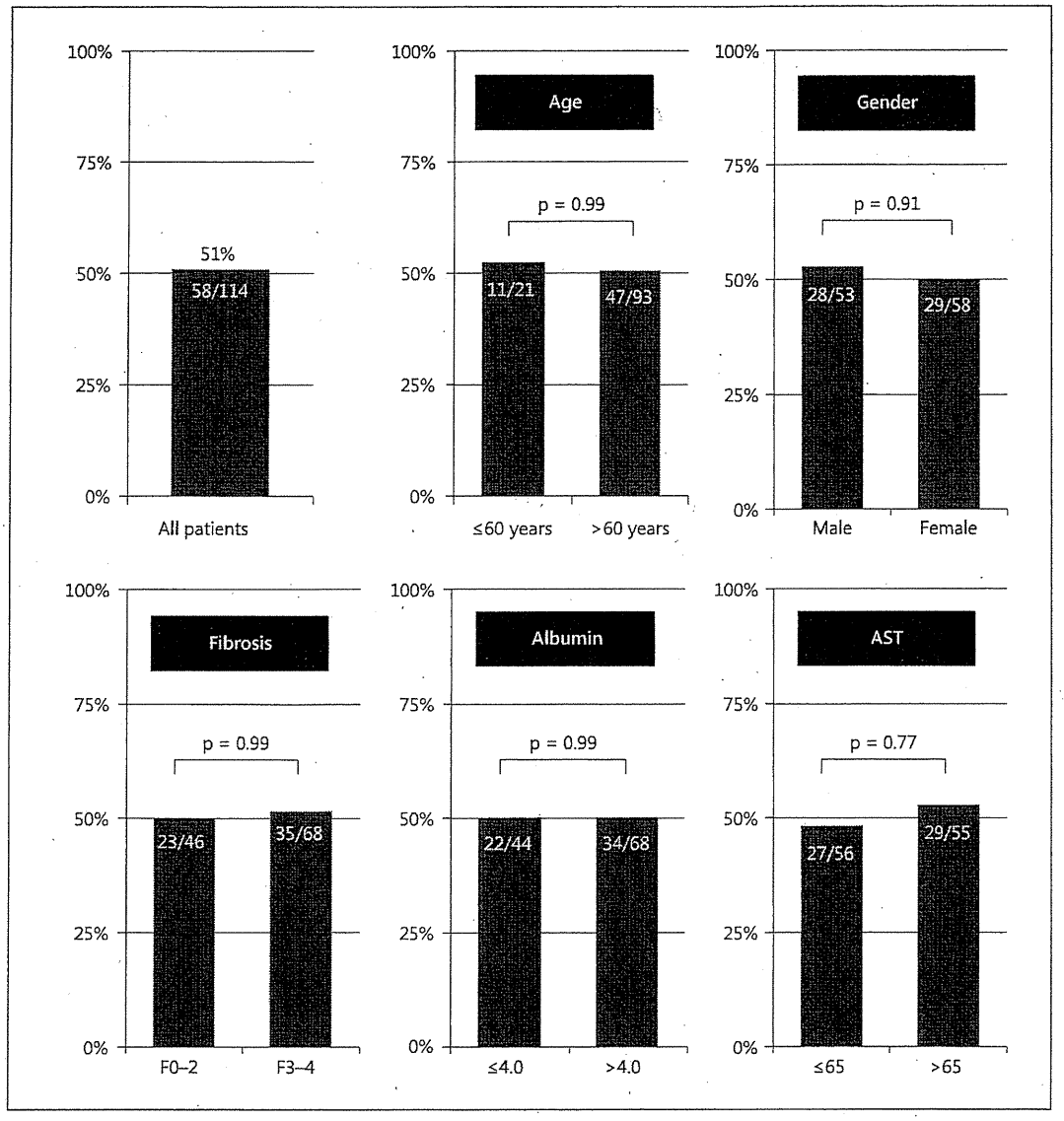

Fig. 4. Percentage of patients with normal SLC22A7 expression according to baseline clinical findings. No significant differences in the percentage of patients with normal SLC22A7 expression were observed after stratification by age, gender, fibrosis stage, albumin and/or AST.

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-

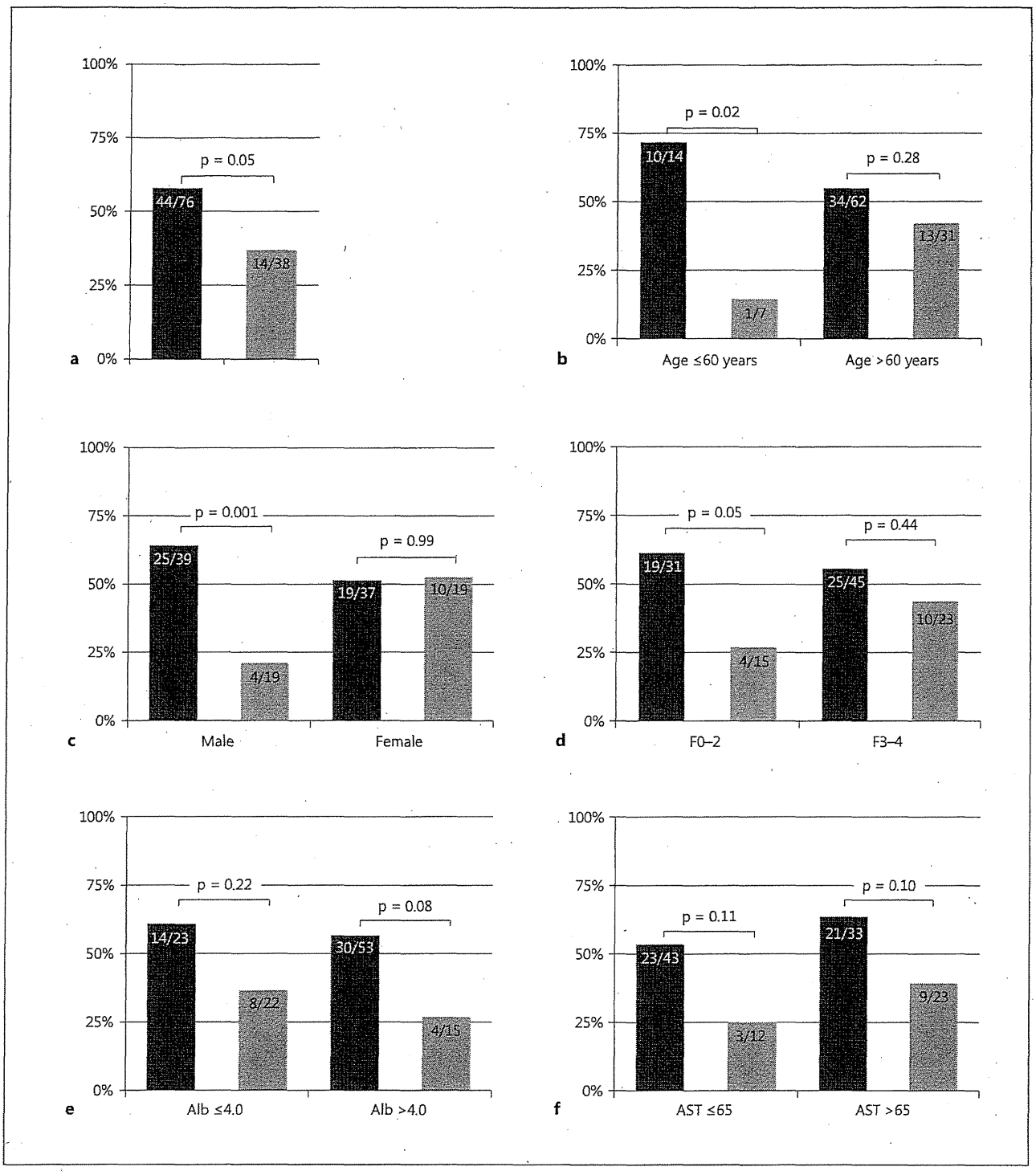

Fig. 5. Percentage of patients with normal SLC22A7 expression and HCC (**a**). SLC22A7 staining was compared between patients who did and did not develop HCC after stratification by age (**b**), gender (**c**), fibrosis stage (**d**), albumin (Alb, **e**) and AST levels (**f**). Light grey and dark grey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

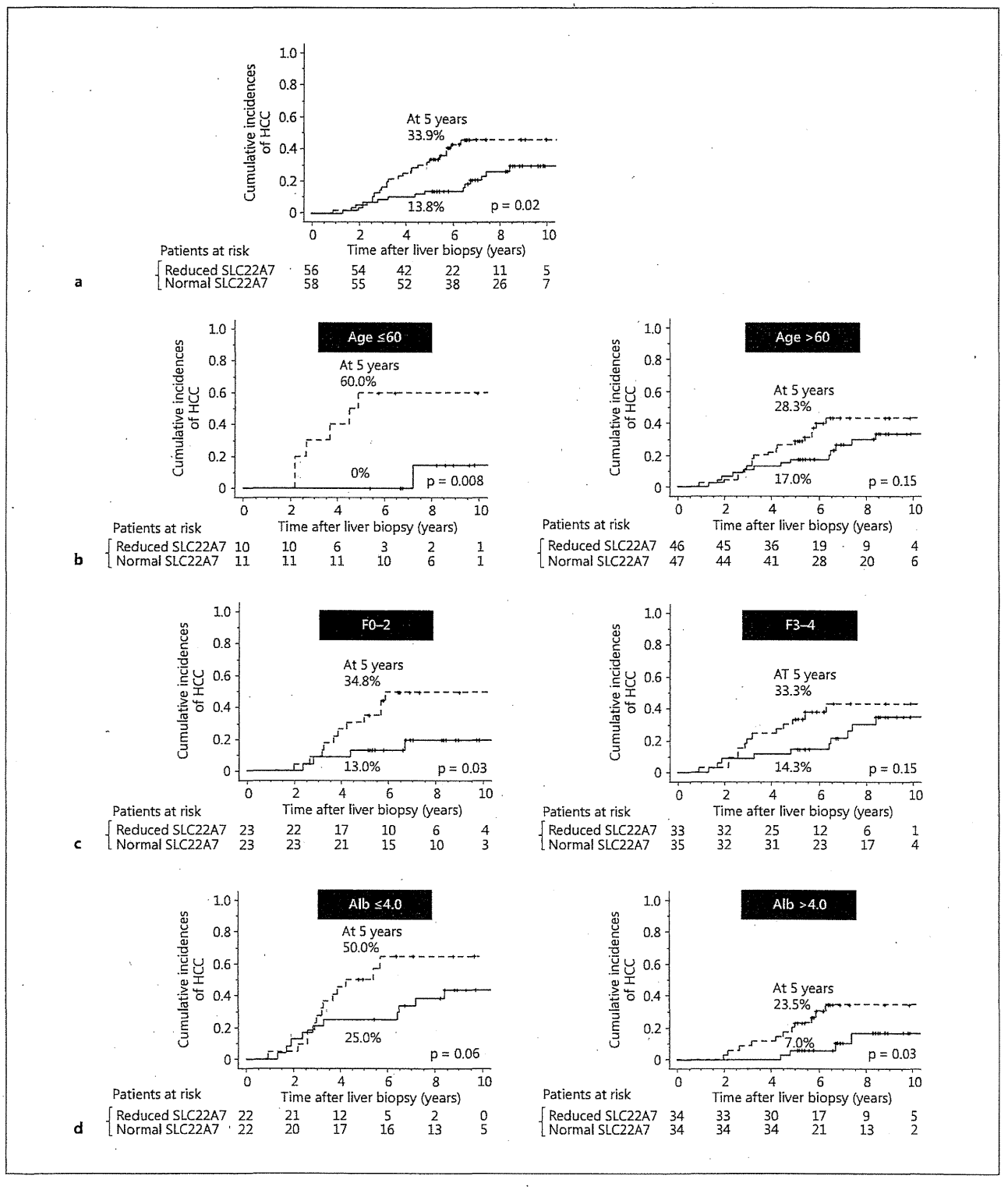

Fig. 6. Cumulative incidence of HCC according to SLC22A7 staining. **a** Comparison of the cumulative incidences of HCC in patients with normal (solid line) and reduced SLC22A7 expression (broken line). **b–d** The cumulative incidences of HCC after stratification by age (**b**), fibrosis stage (**c**)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

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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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Title

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

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Short title

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Keywords

liver transplantation, locoregional therapy, recurrence, α-fetoprotein, prognosis

Tables and Figures

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Disclosure

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Abbreviations

AASLD, American Association for the Study of Liver Diseases; AFP, α-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. α-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 ≤ 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,

158 by transcatheter arterial chemoembolization (TACE), 10 by systemic cytotoxic chemotherapy, 2 by percutaneous microwave coagulation, 4 by percutaneous ethanol injection therapy, 2 by radiation therapy, and 20 by best supportive care. There were no patients who underwent liver transplantation. Of these 554 patients, 323 were treated by RFA as an initial curative therapy for primary HCC and included in the following analyses. Inclusion criteria for RFA were as follows: HCC with solitary tumor ≤ 50mm or three or fewer lesions (none > 30mm), three or fewer lesions without major vascular or biliary invasion, total bilirubin concentration <2.5 mg/dL, platelet count >3 × 10^4/mm^3, and prothrombin activity >50%. Some patients refused hepatic resection and chose RFA voluntarily on the basis of concerns about complications or physician recommendations, which took into account impairment of liver function, HCC location, and cardiopulmonary dysfunction. Patients with ascites uncontrolled by diuretics and/or extrahepatic metastasis were excluded. The reasons why the patients were selected for RFA instead of being offered liver transplantation were a Child-Pugh A classification (n = 256, 79.2%), age >65 years (n = 198, 61.3%), or heart or lung disease complications (n = 6, 1.9%). The number of patients who were classified as Child-Pugh B and who were younger than 66 years of age were 28 (8.7%). In these patients, there was 1 patient who had severe heart disease, and the remaining 27 patients did not have any

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 conducted in accordance with the Declaration of Helsinki.

HCC diagnosis

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 (MRI) or by needle biopsy. For triple-phase dynamic CT scans, arterial, portal, and equivalent phases were set at 35, 70, and 150 s, respectively, after injection of 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 MRI. Liver biopsy was performed when a definite diagnosis was not proved 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 using the percutaneous approach (n = 279)

or general anesthesia using the laparoscopic approach (n = 44), both 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, USA). Ultrasonography was performed with a 3.0–6.0 MHz convex probe using Aloka SSD-5500 (Aloka, Tokyo, Japan), Sonoline Elegra (Siemens, Erlangen, Germany), and Aplio XV (Toshiba Medical Systems, Tokyo, Japan) systems. When the target nodule was > 20mm in diameter, we performed multiple needle insertions and multiple ablations of one 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 1–3 days after RFA. Complete HCC ablation was defined as hypoattenuation of the entire tumor. Patients who were judged as incomplete ablated received additional therapy 1 week after the first ablation, which was continued until the treatment was judged completely effective. Blood was sampled every 2–3 months and tested for indicators of liver function and the markers α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II). A dynamic CT scan

was scheduled every 3–4 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 scan concomitantly 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 AASLD (3). The end of follow-up was tumor progression beyond the Milan criteria, death, or latest medical attendance until 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 initial successful RFA were determined by the Kaplan–Meier method, and the risk factors associated with recurrence exceeding the Milan criteria and death were identified using the Cox

proportional hazards regression model independently for tumor progression and death. Survival analysis was performed on a per patient basis. The starting date of follow-up was defined as the completion date of the initial RFA session. Multivariate analysis was performed using the Cox proportional hazards model, including variables with a marginal p value of <0.05 by univariate analysis. All statistical analyses were performed using StatView 5.0 (SAS Inc., Cary, NC, USA).

RESULTS

The patient characteristics are shown in Table 1. The minimum follow-up period was 7 months, and the median follow-up period was 47.4 months (range, 7–147 months). During follow-up, HCC recurred in 270 of 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, of which 174 (90.1%) 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 recurrence rate exceeding the Milan criteria at 1, 3, and 5 years was 15.1%, 46.0%, and 61.1%, respectively. Major complications were observed in

only 2 cases (0.6%): one was gastric penetration after ablation of segment 2 and the other was hemothorax after ablation of segment 7. Both cases recovered without surgery.

Risk factors for exceeding the Milan criteria and overall survival

The univariate analysis results showed that the higher AFP level (> 100 ng/mL), higher PIVKA-II level (>100 mAU/mL), larger tumor size (diameter > 20mm), and earlier recurrence of intrahepatic lesion (within 1 year after initial RFA) were significantly associated with the risk for recurrence exceeding the Milan criteria (Table 2). Multivariate analysis with the Cox proportional hazards model indicated that the higher AFP level (hazard ratio 1.59, $p = 0.005$), larger tumor size (hazard ratio 1.54, $p = 0.012$), and early recurrence within 1 year after initial RFA (hazard ratio 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 Child–Pugh score. Risk factors associated with overall survival are shown in Table 3. Multivariate analysis with the Cox proportional hazards model indicated that the initial higher AFP level (hazard ratio 2.03, $p = 0.0003$), Child–Pugh B (hazard ratio 2.42, $p < 0.0001$), and early recurrence within 1 year after initial RFA (hazard ratio