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-

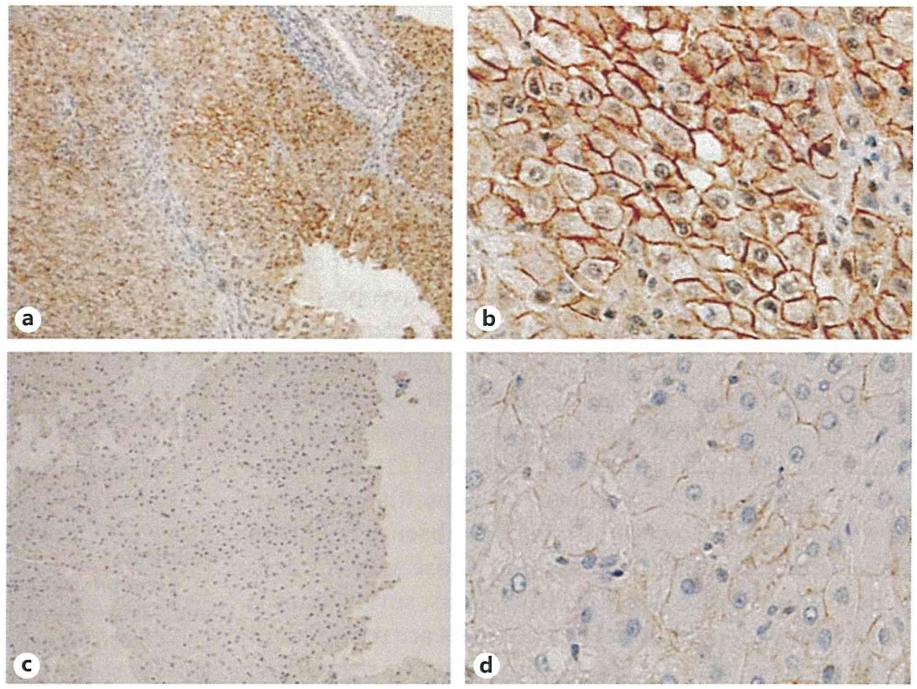

Fig. 1. IHC analysis of SLC22A7 in biopsy specimens. **a**, **b** Normal SLC22A7 expression (≥25% positive cells) **a** ×100. **b** ×400. **c**, **d** Reduced SLC22A7 expression (<25% positive cells). **c** ×100. **d** ×400.

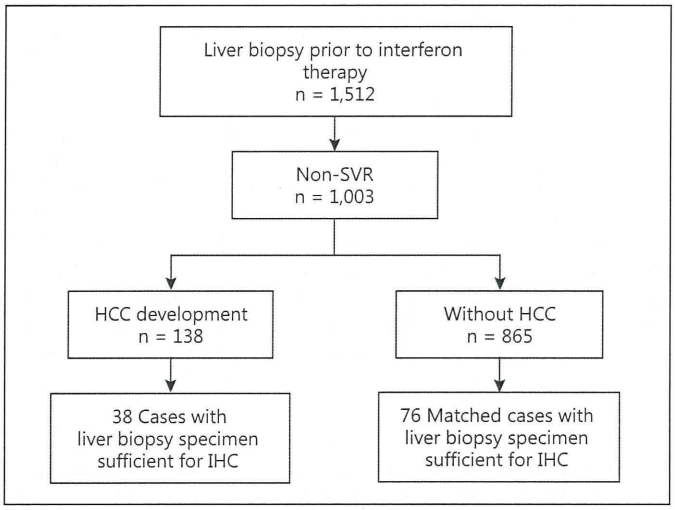

Fig. 2. Consort diagram of stratified analyses.

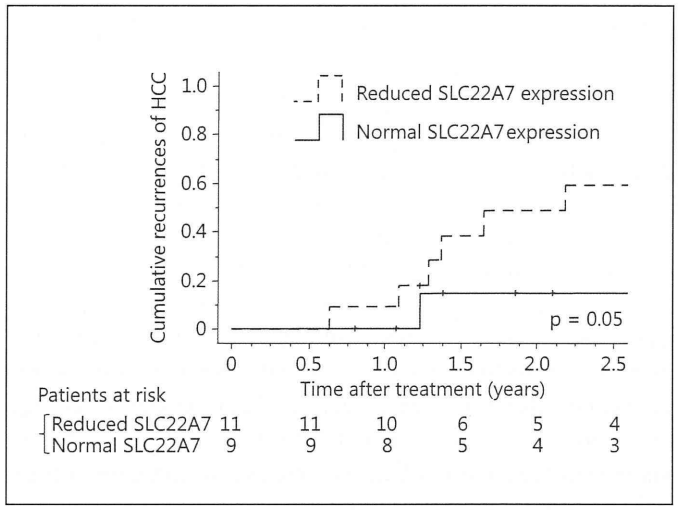

Fig. 3. Cumulative incidence of HCC recurrence after curative RFA was compared between patients with normal and reduced SLC22A7 expression.

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/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.

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

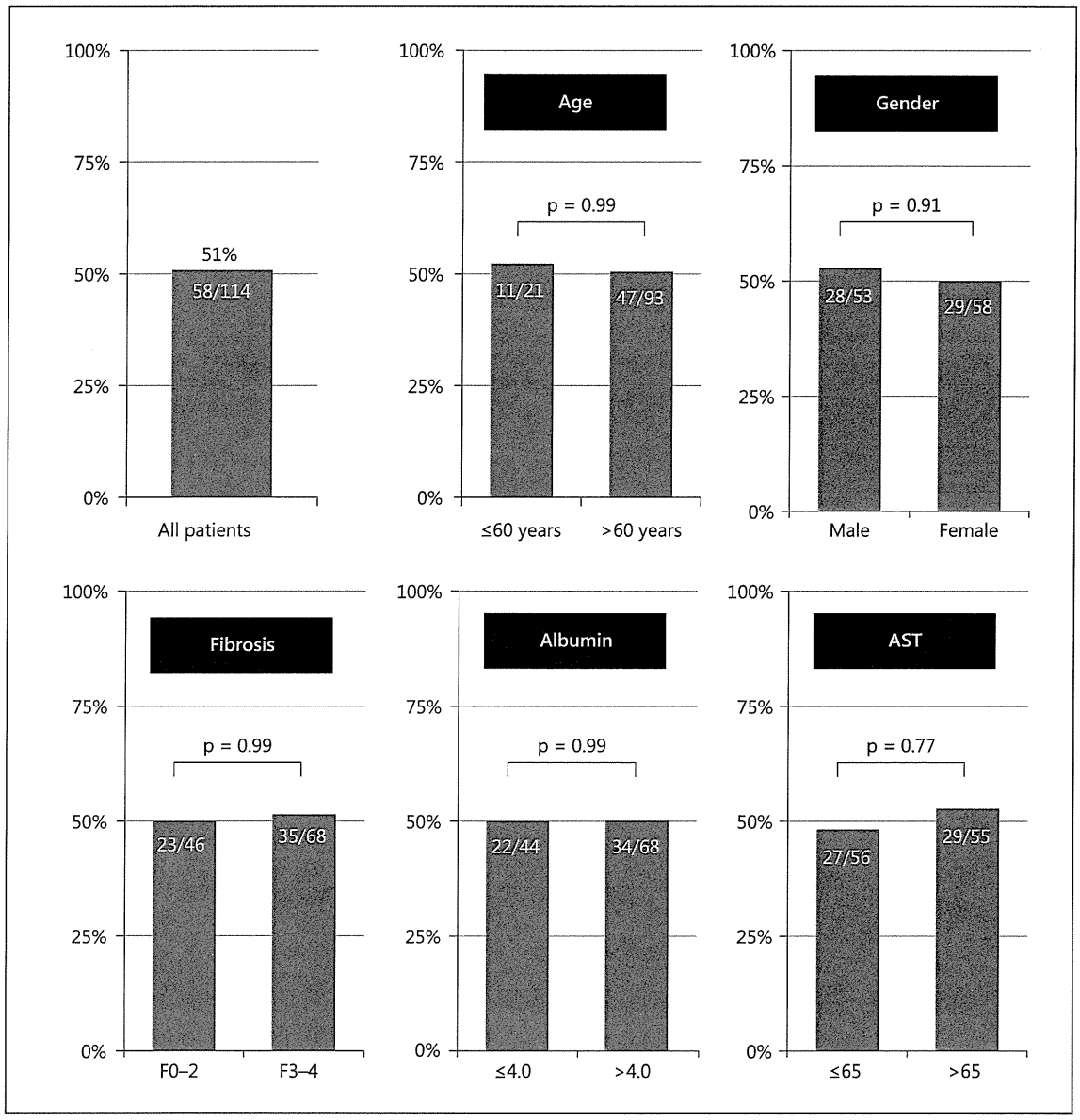

Fig. 4. Percentage of patients with normal SLC22A7 expression according to baseline clinical findings. No significant differences in the percentage of patients with normal SLC22A7 expression were observed after stratification by age, gender, fibrosis stage, albumin and/or AST.

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-

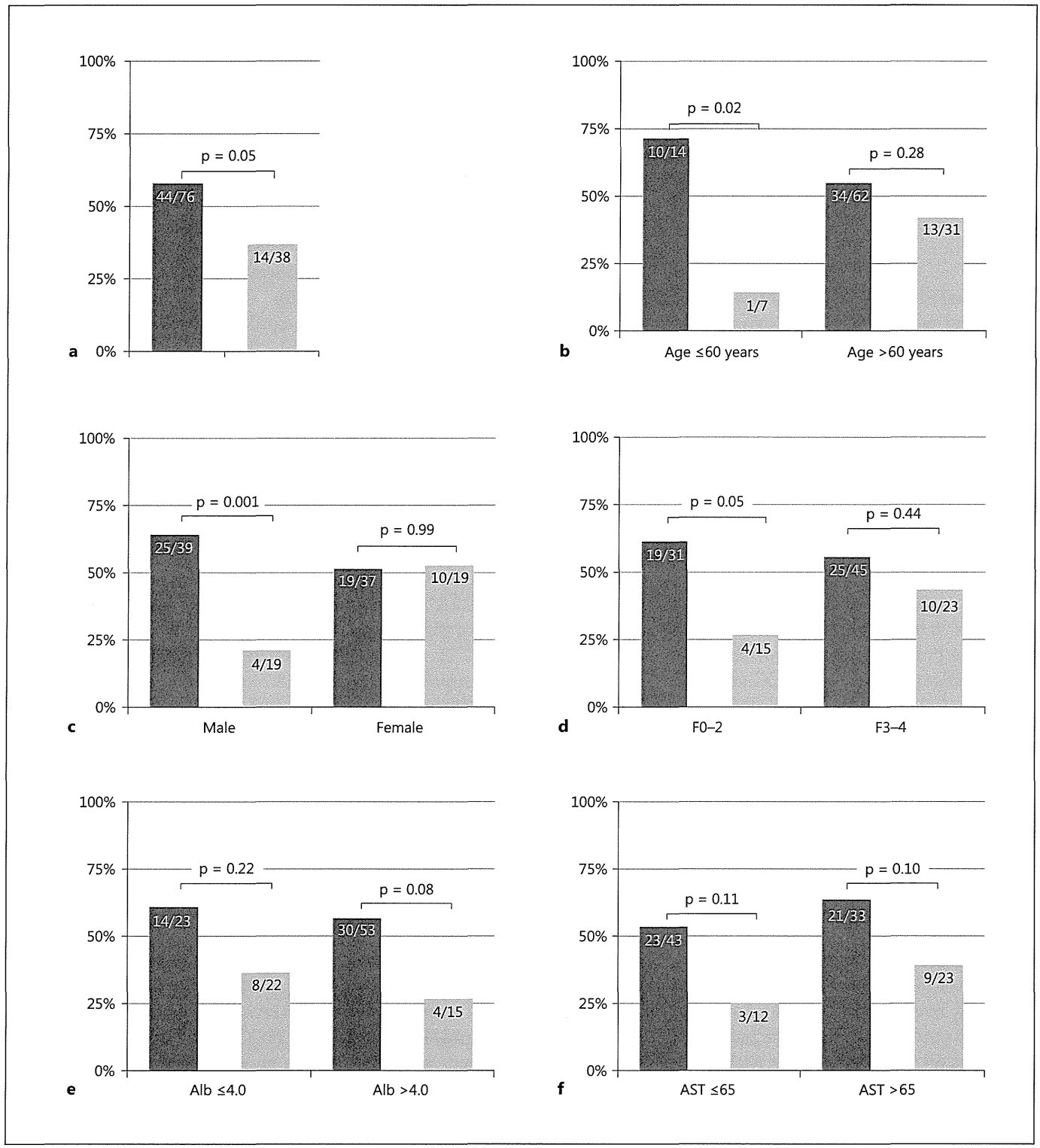

Fig. 5. Percentage of patients with normal SLC22A7 expression and HCC (**a**). SLC22A7 staining was compared between patients who did and did not develop HCC after stratification by age (**b**), gender (**c**), fibrosis stage (**d**), albumin (Alb, **e**) and AST levels (**f**). Light grey and dark grey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

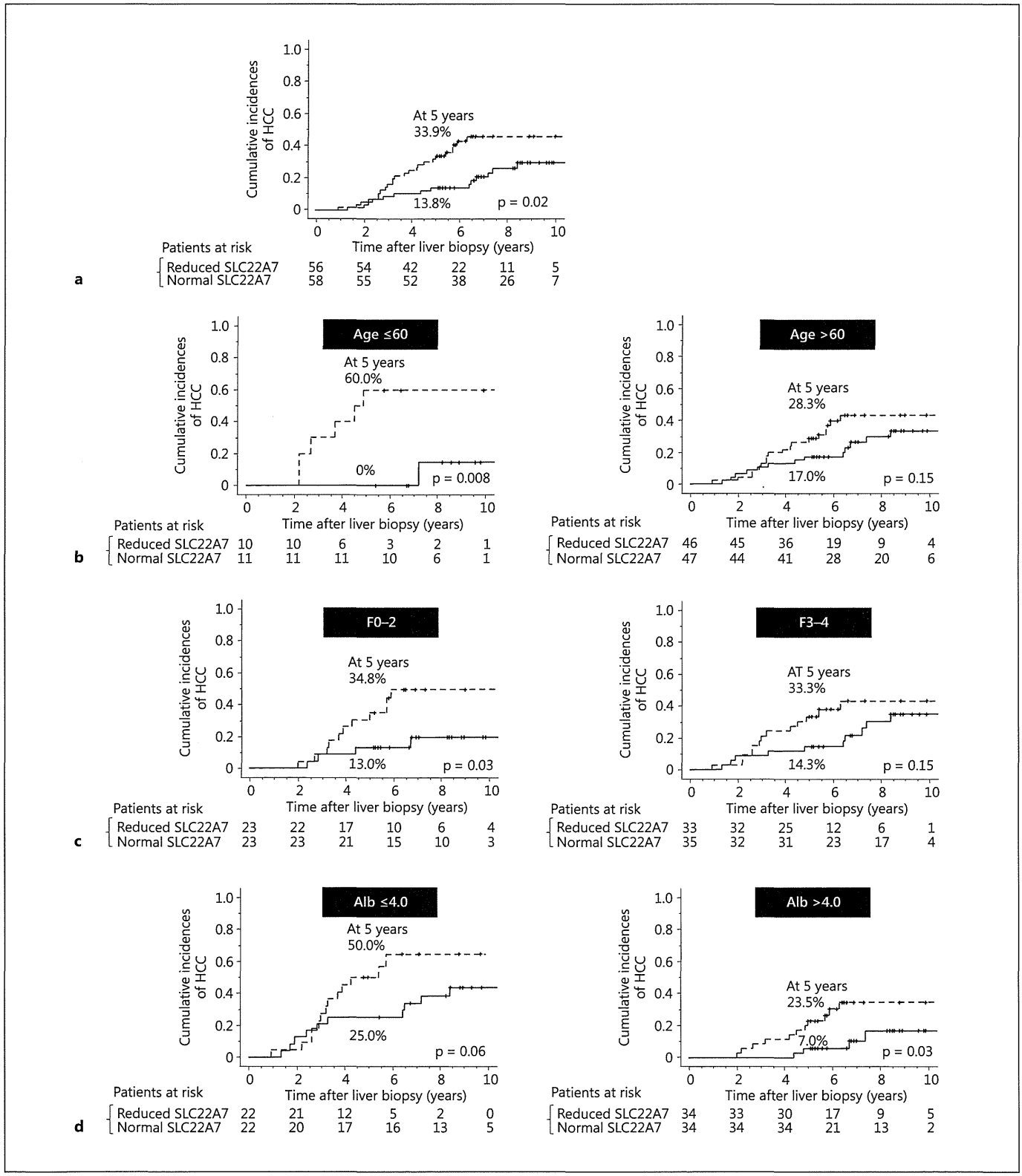

Fig. 6. Cumulative incidence of HCC according to SLC22A7 staining. **a** Comparison of the cumulative incidences of HCC in patients with normal (solid line) and reduced SLC22A7 expression (broken line). **b–d** The cumulative incidences of HCC after stratification by age (**b**), fibrosis stage (**c**)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

Risk factors exceeding the Milan criteria after RFA

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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