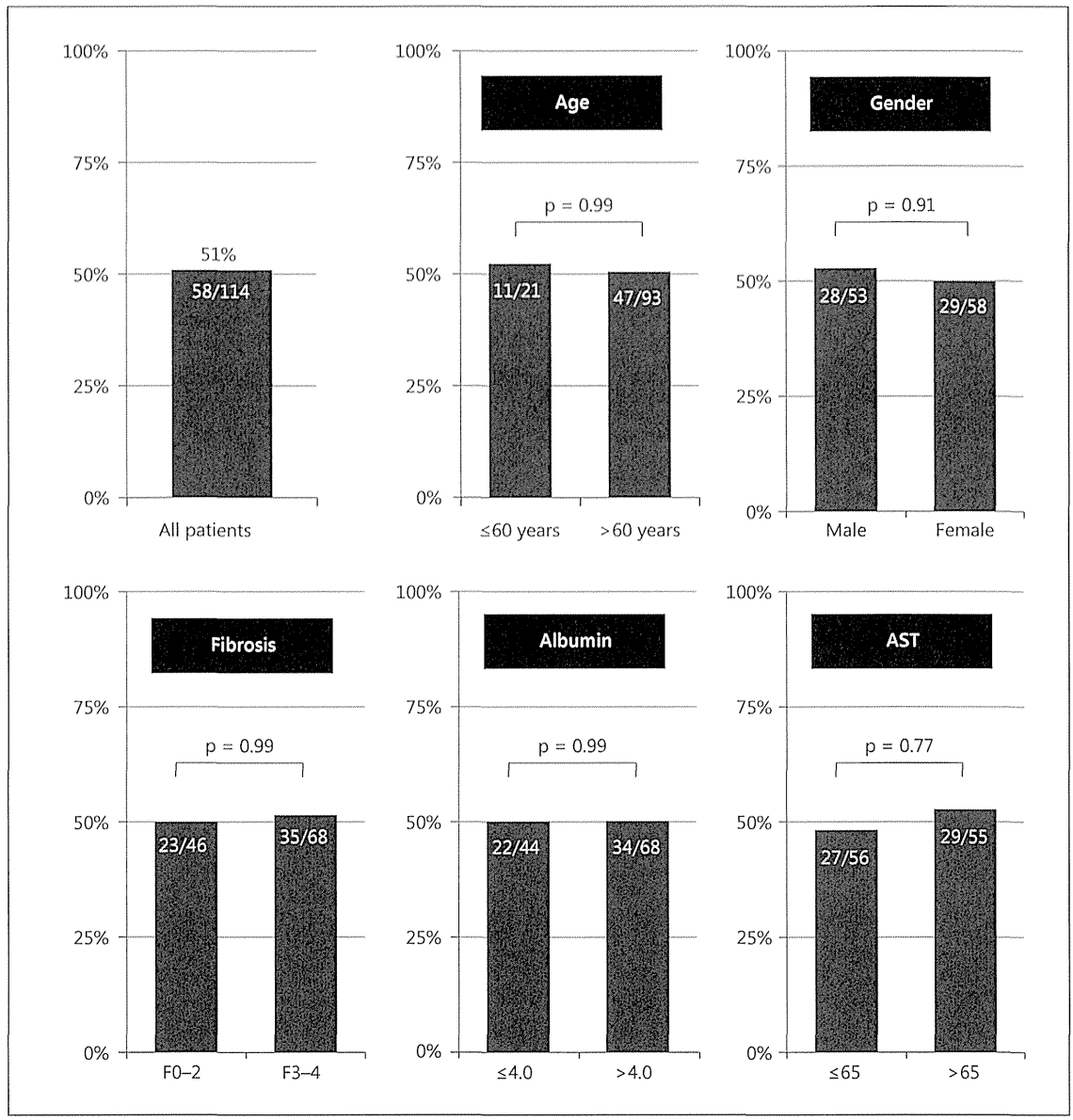

Fig. 4. Percentage of patients with normal SLC22A7 expression according to baseline clinical findings. No significant differences in the percentage of patients with normal SLC22A7 expression were observed after stratification by age, gender, fibrosis stage, albumin and/or AST.

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 revealed 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 ≥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-

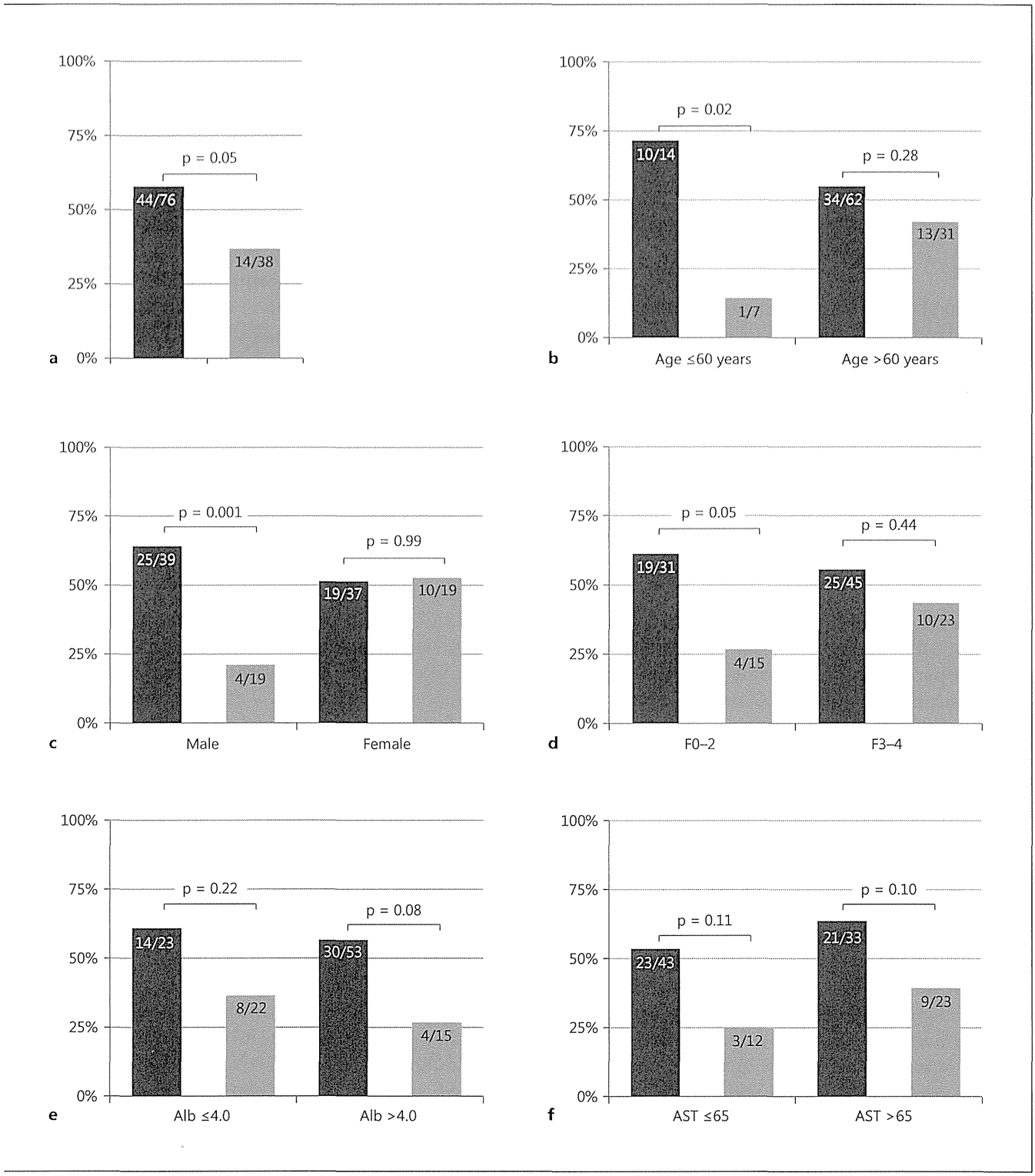

Fig. 5. Percentage of patients with normal SLC22A7 expression and HCC (**a**). SLC22A7 staining was compared between patients who did and did not develop HCC after stratification by age (**b**), gender (**c**), fibrosis stage (**d**), albumin (Alb, **e**) and AST levels (**f**). Light grey and dark grey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

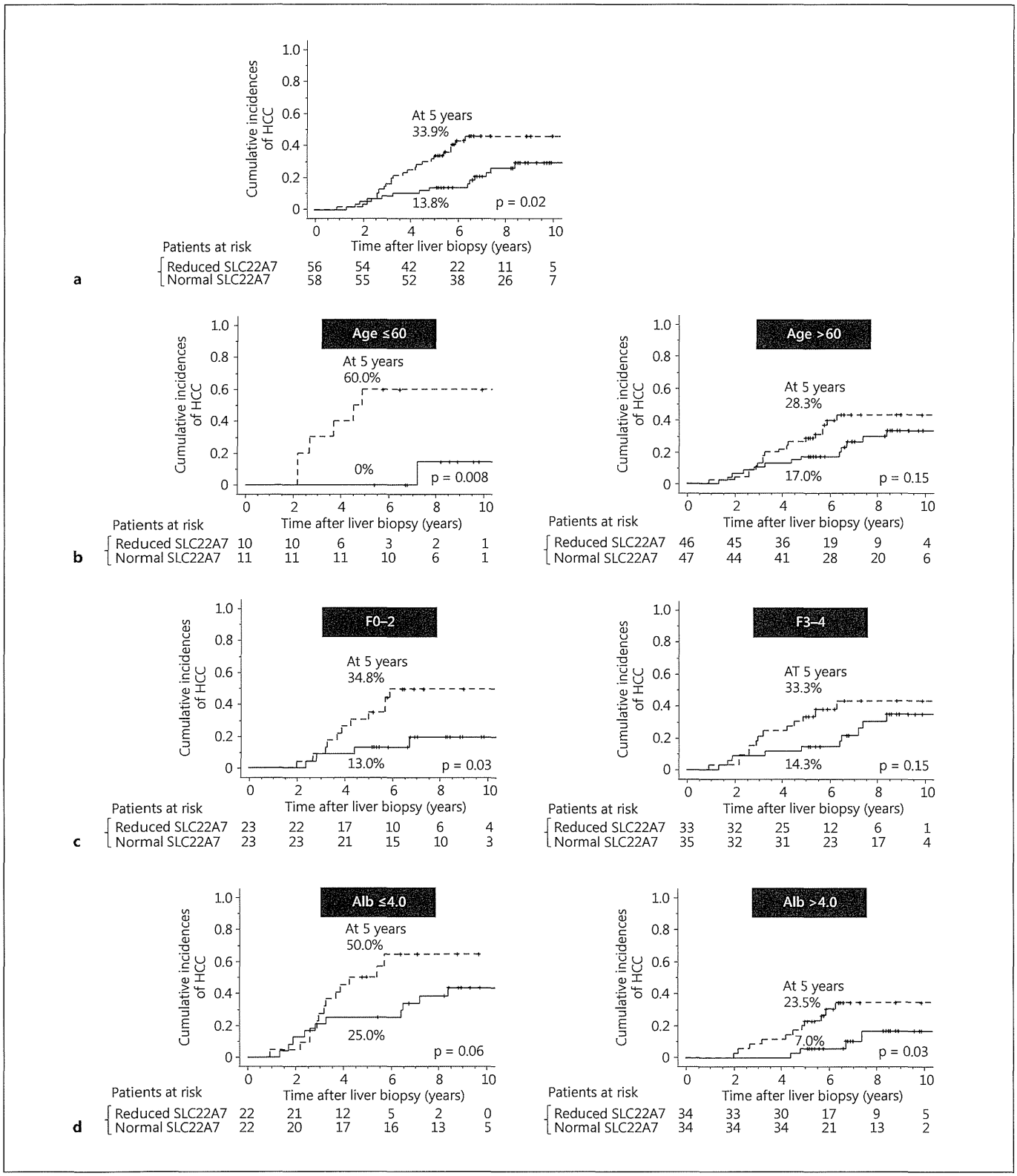

Fig. 6. Cumulative incidence of HCC according to SLC22A7 staining. **a** Comparison of the cumulative incidences of HCC in patients with normal (solid line) and reduced SLC22A7 expression (broken line). **b–d** The cumulative incidences of HCC after stratification by age (**b**), fibrosis stage (**c**)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

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