performed and a pathologic diagnosis was made based on the Edmondson and Steiner criteria. Time to HCC occurrence was defined as the interval between the date of the first US screening and the diagnosis of HCC. Patients were censored at the time of death without HCC development, the last visit when lost to follow up, or the end of the study period. The last observation in this study was made on December 31, 2010. Thus, the time of observation was extended from that of our previous study, which was censored on May 31, 2008 [16].

Transient elastography

Transient elastography was performed using Fibroscan (Echosens, Paris, France) as described previously [16].

Statistical analysis

Data were expressed as means ± standard deviation (SD) unless otherwise indicated. Categorical variables were compared by χ^2tests, whereas continuous variables were compared by the unpaired Student's *t*-test (parametric) or the Mann–Whitney *U*-test (non-parametric). Multivariate logistic regression analysis was used to identify factors that were independently associated with the presence of PLNE. Cumulative HCC incidence was estimated using the Kaplan–Meier method, and the difference between groups was assessed with the log-rank test. In the analysis of risk factors for hepatocarcinogenesis, we tested the following variables in univariate analysis and multivariate Cox proportional hazard regression analysis: age, sex, platelet count, serum albumin concentration, total bilirubin concentration, ALT and aspartate aminotransferase (AST) levels, higher AFP concentration (>10 ng/ml), prothrombin activity, heavy alcohol drinking (alcohol intake >80 g/day), BMI, higher liver stiffness measurement (LSM) (>10 kPa), HCV serotype, HCV viral load (>100 kIU/ml), IFN treatment, achievement of SVR, and presence of PLNE. A *p* value of less than 0.05 on a two-tailed test was considered significant. Data processing and analysis were performed using StatView (ver. 5.0; SAS Institute, Cary, NC, USA) and SPSS (ver. 14.0; SPSS, Chicago, IL, USA) software.

Results

Patient profiles

We detected PLNE in 169 of 846 (20.0 %) patients with chronic hepatitis C. A representative ultrasound image is shown in Fig. 1. The mean (±SD) length of the longest axis was 1.7 (±0.5) cm (range 1.0–3.5 cm). The clinical features of patients with and without PLNE are summarized in Table 1. The proportion of females was significantly higher in the PLNE-positive group than in the PLNE-negative group (63.3 vs. 52.9 %), and BMI was slightly but significantly lower in the PLNE-positive group than in the PLNE-negative group (21.9 ± 2.6 vs. 22.5 ± 2.9). The proportion of HCV serotype 1 patients was higher in the PLNE-positive group than in the PLNE-negative group, with borderline significance. There was a tendency of a higher serum ALT level in the PLNE-positive group, but the difference was without statistical significance. There were no significant differences in other liver function test results, or in liver stiffness and hepatitis C viral load between the two groups. Multivariate logistic regression analysis using the factors of sex, serum ALT, BMI, and HCV serotype revealed that female sex, lower BMI, and HCV serotype 1 were independently associated with the presence of PLNE (Table 2).

Incidence of HCC

The mean follow-up period was 4.8 years, constituting a total observation of 4,021 person-years. During the observation period, 70 (8.3 %) patients were lost to follow up: 15 (8.8 %) patients in the PLNE-positive group and 55 (8.1 %) patients in the PLNE-negative group. There were no patients in whom an enlarged perihepatic LN turned out to be caused by other underlying diseases including metastasis of HCC. The SVR rate in patients who received IFN therapy during the follow-up period was significantly lower in the PLNE-positive group compared with that in the PLNE-negative group [7/34 (20.6 %) vs. 93/172

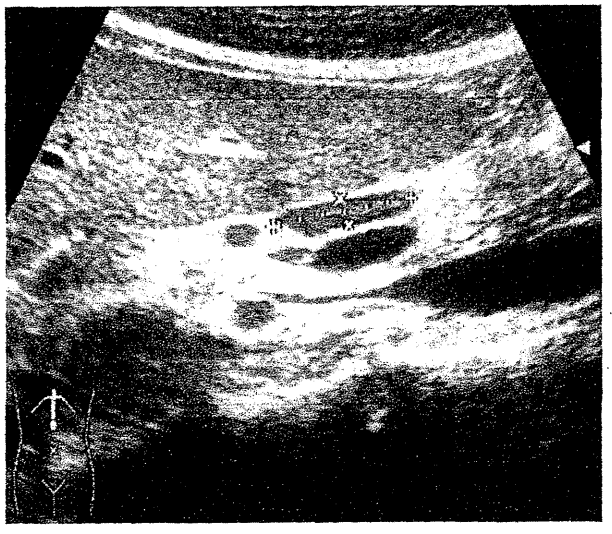

Fig. 1 Representative ultrasound image of enlarged perihepatic lymph node (LN) in a patient with chronic hepatitis C

Table 1 Clinical features of patients with and without PLNE

Variable	PLNE-positive group ($n = 169$)	PLNE-negative group ($n = 677$)	p value
Age (years)	62.4 ± 10.1 (29–83)	62.4 ± 11.5 (17–89)	0.58
Male, n (%)	62 (36.7)	319 (47.1)	0.018
Serum albumin (g/dl)	4.0 ± 0.4 (2.8–4.8)	4.0 ± 0.4 (2.5–5.0)	0.93
Total bilirubin (mg/dl)	0.8 ± 0.3 (0.3–2.1)	0.9 ± 0.5 (0.3–4.6)	0.23
AST (IU/l)	52 ± 34.1 (17–223)	50 ± 33.8 (9–286)	0.16
ALT (IU/l)	57 ± 48.7 (4–374)	53 ± 45.2 (2–503)	0.10
Platelet count ($\times 10^4/\mu l$)	16.1 ± 6.6 (2.1–42.2)	16.1 ± 6.7 (3.2–43.6)	0.89
Prothrombin time (%)	86.0 ± 12.1 (50.3–100.0)	85.7 ± 12.4 (38.9–100.0)	0.88
AFP (ng/ml)	22.0 ± 67.9 (1–592)	13.4 ± 37.1 (1–563)	0.61
BMI (kg/m^2)	21.9 ± 2.6 (14.4–28.7)	22.5 ± 2.9 (15.1–29.8)	0.007
Liver stiffness (kPa)	10.9 ± 7.8 (2.8–42.2)	12.0 ± 10.0 (2.5–75.0)	0.59
Alcohol consumption >80 g/day, n (%)	6 (3.6)	25 (3.7)	0.82
HCV viral load (kIU/ml)	549 ± 646 (5–5000)	651 ± 842 (5–5000)	0.48
HCV serotype 1, n (%)	146 (86.3)	538 (79.5)	0.053
Patients who received IFN, n (%)	34 (20.1)	172 (25.4)	0.18
Patients who achieved SVR, n (%)	7 (4.1)	93 (13.7)	0.0009

PLNE perihepatic lymph node enlargement, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *AFP* α-fetoprotein, *BMI* body mass index, *HCV* hepatitis C virus

Table 2 Factors associated with the presence of PLNE: multivariate analysis

Variable	Odds ratio (95 % confidence interval [CI])	p value
Male sex	0.667 (0.464–0.936)	0.024
ALT level (per 1 IU/l)	1.003 (0.999–1.006)	0.10
BMI (per 1 kg/m^2)	0.919 (0.864–0.978)	0.017
HCV serotype 1	1.64 (1.02–2.66)	0.043

(54.1 %), $p = 0.0005$]. This finding was consistent with previous reports [12, 19].

By the end of the follow-up period, HCC had developed in 121 patients (3.0 % per 1 person-year). The cumulative incidence rates of HCC at 3 and 5 years estimated by the Kaplan–Meier method were 8.9 and 13.7 %, respectively. We then assessed the incidence of HCC stratified by the presence of PLNE. Unexpectedly, the PLNE-positive group revealed a significantly lower incidence of HCC than the PLNE-negative group ($p = 0.019$, log-rank test) (Fig. 2). The cumulative incidence rates at 3 and 5 years were 3.6 and 8.2 %, respectively, in the PLNE-positive group, and 10.1 and 15.1 % in the PLNE-negative group. These results indicate that patients with PLNE have a lower risk of HCC development despite having a lower SVR rate with IFN therapy.

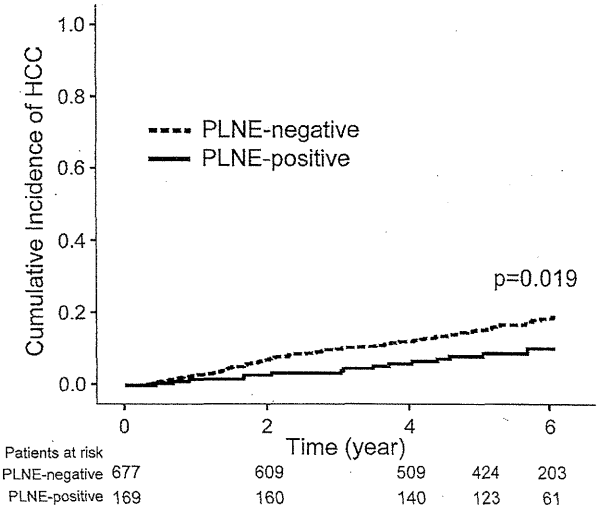

Fig. 2 Cumulative incidence of hepatocellular carcinoma (*HCC*) development stratified by the presence of perihepatic lymph node enlargement (*PLNE*)

Risk analyses

We analyzed the risk factors for HCC development. In the univariate analyses, older age, male sex, lower serum albumin concentration, higher total bilirubin concentration, higher AST level, higher ALT level, lower prothrombin activity, lower platelet count, heavy alcohol drinking, higher BMI, LSM greater than 10 kPa, AFP level greater than 10 ng/ml, HCV serotype 1, not receiving IFN, not achieving SVR, and absence of PLNE were significant risk factors for HCC (Table 3). As we had reported previously, a higher LSM (i.e., greater than 10 kPa) was a strong predictor of HCC development [hazard ratio (HR) 15.4, 95 % confidence interval (CI) 8.6–27.0, $p < 0.0001$)]. Multivariate proportional hazard regression analyses

Table 3 Risk factors for HCC development: univariate and multivariate analyses

Variable	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	*p* value	Hazard ratio (95 % CI)	*p* value
Age (per 1 year age)	1.07 (1.05–1.09)	<0.0001	1.04 (1.01–1.06)	0.002
Male sex	1.45 (1.02–2.08)	0.041	1.49 (1.02–2.17)	0.039
Platelet count (per 10^4/μl)	0.852 (0.823–0.882)	<0.0001	0.965 (0.926–1.005)	0.089
Total bilirubin (per 1 mg/dl)	1.88 (1.45–2.45)	<0.0001	0.825 (0.567–1.2)	0.32
Serum albumin level (per 1 g/dl)	0.12 (0.084–0.17)	<0.0001	0.441 (0.263–0.739)	0.002
AST level (per 1 IU/l)	1.01 (1.007–1.014)	<0.0001	1.002 (0.991–1.013)	0.71
ALT level (per 1 IU/l)	1.004 (1.002–1.007)	0.002	1.0 (0.991–1.013)	0.94
AFP level >10 ng/ml	6.76 (4.69–9.8)	<0.0001	1.9 (1.22–2.97)	0.005
Prothrombin time (per 1 %)	0.973 (0.966–0.979)	<0.0001	0.989 (0.976–1.001)	0.072
Alcohol consumption >80 g/day	2.73 (1.43–5.24)	0.002	3.53 (1.76–7.09)	0.0004
BMI (per 1 kg/m^2)	1.09 (1.03–1.16)	0.006	1.09 (1.01–1.17)	0.025
Liver stiffness >10 kPa	15.4 (8.6–27.0)	<0.0001	4.41 (2.24–8.7)	<0.0001
HCV serotype 1	1.76 (1.03–3.03)	0.04	1.36 (0.774–2.38)	0.29
HCV-RNA >100 kIU/ml	1.36 (0.87–2.13)	0.18	1.24 (0.781–1.97)	0.36
Patients treated with IFN	0.44 (0.262–0.75)	0.002	0.59 (0.315–1.11)	0.1
Patients with SVR	0.175 (0.055–0.549)	0.003	0.621 (0.169–2.28)	0.47
Presence of PLNE	0.53 (0.31–0.91)	0.02	0.551 (0.31–0.978)	0.042

HCC hepatocellular carcinoma, *IFN* interferon, *SVR* sustained viral response

revealed that older age, male sex, lower serum albumin concentration, AFP level greater than 10 ng/ml, heavy alcohol drinking, higher BMI, LSM greater than 10 kPa, and absence of PLNE were independent risk factors for HCC (Table 3). These results suggest that the presence of PLNE is an independent negative predictor of HCC development in chronic hepatitis C patients.

Subgroup analysis of non-obese patients

To further rule out the possibility that obesity acted as a confounder in the association between the presence of PLNE and HCC development, we reanalyzed the contribution of PLNE to HCC development in a subgroup of non-obese patients, defined as those with BMI <25 kg/m^2 ($n = 695$), because we could clearly visualize the liver hilum in such individuals. As shown in Fig. 3, the PLNE-positive group had a significantly lower incidence of HCC than the PLNE-negative group even in the non-obese subgroup ($p = 0.02$). Thus, we further confirmed that the presence of PLNE was negatively associated with HCC development independently of obesity.

Significance of the size of perihepatic LNs

To examine the significance of the size of perihepatic LNs, we divided patients with PLNE into two groups: a smaller LN group (longest axis of LN 1 cm to 2 cm, $n = 122$) and a larger LN group (longest axis of LN ≥2 cm, $n = 47$).

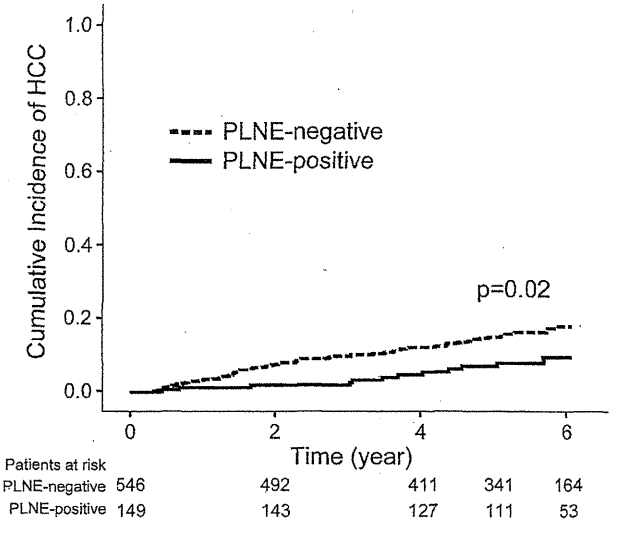

Fig. 3 Cumulative incidence of HCC development stratified by the presence of PLNE: subgroup analysis of non-obese patients (body mass index [BMI] <25 kg/m^2)

The characteristics of each group are summarized in Table 4. The proportion of male patients and the LSM value tended to be higher in the larger LN group than in the smaller LN group, but the difference was not statistically significant for either factor. There were no significant differences in other factors between the two groups. Furthermore, there was no significant difference in HCC incidence rates between the two groups (Fig. 4), although the larger LN group revealed a slightly higher incidence of

Table 4 Comparison of clinical features between patients with small perihepatic lymph nodes (LNs) and those with large perihepatic LNs

Variable	LN size 1 cm to <2 cm, (n = 122)	LN size ≥2 cm (n = 47)	p value
Age (years)	62.7 ± 10.4 (29–83)	61.5 ± 9.2 (32–77)	0.32
Male, n (%)	40 (32.8)	22 (46.8)	0.092
Serum albumin (g/dl)	4.0 ± 0.3 (3.0–4.8)	4.0 ± 0.4 (2.8–4.8)	0.96
Total bilirubin (mg/dl)	0.8 ± 0.3 (0.3–1.6)	0.8 ± 0.4 (0.3–2.1)	0.98
AST (IU/l)	53 ± 36.0 (17–223)	50 ± 28.7 (17–181)	0.83
ALT (IU/l)	56 ± 49.0 (4–374)	59 ± 48.2 (6–308)	0.48
Platelet count ($\times 10^4/\mu$l)	16.3 ± 6.2 (2.1–36.4)	15.4 ± 7.4 (4.8–42.2)	0.25
Prothrombin time (%)	86.7 ± 11.7 (57.4–100.0)	84.2 ± 13.0 (50.3–100.0)	0.27
AFP (ng/ml)	13.0 ± 27.7 (1–168)	45.3 ± 118.6 (1–592)	0.19
BMI (kg/m^2)	21.8 ± 2.6 (16.8–28.0)	22.0 ± 2.6 (14.4–28.7)	0.75
Liver stiffness (kPa)	10.2 ± 7.1 (2.8–37.4)	12.5 ± 9.3 (4.2–42.2)	0.064
Alcohol consumption >80 g/day, n (%)	5 (4.1)	1 (2.1)	0.54
HCV viral load (kIU/ml)	658 ± 788	504 ± 582	0.17
HCV serotype 1, n (%)	107 (87.7)	39 (83.0)	0.58
Patients who received IFN, n (%)	24 (19.6)	10 (21.2)	0.98
Patients who achieved SVR, n (%)	5 (4.1)	2 (4.3)	0.99

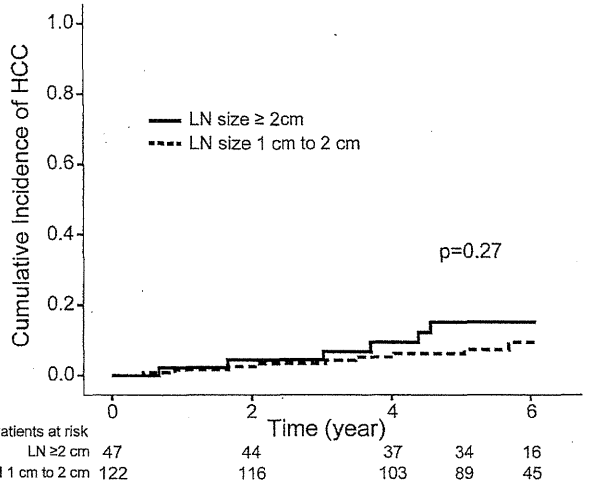

Fig. 4 Cumulative incidence of HCC development in patients with PLNE stratified by the size of perihepatic LNs: i.e., smaller (longest axis of LN 1 to <2 cm) and larger (longest axis of LN ≥2 cm)

HCC. These results suggest that the size of perihepatic LNs in chronic hepatitis C patients may not be clinically as important as the presence of PLNE itself.

Discussion

Although PLNE is a common finding in patients with chronic hepatitis C, its clinical significance has remained unclear. In the present study, we reevaluated the clinical relevance of PLNE in a large cohort of chronic hepatitis C patients. We found, by prospective analysis, that patients with PLNE had a lower risk of HCC development than those without PLNE. To our knowledge, this is the first study reporting a negative association between the presence of PLNE and HCC development.

Before we started this study, we expected that patients with PLNE would have a higher risk of HCC development, based on previous reports showing positive associations between PLNE and liver inflammation and fibrosis [8, 13–15]. However, in our study, neither inflammatory markers, such as serum AST and ALT levels, nor fibrosis markers, such as the platelet count and LSM, had statistically significant associations with the presence of PLNE. On the contrary, patients with PLNE revealed a significantly lower risk of HCC development. One possible explanation for this result is that obesity may affect the ability of US to detect perihepatic LNs, although patients with severe obesity were excluded from the study. To rule out the effect of confounders, especially obesity, we performed multivariate analysis and subgroup analysis of non-obese patients, and the results showed that the presence of PLNE was an independent negative predictor of HCC development. Additionally, of the 846 patients enrolled in this study, 175 patients underwent abdominal computed tomography (CT) within one year from the date of the US examination. The concordance rate for the diagnosis of PLNE between CT and US in these patients was 91.4 % (160/175). Therefore, we consider that the diagnostic accuracy of US for PLNE was acceptable in this study.

Although the mechanism of PLNE in patients with hepatitis C is still unknown, hyperplasia of regional LNs is generally considered to reflect inflammatory responses in the adjacent organs. The volume of perihepatic LNs has been reported to significantly decrease after antiviral

therapy, especially in patients with an SVR, supporting the hypothesis that PLNE reflects the inflammatory response to HCV [19–21]. In fact, PLNE was reported to be associated with CD8 lymphocyte counts in the peripheral blood [10]. Furthermore, HCV-specific IFN-γ production and proliferative responses of T cells were found most commonly in perihepatic LNs rather than in liver tissue or in the peripheral blood, indicating that there was ongoing T-cell activation in perihepatic LNs [22]. Our results, taken together with these previous reports, suggest that the presence of PLNE may reflect an adequate host immune response to HCV. T-cell immunity is very important in the control of HCV infection and in the prevention of hepatocarcinogenesis [23–25], and a T-cell response that is too weak may accelerate hepatocarcinogenesis, as seen in patients co-infected with HCV and human immunodeficiency virus [26, 27]. Thus, a weak T-cell response may be one explanation of the higher risk of HCC development in patients without PLNE. On the other hand, too strong an anti-HCV T-cell response may induce hepatocellular damage and lead to subsequent hepatocarcinogenesis [28], so patients with larger perihepatic LNs may have a slightly higher tendency to develop HCC. However, from the present type of observational study, we cannot evaluate a causal relationship between PLNE and hepatocarcinogenesis, so further studies are needed to clarify this point.

As mentioned above, several studies have shown that PLNE was positively associated with the degree of liver inflammation or fibrosis [8, 13–15], but, in the present study we could not find such associations, except for slight serum ALT elevation. However, because of ethical concerns regarding the performance of liver biopsy, we did not assess liver histology, so we cannot conclude whether or not PLNE is really associated with liver inflammation and fibrosis. Of note, the reported relationships of PLNE to liver function tests and liver inflammation and fibrosis are inconsistent among studies [7–15]. One reason may be that these findings were based on relatively small samples. Another reason is that there is a lack of established criteria for the diagnosis of PLNE. The lack of definite criteria may also contribute to the wide variation in the prevalence of PLNE among studies (from 20 to 100 %) [8–10, 21]. We defined PLNE as an LN that was at least 1 cm in the longest axis, and this definition was based on the report by Grier et al. [21] and our preliminary investigation in healthy subjects. Some studies have used more detailed measurements of LNs with calculations of node volume and shape [8, 10, 20]. These methods are certainly more accurate in terms of the assessment of nodal volume, but may be too complicated in the clinical setting, as discussed by Grier et al. [21]. We used a simpler method, because our study included a large number of patients and was conducted to examine the significance of PLNE in daily clinical practice. Admittedly, a more detailed method would be appropriate to elucidate more clearly the involvement of PLNE in the pathophysiology of hepatitis and hepatocarcinogenesis.

In the present study, female sex, lower BMI, and HCV serotype 1 were independently associated with the presence of PLNE. Soresi et al. [29] also reported that PLNE was observed significantly more often in female patients than in male patients with chronic hepatitis C. Although we cannot clarify the mechanism underlying this association, this finding may be interesting from the point of view of gender differences in immune systems and hepatocarcinogenesis. In the study by Soresi et al., BMI in patients with PLNE tended to be lower than that in patients without PLNE, although the difference was not statistically significant [29], and this finding may be in line with our present results. Regarding BMI in patients with chronic HCV infection, an anti-HCV specific immune response was reportedly associated with lower BMI through the expression of adiponectin, one of the major adipokines [30]. Thus, the active immune response to HCV in patients with lower BMI might cause PLNE. Recent studies have reported that obesity and obesity-induced dysregulation of adipokines play important roles in hepatocarcinogenesis [31–33], so the examination of adipokine expression may help to explain the relationship of PLNE to BMI and hepatocarcinogenesis.

Another important finding in our study was that the SVR rate in patients who received IFN therapy was significantly lower in patients with PLNE than in patients without PLNE. This finding is consistent with previous reports [12, 19]. Although the proportion of individuals with HCV serotype 1 was higher in our patients with PLNE than in patients without PLNE, subgroup analysis of the patients with HCV serotype 1 also revealed a significantly lower SVR rate in patients with PLNE than in patients without PLNE (data not shown). Therefore, further analyses are planned to clarify the relationship of PLNE to HCV serotype and response to IFN therapy.

In conclusion, the presence of PLNE is an independent negative predictor of HCC development in chronic hepatitis C patients. This study may provide new insights into daily clinical practice and the pathophysiology of HCV-induced hepatitis and hepatocarcinogenesis.

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Conflict of interest The authors have no conflicts of interest regarding this study.

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Frequency, Risk Factors and Survival Associated with an Intrasubsegmental Recurrence after Radiofrequency Ablation for Hepatocellular Carcinoma

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Abstract

Background: In the treatment of hepatocellular carcinoma (HCC), hepatic resection has the advantage over radiofrequency ablation (RFA) in terms of systematic removal of a hepatic segment.

Methods: We enrolled 303 consecutive patients of a single naïve HCC that had been treated by RFA at The University of Tokyo Hospital from 1999 to 2004. Recurrence was categorized as either intra- or extra-subsegmental as according to the Couinaud's segment of the original nodule. To assess the relationship between the subsegments of the original and recurrent nodules, we calculated the kappa coefficient. We assessed the risk factors for intra- and extra-subsegmental recurrence independently using univariate and multivariate Cox proportional hazard regression. We also assessed the impact of the mode of recurrence on the survival outcome.

Results: During the follow-up period, 201 patients in our cohort showed tumor recurrence distributed in a total of 340 subsegments. Recurrence was categorized as exclusively intra-subsegmental, exclusively extra-subsegmental, and simultaneously intra- and extra-subsegmental in 40 (20%), 110 (55%), and 51 (25%) patients, respectively. The kappa coefficient was measured at 0.135 (95% CI, 0.079–0.190; $P<0.001$). Multivariate analysis revealed that of the tumor size, AFP value and platelet count were all risk factors for both intra- and extra-subsegmental recurrence. Of the patients in whom recurrent HCC was found to be exclusively intra-subsegmental, extra-subsegmental, and simultaneously intra- and extra-subsegmental, 37 (92.5%), 99 (90.8%) and 42 (82.3%), respectively, were treated using RFA. The survival outcomes after recurrence were similar between patients with an exclusively intra- or extra-subsegmental recurrence.

Conclusions: The effectiveness of systematic subsegmentectomy may be limited in the patients with both HCC and chronic liver disease who frequently undergo multi-focal tumor recurrence.

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Introduction

Hepatic resection is regarded as the most appropriate first-line treatment for patients with solitary hepatocellular carcinoma (HCC) who are non-cirrhotic or cirrhotic without portal hypertension [1]. Hepatic resection is also indicated for HCC patients with more advanced cirrhosis in countries like Japan where the option of performing a liver transplantation is limited by the scarcity of cadaveric donor organs [2]. As a surgical procedure, anatomical resection, which is the systematic removal of a hepatic segment containing tumor tissue, is considered to be preferable based on the concept that tumor cells disseminate through the portal vein [3–8].

Percutaneous tumor ablation methods, such as ethanol injection and microwave coagulation, have played an important role as nonsurgical treatments that can achieve high local cure rates without reducing background liver function [9–12]. Radiofrequency ablation (RFA) is currently considered to be the most effective first-line percutaneous ablation protocol because of its greater efficacy in terms of local cure compared with ethanol injection [13–16]. The survival outcomes for patients who achieved a complete response by RFA are comparable to that among patients treated by hepatic resection [17–20].

Hepatic resection is supposed to have the advantage over RFA as an effective intervention as it involves the systematic removal of a hepatic segment containing the tumor. Indeed, microscopic

satellite nodules, not detected by radiological examination prior to resection, are often observed in the resected specimen [5,6,21]. However, this does not necessarily mean that microscopic lesions will have been confined to the resected segment. Indeed, even after anatomical resection, the cumulative recurrence rate at 5 years is as high as 50–70% [6–8], and it is not known to what extent anatomical resection can reduce HCC recurrence as compared with RFA.

Whereas RFA can reliably eliminate target nodules together with some of the surrounding tissue, most of the liver parenchyma of the tumor-bearing segment is left unablated. In contrast to anatomical resection, it is possible to observe and analyze intra- and extra-subsegmental recurrence by following up patients after ablation. The aim of our present study was to assess the frequency, risk factors and survival outcomes associated with intra-subsegmental HCC recurrence after RFA in comparison with extra-subsegmental recurrence.

Patients and Methods

Patients

This retrospective study was conducted according to the ethical guidelines for epidemiological research designed by the Japanese Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour, and Welfare. The study design was included in a comprehensive protocol of retrospective study at the Department of Gastroenterology, The University of Tokyo Hospital approved by The University of Tokyo Medical Research Center Ethics Committee (approval number 2058). The following statements were posted at a website (http://gastro.m.u-tokyo.ac.jp/med/0602A.htm) and participants who do not agree to the use of their clinical data can claim deletion of them.

Department of Gastroenterology at The University of Tokyo Hospital contains data from our daily practice for the assessment of short-term (treatment success, immediate adverse events etc.) and long-term (late complications, recurrence etc.) outcomes. Obtained data were stored in an encrypted hard disk separated from outside of the hospital. When reporting analyzed data, we protect the anonymity of participants for the sake of privacy protection. If you do not wish the utilization of your data for the clinical study or have any question on the research content, please do not hesitate to make contact with us.

From 1999 to 2004 a total of 569 patients with HCC underwent RFA as the initial treatment for naïve HCC. Of them, 304 patents had a single nodule. We enrolled 303 of these patients in our current study excluding one patient who could not achieve complete ablation. The inclusion criteria for RFA had been as follows: a total bilirubin level of less than 3 mg/dL, a platelet count of no less than $50\times10^3/mm^3$ and prothrombin activity levels of no less than 50%. Patients with a portal vein tumor thrombosis, refractory ascites, or extrahepatic metastasis were excluded. In general, we performed RFA on patients with three or fewer lesions of 3 cm or less in diameter. However, we also performed ablation on patients beyond these criteria if it was predicted to be clinically effective [22,23]. We enrolled patients who underwent transcatheter arterial chemoembolization (TACE) prior RFA when the treatments were sequentially performed.

Diagnosis of HCC

HCC was diagnosed using dynamic computed tomography (CT), with a consideration of hyperattenuation in the arterial phase with washout in the late phase as a definite sign of this disease [24]. Most nodules were also confirmed histopathologically via an ultrasound-guided biopsy.

Treatment and evaluation

All patients received dynamic CT with a slice thickness of 5 mm within one month prior to ablation for comparison. The interval between the initiation of contrast material infusion and CT image recording was 30 and 120 sec for single detector-row spiral CT (Highspeed Advantage; GE Medical Systems; Milwaukee, WI) and 25, 40 and 120 sec for multidetector-row CT (LightSpeed QX/i GE Medical Systems). The images were presented after axial reconstruction with a slice thickness of 5 mm. RFA was performed on an in-patient basis using a cooled-tip electrode (Covidien, Mansfield, MA) under real-time ultrasound guidance. After 1 to 2 sessions of RFA, dynamic CT was performed to evaluate the treatment efficacy. During the treatment evaluation, we compared the CT findings for early and late phase before ablation and late phase after ablation. A lesion was judged to be completely ablated when the non-enhanced area shown in the late phase of CT post-ablation covered the entire lesion shown in both early and late phase of CT pre-ablation with a safety margin in the surrounding liver parenchyma. We confirmed complete ablation in all slices on which the target nodule was visualized. Patients received additional sessions until complete ablation was confirmed in each nodule. Finally, 303 of the 304 patients enrolled in this study were judged to be completely ablated.

Assessment of tumor recurrence

The follow-up regimen consisted of blood tests and monitoring of tumor markers in an outpatient setting. Ultrasonography and

Table 1. Baseline Characteristics of the HCC Patients analyzed in this study (n = 303).

Variable	n(%)
Age (y)	
mean ± SD	67.5±8.2
Range	44–91
Male sex	191 (63.0)
Viral infection	
HBsAg positive only	28 (9.2)
anti HCVAb positive only	225 (74.3)
Both positive	5 (1.7)
Both negative	35 (11.6)
Alcohol consumption >80 g/day	43 (14.2)
Child-Pugh classification	
Class A	213 (70.3)
Class B	75 (24.8)
Class C	6 (2.0)
Size of tumor (cm)	
mean ± SD	2.5±1.1
≤2.0	106 (35.0)
2.1–3.0	121 (40.0)
>3.0	76 (25.1)
AFP >100 ng/mL	68 (22.4)
DCP >100 mAU/mL	39 (12.9)
AFP-L3 >15%	44 (14.5)

AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; Anti-HCVAb, anti-hepatitis C virus antibody; DCP, des-gamma-carboxy prothrombin; HBsAg, hepatitis B surface antigen.

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dynamic CT were also performed every four months. Tumor recurrence was defined as a newly developed lesion on a dynamic CT that showed hyperattenuation in the arterial phase with washout in the late phase. The nomenclature used for the hepatic segments conformed to *The General Rules for the Clinical and Pathological Study of Primary Liver Cancer, Second English Edition* [25]. According to these rules, subsegments 1 to 8 correspond to Couinaud's segment 1 to 8, respectively [26]. All images were independently reviewed by two experienced radiologists (M.A. and J.S.), and a consensus reading was subsequently performed. Recurrence was categorized as either intra- or extra-subsegmental based on the subsegment of the original nodule. When a tumor was located on two or more subsegments, the subsegment where the major part of the tumor was present was adopted. Local tumor progression and neoplastic seeding through a needle tract were considered to be an intrasubsegmental recurrence. Extrahepatic recurrence was defined as extrasubsegmental.

Treatment of recurrent HCC and Survival Outcomes

When HCC recurrence was identified, patients who met the same criteria used for primary HCC underwent RFA. Survival analysis was performed on a per patient basis. Patients without an indication for RFA due to a multiplicity of recurrent nodules underwent TACE if liver function was categorized as Child-Pugh class B or better. Patients with localized portal tumor invasion were treated by radiotherapy [27]. Patients with tumor invasion to the first branch or main tract of the portal vein were treated with intra-arterial 5-fluorouracil and systemic interferon-α combination therapy [28]. Those with extrahepatic tumor metastasis received systemic chemotherapy if they had well-preserved liver function and a good performance status. Survival time was defined as the interval between the diagnosis of recurrence and the last visit to the outpatient clinic or death up to December 31, 2010. We also analyzed overall survival after the initial RFA. For the analysis start date was set at the day when we perform the first RFA for each patient.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) unless otherwise indicated. To assess whether the location of recurrent nodules was independent of the subsegment of the original nodule, we calculated the kappa coefficient and its 95% confidence interval (CI) [29]. A coefficient of 1 indicates that the subsegments of the original and recurrent nodules are identical, whereas a kappa coefficient of 0 indicates that tumor recurrence occurs completely at random. P values were also calculated on the null hypothesis of kappa equal to zero.

To assess the exclusively intra-subsegmental recurrence rate separately from all kinds of recurrence, we used cumulative incidence estimation with competing risk methods [30]. On this analysis, all types of recurrence were categorized as exclusively intra-subsegmental recurrence, exclusively extrasubsegmental recurrence, or simultaneously intra- and extra-subsegmental recurrence. The hazard function of each type of recurrence was estimated using kernel-based methods described by Muller and Wang [31].

We assessed the risk factors for intra- and extra-subsegmental recurrence independently using univariate and multivariate Cox proportional hazard regression. In assessing the risk factor for intra-subsegmental recurrence, patients with exclusively extra-subsegmental recurrence were treated as censored data and vice versa. The following factors were used for these analyses: age, gender, hepatitis B surface antigen positivity, hepatitis C antibody positivity, platelet count, alanine aminotransferase (ALT), tumor size, alpha-fetoprotein (AFP), des-gamma-carboxyprothrombin (DCP) and lens culinaris agglutinin-reactive fraction of AFP

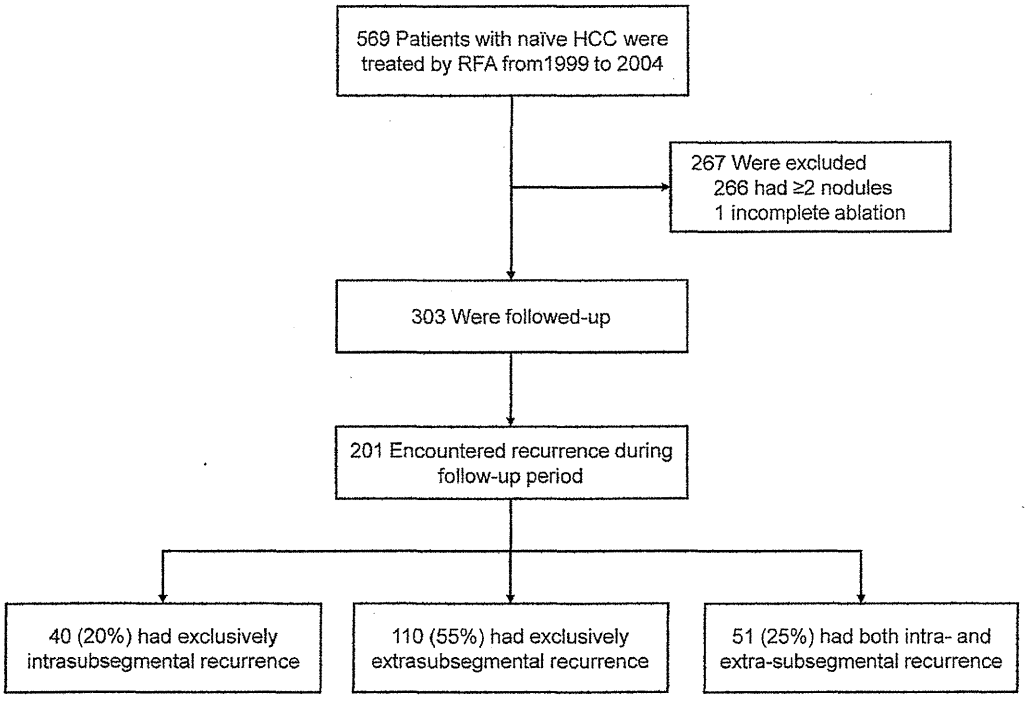

Figure 1. Patient enrollment flow.
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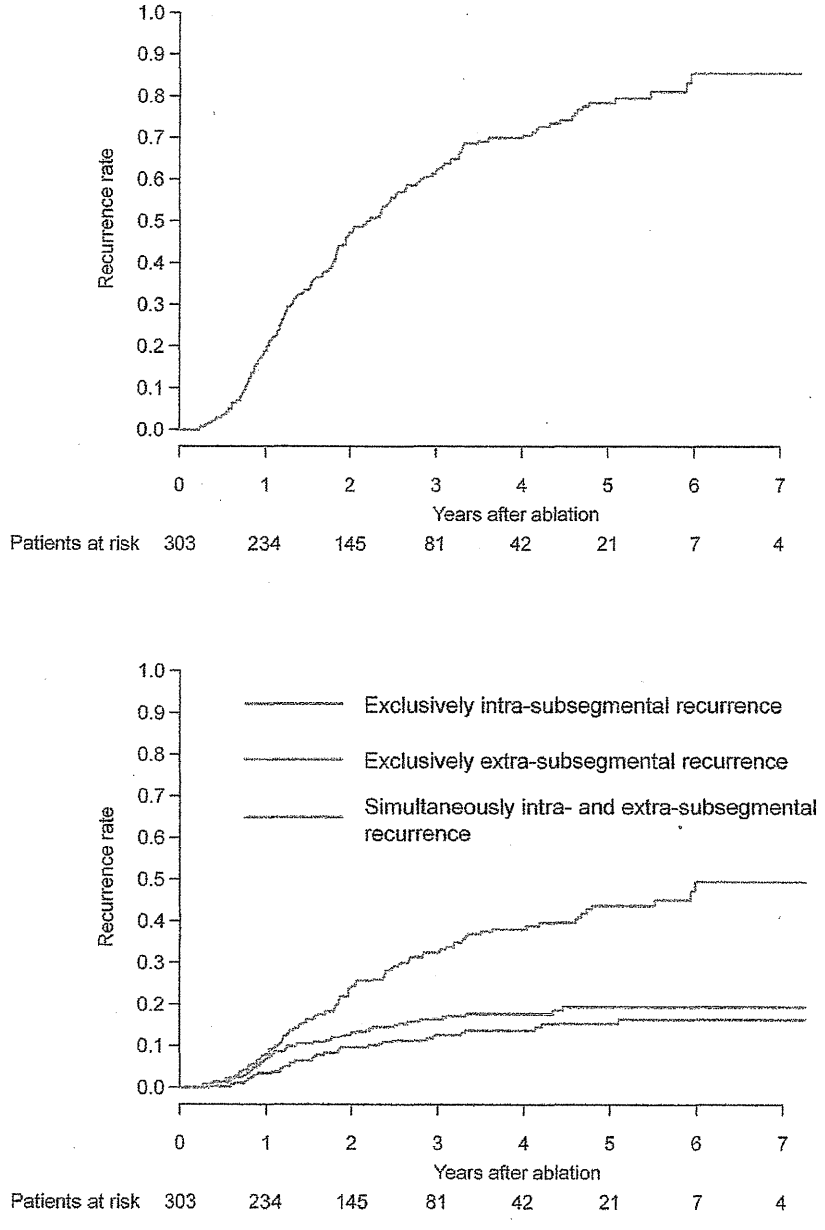

Figure 2. Figure 2A: Overall recurrence. Figure 2B: Recurrence rates of according to the mode of recurrence.
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(AFP-L3). Factors showing statistical significance as a predictor in univariate analysis were further analyzed using a multivariate Cox proportional hazard regression model with stepwise selection of variables based on the Akaike information criterion (AIC).

We plotted survival curves according to the mode of recurrence (i.e., intra-, extra-subsegmental or both) using the Kaplan-Meier method. Statistical significance among these three groups was assessed using the log-rank test. We also calculated adjusted hazard ratios for survival according to the mode of recurrence using multivariate Cox proportional hazard regression with factors that showed statistical significance in a univariate analysis of survival. Differences with a P value of less than 0.05 were considered statistically significant. All statistical analyses were performed with S-Plus Ver. 7 (TIBCO Software Inc., Palo Alto, CA) and R 2.13.0 (http://www.R-project.org).

Results

Patient profiles

The enrolled HCC patient cohort in this study consisted of 191 males and 112 females with a mean age of 67.5 years (Table 1). The mean tumor size was 2.5 ± 1.1 cm in diameter. The number of the nodules distributed in subsegments 1 to 8 was 7 (2.3%), 12 (4.0%), 30 (9.9%), 43 (14.2%), 37 (12.2%), 32 (10.6%), 46 (15.2%), and 96 (31.7%), respectively. One hundred one patients underwent TACE before RFA. The median (range) interval between TACE and RFA was 23 (6–71) days.

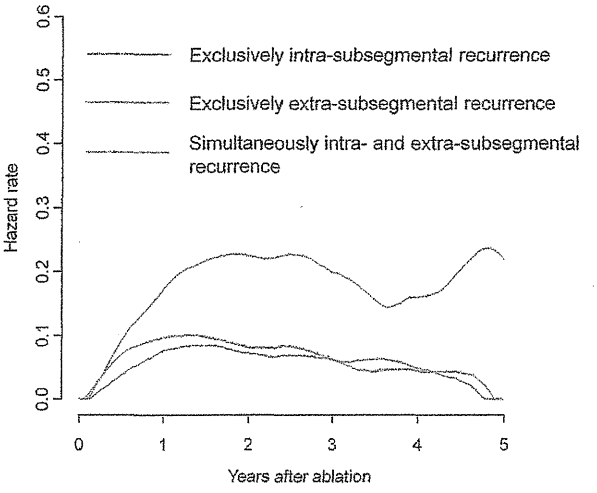

Figure 3. The estimated hazard function over time according to the mode of recurrence.
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HCC recurrence

During the follow-up period (mean, 2.3 years; range 0.2 to 7.3 years), tumor recurrence in the HCC patient cohort was identified in 201 cases. The recurrent nodules were distributed in a total of 340 subsegments. Recurrent nodules were exclusively intra-subsegmetal in 40 patients (20%), and exclusively extra-subsegmental in 110 patients (55%, Fig. 1). Simultaneous intra- and extra-subsegmental recurrence was observed in the remaining 51 patients (25%). The diagnosis of recurrence revealed that 104, 39, 22, 17 and 19 patients had 1, 2, 3, 4–5, and >5 tumors, respectively. Local tumor progression was identified in 10 patients, among which two individuals had simultaneous extra-subsegmental recurrent nodules. Two patients with extrahepatic recurrence (one lymph node and one left adrenal grand) were categorized as extra-subsegmental. Neoplastic seeding, which was categorized as intra-subsegmental recurrence, was observed as the first recurrence in two patients. Details of the distribution of original and recurrent nodules based on subsegments are listed in Table 2. The observed proportion of recurrent nodules in the same subsegment as the original nodule was 0.268, whereas the expected probability that the subsegments of original and recurrent nodules were the same, assuming a random distribution, was 0.154. The kappa coefficient was calculated as 0.135 (95% CI, 0.079–0.190; P<0.001). When patients with a local tumor progression or neoplastic seeding were excluded from this calculation, the kappa statistic decreased to 0.101 (95% CI, 0.046–0.156; P<0.001). The cumulative rates of overall recurrence at 1, 3, and 5 years were 19.6%, 61.8%, and 78.3%, respectively (Fig. 2A). Cumulative rates of exclusively intra-subsegmental, exclusively extrasubsegmental and simultaneously intra- and extra-subsegmental recurrence were 3.4%, 8.1%, and 7.1% at 1 year, 12.7%, 32.7%, and 16.4% at 3 years, and 15.3%, 43.6%, and 19.4% at 5 years, respectively (Fig. 2B). The estimated hazard function curves according to the three types of recurrence showed a similar pattern over the first 4 years. Then only the hazard rate of exclusively extra-subsegmental recurrence increased whereas the hazard rate of the other two types of recurrence decreased (Fig. 3).

Risk factors related to intra- and extra-subsegmental recurrence

Univariate Cox proportional regressions revealed that the following factors were significantly associated with intra-subsegmental recurrence: tumor size, AFP, DCP, AFP-L3, platelet count and anti-HCV antibody positivity. The final model for predicting intra-subsegmental recurrence with stepwise variable selection included tumor size, AFP, platelet count and anti-HCV antibody positivity (Table 3). Factors related to extra-subsegmental recurrence that were found to be significant by univariate Cox proportional hazard regression were age, platelet count, tumor size, AFP and AFP-L3. Multivariate analysis with step-wise variable selection showed that the risk factors for extra-subsegmental recurrence were age, platelet count, tumor size, and AFP (Table 4).

Treatment of recurrent HCC and associated survival outcomes

Among the 40, 110 and 51 patients in whom recurrent HCC was found to be exclusively intra-subsegmental, exclusively extra-subsegmental, and simultaneously intra- and extra-subsegmental, 37 (92.5%), 99 (90.8%) and 42 (82.3%), respectively, were treated using RFA. Of the three patients with an exclusively intra-segmental recurrence, one individual was treated by hepatic resection and one patient was treated by TACE. The remaining patient received best supportive care because of deterioration in liver function. During the follow up period up to December 31,

Table 2. Distribution of the Original and Recurrent Tumors Divided by Subsegment.

	Subsegment of recurrent tumor, n								
Subsegment of original tumor, n	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**sum**
S1	2		2	1			1	2	8
S2	1		1		4	1	1		8
S3		7	10	8	3	3	4	12	47
S4		8	7	11	3	3	6	6	44
S5		2	2	5	10	2	4	7	32
S6	2	1		4	4	10	5	5	31
S7	3	8	11	9	9	9	15	7	71
S8	4	9	9	10	6	13	15	33	99
sum	12	35	42	48	39	41	51	72	340

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Table 3. Univariate and Multivariate Analysis of Intrasubsegmental Recurrences (n = 303).

	Univariate		Multivariate	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age per year	1.00 (0.97–1.02)	0.77		
Male gender	1.05 (0.68–1.63)	0.82		
HBsAg, positive	0.64 (0.30–1.40)	0.27		
anti-HCVAb, positive	2.08 (1.13–3.84)	0.02	2.04 (1.09–3.81)	0.03
Platelet count, $\times 10^4/\mu L$	0.95 (0.91–0.99)	0.009	0.97 (0.93–1.01)	0.09
ALT >80 IU/L	0.99 (0.58–1.71)	0.98		
Size per 1 cm	1.29 (1.08–1.55)	0.006	1.28 (1.06–1.54)	0.009
log(AFP)	1.93 (1.53–2.45)	<0.001	1.29 (1.16–1.44)	<0.001
log(DCP)	1.66 (1.18–2.33)	0.003		
AFP-L3>15%	2.02 (1.20–3.41)	0.009		

HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; Anti-HCVAb, anti-hepatitis C virus antibody; ALT, alanine aminotransferase, AFP, alpha-fetoprotein; DCP, des-gamma-carboxyprothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.

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Table 4. Univariate and Multivariate Analysis of Extrasubsegmental Recurrences (n = 303).

	Univariate		Multivariate	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age per year	1.02 (1.00–1.04)	0.03	1.03 (1.01–1.05)	0.001
Male gender	1.13 (0.83–1.56)	0.44		
HBsAg, positive	0.91 (0.30–1.40)	0.69		
anti-HCVAb, positive	1.49 (1.00–2.20)	0.049		
Platelet count, $\times 10^4/\mu L$	0.94 (0.91–0.97)	<0.001	0.94 (0.92–0.97)	<0.001
ALT >80 IU/L	1.05 (0.72–1.56)	0.78		
Size per 1 cm	1.32 (1.16–1.51)	<0.001	1.39 (1.21–1.60)	<0.001
log(AFP)	1.53 (1.27–1.85)	<0.001	1.37 (1.12–1.68)	0.03
log(DCP)	1.29 (0.96–1.73)	0.1		
AFP-L3>15%	1.66 (1.09–2.52)	0.018		

HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; Anti-HCVAb, anti-hepatitis C virus antibody; AST, aspartate aminotransferase; ALT, alanine aminotransferase, AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.

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2010, 130 patients died and 9 patients were lost to follow-up. The median survival time (95% CI) was 5.72 (3.51-NA) years in patients with exclusively intra-subsegmental recurrence, 4.95 (4.19–5.76) years in patients with exclusively extra-subsegmental recurrence, and 2.43 (1.90–4.26) years n patients with simultaneously intra- and extra-subsegmental recurrence, respectively ($P<0.001$ by log-rank test, Fig. 4). Univariate Cox regression analysis revealed that patients with simultaneously intra- and extra-subsegmental recurrences had a significantly poorer survival than those with an exclusively intra-subsegmental recurrence (hazard ratio, 2.39; 95% CI, 1.32–4.02; $P = 0.001$), whereas this difference became non-significant (HR, 1.91; 95% CI, 0.96–3.80; $P = 0.07$) when adjusted using other significant factors in univariate analysis (Table 5). No differences in the survival outcomes between patients with exclusively intra- and extra-subsegmental recurrences were observed by univariate and multivariate analysis. Finally overall survival rates after the initial RFA at 1, 3, 5, 7 and 10 years were 96.7%, 81.4%, 62.4%, 49.0%, and 31.1%, respectively.

Discussion

Recurrences of HCC are more complicated than those of other solid tumors as they can arise in two distinct forms: de novo carcinogenesis and intrahepatic metastasis [32]. Systematic subsegmentectomy may be effective in treating such patients if the distribution of the hematogenous spread of cancer cells correlates with the physical distance from the original tumor or local portal venous flow. Indeed, in the present study we showed from our data that the location of recurrent nodules was weakly but significantly related to that of the original tumor, even after the exclusion of local tumor progression from the analysis. Given that exclusively intrasubsegmental recurrence in this study could be prevented by subsegmentectomy, through a simple calculation, one fifth of patients who received locally curative RFA might have benefitted if they had received systematic subsegmentectomy. However, it should be mentioned in this regard that those patients who had avoided an intra-subsegmental recurrence owing to a systematic subsegmentectomy would have subsequently encountered tumor recurrence in the remnant liver, and the actual risk reduction of recurrence would therefore be smaller. Actually recurrence-free survival at 10 years after systematic subsegmentectomy was reported to be only 9.4% in a previous nation-wide survey [33].

The risk factors related to de novo carcinogenesis and hematogenous intrahepatic metastasis would be expected to be different. The factors responsible for HCC development, such as fibrosis stage, age, gender, and presence of viral hepatitis, may also affect de novo carcinogenesis [34,35]. On the other hand, factors related to the primary tumor, such as the size and number of tumor nodules, pathological grade(s), the presence of vascular invasion, and positivity of tumor markers, may affect the possibility of intrahepatic occult metastasis at the time of initial treatment. We speculated that there would be differences between the risk factors for intra- and extrasubsegmental recurrence since the former would more strongly correlate with hematogenous intrahepatic metastasis. However the risk factors related to intra- and extrasubsegmental recurrence were found to be quite similar except that old age was a risk factor for only extrasubsegmental recurrence.

Previous reports suggested the hazard function of de novo carcinogenesis and hematogenous intrahepatic metastasis would be different [36,37]. The hazard function of the former is assumed to be gradually increasing over time whereas that of the latter has a peek within two years. And the actual hazard function represents the sum of the two curves. The estimated hazard function of exclusively extra-subsegmental recurrence in this study seemed compatible with the previous reports. However we should be careful to interpret the results because the number at risk at year 4 or 5 was limited.

A previous large scale cohort study of the prognosis of patients with HCC treated by liver transplantation has reported that microvascular invasion is the most important predictor of a poor outcome [38]. This suggests that even if the whole liver is removed, there may be remaining circulating tumor cells that have resulted from tumor nodule invasion of the microvessels. It has also been reported that microsatellite metastatic nodules sur-

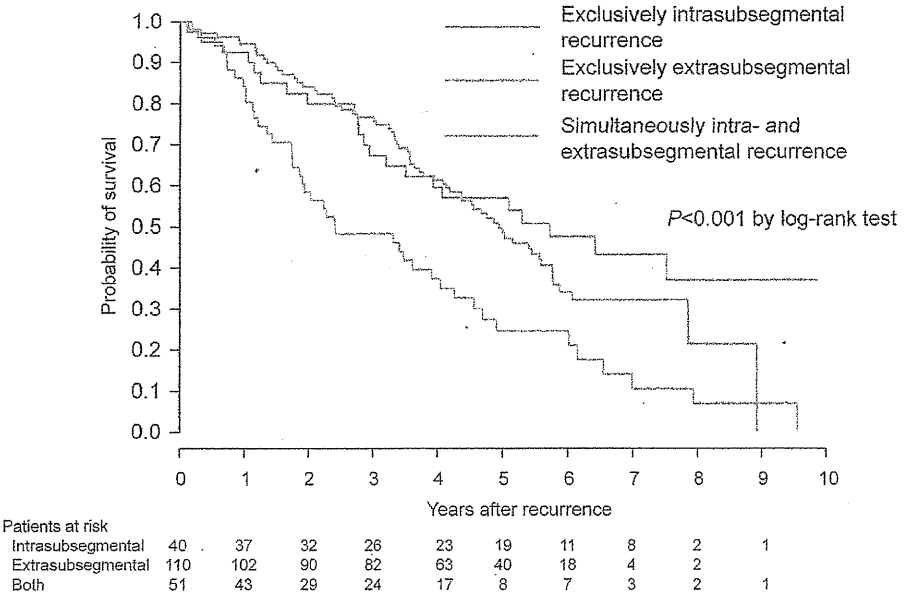

Figure 4. Cumulative survival probability after the diagnosis of recurrence according to the mode of recurrence.
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rounding the main tumor are associated with microvascular invasion and indicate a higher risk of tumor recurrence after liver transplantation [39]. Hence, the impact of removing a tumor-bearing subsegment, including microvascular invasions or micro-satellite nodules, which is thought to be a major advantage of resection over RFA, might be more limited than previously considered.

In this study factors that were supposed to be related to de novo carcinogenesis (e.g., lower platelet count and HCV infection) were risk factors for intra-subsegmental recurrence as well as extra-subsegmental recurrence. The risk of recurrence due to de novo carcinogenesis might be reduced by a subsegmentectomy according to the resected liver volume. However, as most patients with HCC have chronic liver disease, removal of non-cancerous liver

Table 5. Univariate and Multivariate Analysis of Survival after Recurrence*.

	Univariate		Multivariate	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Exclusively extra-subsegmental recurrence vs. exclusively intra-subsegmental recurrence	1.18 (0.73–1.92)	0.50	1.28 (0.76–2.16)	0.35
Simultaneously intra- and extra-subsegmental recurrence vs. exclusively intra-subsegmental recurrence	2.39 (1.32–4.02)	0.001	1.91 (0.96–3.80)	0.07
Age, per 1year	1.02 (1.00–1.05)	0.04	1.04 (1.02–1.07)	0.001
Male gender	1.06 (0.75–1.52)	0.73		
HBsAg, positive	0.74 (0.40–1.38)	0.34		
anti-HCVAb, positive	1.24 (0.77–1.99)	0.39		
Child-Pugh Score, per 1 point	1.45 (1.28–1.63)	<0.001	1.44 (1.27–1.63)	<0.001
Platelet count, per $10^4/\mu L$	0.98 (0.95–1.01)	0.15		
ALT >80 IU/L	0.81 (0.49–1.33)	0.40		
Size >2cm	1.57 (1.11–2.23)	0.01	1.54 (1.06–2.23)	0.02
Multinodular	1.66 (1.18–2.35)	0.004	1.02 (0.63–1.66)	0.92
log(AFP)	1.45 (1.20–1.85)	<0.001	1.13 (1.01–1.26)	0.04
log(DCP)	1.61 (1.21–2.15)	0.001	1.21 (1.06–1.38)	0.004
AFP-L3 >15%	1.93 (1.25–2.98)	0.003	1.17 (0.70–1.96)	0.56

*Clinical data at the diagnosis of recurrence were adopted.
HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; Anti-HCVAb, anti-hepatitis C virus antibody; AST, aspartate aminotransferase: ALT, alanine aminotransferase, AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.
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parenchyma may have a negative impact on long-term survival, especially for those individuals with impaired liver regeneration. Therefore, the key issue is to what extent the liver parenchyma should be removed to sufficiently treat the patient on a case by case basis. It may be speculated that extensive resection could be tolerable and beneficial to those who have a well-preserved capacity for liver regeneration [40].

There is no doubt that tumor recurrence deteriorates the long-term prognosis for HCC patients. However it is also true that there are effective, sometimes potentially curative treatments for recurrent HCC. The re-resection after recurrence of HCC is indicated in 10–30% of patients [41–43] and percutaneous ablation can be repeatedly performed [20,44,45]. Indeed, 37 of 40 patients analyzed in this study who had recurrent nodules confined to the same subsegment as the original tumor were successfully re-treated with RFA. No differences in the survival outcomes were observed between patients with solely intra- or extra-subsegmental recurrences. Hence, the impact of the first recurrence on overall survival may be smaller for HCC compared with other gastrointestinal malignancies such as stomach cancer or colorectal cancer.

We would touch upon the possibility that TACE prior RFA might affect the results. Approximately one third of patients underwent TACE prior RFA. TACE prior RFA can increase the ablated area through the blockade of arterial flow and may decrease the risk of local tumor progression. However the influence would be minimal because local tumor progression was found in only 10 patients and the location of recurrent nodules was weakly but significantly related to that of the original tumor, even after the exclusion of local tumor progression.

In conclusion, the vast majority of recurrent nodules in HCC patients were found to be independent of the subsegment of the original tumor. In addition, whether these recurrences were intra-subsegmental or extrasubsegmental had no impact on the survival outcomes.

Author Contributions

Conceived and designed the experiments: RT SS MA MO KO HY KK. Performed the experiments: RT SS MA JS YK RM HN YA TG. Analyzed the data: RT HY. Contributed reagents/materials/analysis tools: RT. Wrote the paper: RT SS HY KK.

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Identification of a Functional Variant in the *MICA* Promoter Which Regulates *MICA* Expression and Increases HCV-Related Hepatocellular Carcinoma Risk

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Abstract

Hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma (HCC) in Japan. We previously identified the association of SNP rs2596542 in the 5′ flanking region of the *MHC class I polypeptide-related sequence A* (*MICA*) gene with the risk of HCV-induced HCC. In the current study, we performed detailed functional analysis of 12 candidate SNPs in the promoter region and found that a SNP rs2596538 located at 2.8 kb upstream of the *MICA* gene affected the binding of a nuclear protein(s) to the genomic segment including this SNP. By electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay, we identified that transcription factor Specificity Protein 1 (SP1) can bind to the protective G allele, but not to the risk A allele. In addition, reporter construct containing the G allele was found to exhibit higher transcriptional activity than that containing the A allele. Moreover, SNP rs2596538 showed stronger association with HCV-induced HCC ($P = 1.82 \times 10^{-5}$ and OR = 1.34) than the previously identified SNP rs2596542. We also found significantly higher serum level of soluble MICA (sMICA) in HCV-induced HCC patients carrying the G allele than those carrying the A allele ($P = 0.00616$). In summary, we have identified a functional SNP that is associated with the expression of MICA and the risk for HCV-induced HCC.

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Introduction

Hepatocellular carcinoma (HCC) is one of the common cancers in the world. It is well-known to be associated with the chronic infection of Hepatitis B (HBV) and Hepatitis C (HCV) viruses. In Japan, nearly 70% of HCC patients are infected with HCV [1]. The annual rate of developing HCC among patients with HCV-related liver cirrhosis in Japan is estimated to be about 4–8 percent [2]. Recent analyses have identified various genetic factors that are related with viral induced liver diseases [3–5]. In our previous two-stage genome-wide association study (GWAS) using a total number of 1,394 cases and 5,486 controls, a SNP rs2596542 located on chromosome 6p21.33 was shown to be significantly associated with HCV-induced HCC ($P = 4.21 \times 10^{-13}$ and OR = 1.39) [6]. This SNP is located within the class I major histocompatibility complex (MHC) region and is at about 4.8 kb upstream of *MHC class I polypeptide-related sequence A* (*MICA*) gene. We also identified that the risk A allele of SNP rs2596542 was strongly associated with the low expression of soluble MICA (sMICA) in the serum of HCV-related HCC patients [6].

MICA is a membrane protein which is up-regulated in various tumor cells and also induced in response to various cellular stresses such as infection, hypoxia, and heat shock [7]. It is an important component of the innate immune response, as MICA can bind to the NKG2D receptor and subsequently activate natural killer (NK) cells, CD8+ cells, and γδ T cells [8,9]. Moreover, membrane MICA can be shed by metalloproteinases, including MMP9, ADAM10, and ADAM17, and secreted into serum as a soluble form [10,11]. Since these metalloproteinases are often activated in HCC, the expressions of both membrane-bound MICA and sMICA are increased [12,13]. SNP rs2596542 was found to be associated with the progression from chronic hepatitis C (CHC) to HCC and also with serum sMICA level. Hence, both rs2596542 and sMICA would be possible prognostic biomarkers for CHC patients. However, their underlying molecular mechanisms were not fully elucidated so far.

We hypothesize that *MICA* variations could affect sMICA level by either one or both of the following two possible mechanisms: (1) the genetic variation(s) in the coding region affecting the protein stability and (2) the transcriptional regulation. Previously, variable

numbers of tandem repeats (VNTRs) in exon 5 of *MICA* were identified to affect MICA subcellular localization and serum MICA level [14]. The exon 5 of *MICA* encodes the transmembrane domain and the insertion of an extra G nucleotide in the domain would result in a premature stop codon that would generate MICA protein without a transmembrane domain and subsequently affect sMICA level [14]. However, our previous results indicated that MICA VNTR was not significantly associated with the sMICA level or HCC risk [6]. Therefore, in the current study, we have tried to investigate whether the *MICA* variations would affect the *MICA* transcription in the liver cancer cells. Through the functional analysis of genetic variations in the *MICA* promoter region, we here report a causative SNP rs2596538 that increases the binding affinity of the transcription factor Specificity Protein 1 (SP1) and the risk of progression of the disease.

Materials and Methods

Samples and genotyping

DNA samples for direct sequencing (50 HCV-related HCC cases), imputation analysis (721 HCV-related HCC cases and 5,486 HCV-negative controls), and serum samples for sMICA ELISA (246 HCV-related HCC) were obtained from BioBank Japan [15,16]. Genotyping of SNPs from 1,394 HCC patients and measurement of sMICA expression by ELISA were performed in the previous study [6]. Genotyping of SNP rs2596542 in 1,043 CHC was performed previously in RIKEN using Illumina HumanHap610-Quad BeadChip [17]. All CHC subjects had abnormal levels of serum alanine transaminase for more than 6 months and were positive for both HCV antibody and serum HCV RNA. The SNP rs2596542 in liver cirrhosis samples without hepatocellular carcinoma from BioBank Japan (n = 420) and the University of Tokyo (n = 166) were genotyped using Illumina HumanHap610-Quad BeadChip or invader assay [18]. All subjects were either subjected to liver biopsy or diagnosed by non-invasive methods including hepatic imaging, biochemical data, and the presence/absence of clinical manifestations of portal hypertension [18]. The samples used in the current project were listed in Table S1. Case samples with HBV co-infection were excluded from this study. The subjects with cancers, chronic hepatitis B, diabetes or tuberculosis were excluded from non-HCV controls. All subjects were Japanese origin and provided written informed consent. This research project was approved by the ethical committees of the University of Tokyo and RIKEN.

Imputation study

The imputation study was performed by using a hidden Markov model programmed in MACH [19] and haplotype information from 1000 genomes database [20]. The imputation results were confirmed by direct DNA sequencing in 50 randomly selected samples.

Cell culture

Human liver cancer cell lines HLE and HepG2 were purchased from JHSF (Osaka, Japan) and ATCC. These cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum. Cells were cultured at 37°C with 5% CO_2.

EMSA

HLE cells were grown in 15 cm culture plate until they reached 95% confluency. The plate was then sealed with parafilm and immersed in a water bath at 42.5°C for 1.5 hours [21]. Nuclear extracts from these cells were prepared according to the standard protocol [22]. EMSA was carried out using DIG Gel Shift Kit, 2nd Generation (Roche) according to the manufacturer's instructions. The sequences of the 12 probes were listed in the Table S2. In brief, 30 fmol of labeled probes were hybridized with 5 μg nuclear extract for 15 minutes at room temperature. The mixtures were then loaded into a 6% TBE gel, separated by electrophoresis at 4°C and transferred onto a nylon membrane. The membrane was then hybridized with anti-digoxigenin-AP antibody and developed by CSPD solution. For competition study, nuclear extracts were incubated with non-labeled oligonucleotides first before adding labeled probe. For supershift assay, SP1 antibody (SC-59X, Santa Cruz Biotechnology) was added into the nuclear extract and incubated on ice for 30 minutes first before adding labeled probe. The mixtures were then separated by electrophoresis using 4% TBE gel. All EMSAs were repeated twice for reconfirmation of the results.

ChIP

The HLE cells (G allele homozygote) and HepG2 cells (heterozygote) were used in the ChIP assay. The plasmid pCAGGS-SP1 was transfected into both cells by using FuGENE6 Transfection Reagent (Roche). The ChIP assays were carried out using Chromatin Immunoprecipitation Assay Kit (Millipore) according to the manufacturer's protocol. In brief, the cells were treated with formaldehyde to crosslink DNA-protein complexes at 48 hours post-transfection. DNA-protein complexes were then sheared by sonication and immunoprecipitated by rabbit polyclonal anti-SP1 antibody (SC-59X, Santa Cruz Biotechnology). The resulting DNAs were analyzed by PCR (Table S2). In order to determine the binding specificity of SP1 to the SNP rs2596538 allele, the PCR products from HepG2 cells were further subcloned into pCR 2.1 vector and sequenced to assess G to A ratio in both input DNA and immunoprecipitant.

Dual luciferase reporter assay

Three copies of 31 bp DNA fragments equivalent to the EMSA oligonucleotides of SNP rs2596538 were cloned into pGL3-promoter vector (Promega). The plasmids were co-transfected with pCAGGS-SP1 and pRL-TK plasmids (Promega) into HLE cells by FuGENE6 Transfection Reagent (Roche). The pCAGGS-SP1 plasmid provided the expression of transcription factor SP1, and pRL-TK plasmid served as internal control for transfection efficiency [23]. The cells were lysed at 48 hours post-transfection, and relative luciferase activities were measured by Dual Luciferase Assay System (Toyo B-Net).

Western blotting

Cancer cell lysates were prepared by using pre-chilled RIPA buffer, and 25 μg of each lysate was loaded into the gel and separated by SDS-PAGE. Western blotting was performed according to the standard protocol. Rabbit anti-MICA antibody (ab63709, abcam: 1/1000) and rabbit anti-SP1 antibody (17-601, Upstate Biotechnology: 1/500) were used in the experiment.

Statistical analysis

The case-control association was analyzed by Student's *t*-test and Fisher's exact test as appropriate. The association of allele dependent sMICA expression was studied by Kruskal-Wallis test using R statistical environment version 2.8.1. The LD and coefficients (D' and r^2) were calculated by Haploview version 4.2 [24].

Table 1. Association of rs2596542 with the progression from CHC to LC and HCC.

	Case MAF	Control MAF	P^*	OR	95% C.I.
LC vs CHC	0.3797	0.3442	0.04842	1.166	1.01–1.35
HCC vs LC	0.4012	0.3797	0.20296	1.094	0.95–1.26

MAF, minor allele frequency; OR, odds ratio for minor allele. C.I., confidence interval. SNP rs2596542 was analyzed in 1,043 chronic hepatitis C (CHC), 586 liver cirrhosis without hepatocellular carcinoma (LC) and 1,394 HCV-induce hepaticellular carcinoma patients (HCC). *calculated by Armitage trend test.
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Results

Analyses of SNP rs2596542 in HCV-infected patients at different disease stages

Since the development of HCC consists of multiple steps, we investigated the role of SNP rs2596542 with disease progression. SNP rs2596542 was genotyped in patients at three different disease categories of CHC (chronic hepatitis C) without liver cirrhosis (LC) or HCC, LC without HCC, and HCC. The statistical analysis indicated that SNP rs2596542 was significantly associated with disease progression from CHC to LC with P-value of 0.048 and odds ratio of 1.17 (Table 1). The risk allele frequency among HCC patients (40.1%) was higher than that among LC patients (38.0%), but the association was not statistically significant (P-value of 0.203 and odds ratio of 1.09). These results suggested the involvement of *MICA* with both liver fibrosis and hepatocellular carcinogenesis.

HCV-HCC risk is not associated with MICA copy number variation

A previous report has indicated the deletion of the entire *MICA* locus in 3.2% of Japanese population [25] and this deletion was shown to be associated with the risk of nasopharyngeal carcinoma (NPC), especially in male [26]. To identify the functional SNP that may affect *MICA* mRNA expression, we analyzed the relation between the *MICA* copy number variation (CNV) and the HCC susceptibility. We quantified this CNV by real-time PCR in 375 HCV-related HCC patients and 350 HCV-negative controls. As shown in Table S3, we found no difference in the copy numbers between HCC cases and controls, indicating that this CNV is unlikely to be causative genetic variation for the risk of HCC.

Direct sequencing of 5′ flanking region of *MICA*

We then focused on the variations in the 5′ flanking region of the *MICA* gene which may be associated with its promoter activity. We had conducted direct DNA sequencing of the 5-kb promoter region which included the marker SNP rs2596542 using genomic DNAs of 50 HCC subjects and identified 11 SNPs showing strong linkage disequilibrium with the marker SNP rs2596542 ($D'>0.953$ and $r^2>0.832$) (Fig. S1, Table 2).

Allele specific binding of nuclear protein to genomic region including SNP rs2596538

To investigate whether these genetic variations would affect the binding affinity of some transcription factors, we had conducted the electrophoretic mobility shift assay (EMSA) using the nuclear extract of HLE human hepatocellular carcinoma cells. Since MICA is a stress-inducible protein [21], we first treated the cells with heat shock treatment at 42°C for 90 minutes and confirmed significant induction of MICA expression as shown in Fig. 1a. Then we performed EMSA using 24 labeled-oligonucleotides corresponding to each allele of the 12 candidates' SNPs. The results of EMSA demonstrated that an oligonucleotide corresponding to a G allele of SNP rs2596538 exhibited stronger binding affinity to a nuclear protein(s) than that to an A allele (Fig. 1b). We then confirmed the specific binding of nuclear proteins to the G allele by competitor assay using non-labeled oligonucleotides (Fig. 1c). The self (G allele) oligonucleotides inhibited the formation of DNA-protein complex in a dose-dependent manner, but the non-self (A allele) oligonucleotides showed no inhibition effect. Taken together, some nuclear protein(s) in hepatocellular carcinoma cells would interact with a DNA fragment including the G allele of SNP rs2596538.

Table 2. Linkage disequilibrium between 11 candidate SNPs and SNP rs2596542.

SNP ID	Relative position[a]	A1	A1 frequency	D'	r^2
rs2596542	−4815	A	0.36		
rs2428475	−4788	G	0.36	1	1
rs28366144	−4586	T	0.36	1	1
rs2428474	−4387	G	0.39	1	0.88
rs2251731	−4045	A	0.39	1	0.88
rs2844526	−3703	C	0.38	1	0.918
rs2596541	−3572	A	0.38	1	0.918
rs2523453	−3285	G	0.38	1	0.918
rs2544525	−3259	C	0.38	1	0.918
rs2523452	−2870	G	0.34	0.953	0.832
rs2596538	−2778	A	0.34	0.953	0.832
rs2844522	−2710	C	0.34	0.953	0.832

Note: Direct DNA sequence of 5-kb promoter region of MICA from 50 HCV-HCC subjects. D' and r^2 were calculated by comparing the genotypes of these SNPs to the marker SNP rs2596542 by Haploview. A1, minor allele; [a]Relative position to exon 1 of the *MICA* gene.
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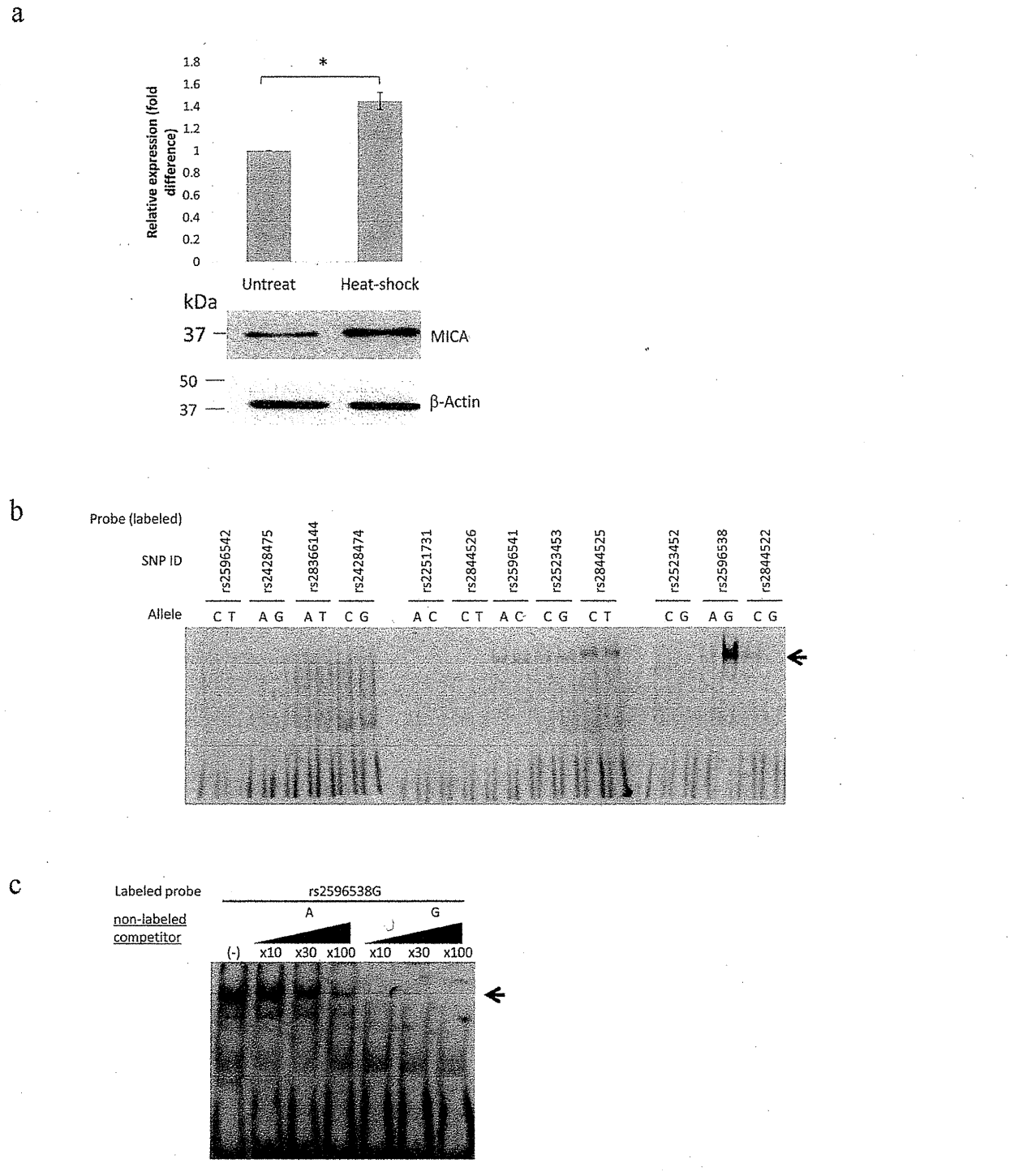

Figure 1. SNP rs2596538 affects the binding affinity of nuclear proteins. (**A**) Real-time quantitative PCR (upper) and Western blotting (lower) of MICA before and after heat shock treatment in HLE cells. *B2M* and β-actin are served as internal and protein loading control. (**B**) EMSA using 31 bp labeled probes flanking each SNP located within the 4.8 kb region upstream of *MICA* transcription start site. A black arrow indicates the shifted band specific to G allele of SNP rs2596538. (**C**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat treated HLE cells. Non-labeled A or G allele of SNP rs2596538 at different concentrations are used as competitors. Pointed arrow indicates shifted band. *$P<0.05$ by Student's *t*-test.

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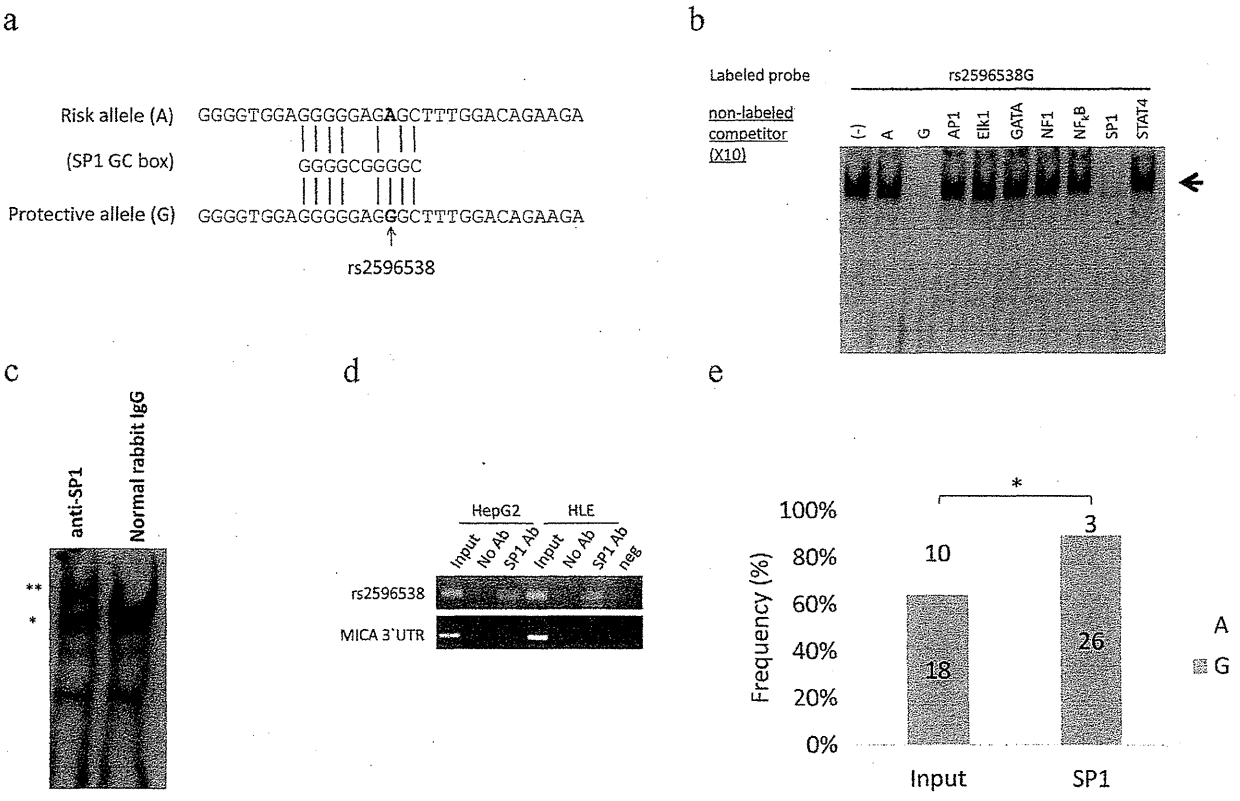

Figure 2. Binding of transcription factor SP1 to G allele of SNP rs2596538. (**A**) Multiple alignment of a GC box and DNA sequence of A or G probe of SNP rs2596538 used in EMSA. (**B**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat treated HLE cells. Non-labeled consensus oligonucleotides of seven transcription factors are used as competitors. Pointed arrow indicates shifted band. (**C**) EMSA using the labeled G allele of SNP rs2596538 and nuclear extract from heat shock treated HLE cells in the presence of anti-SP1 antibody or normal rabbit IgG. Asterisks on the left side indicate the shifted (*) and super-shifted bands (**). Normal rabbit IgG serves as a negative control. (**D**) ChIP assay using HepG2 and HLE cell lines were ectopically expressed with SP1 protein. DNA-protein complex was immunoprecipitated with anti-SP1 antibody followed by PCR amplification using a primer pair flanking SNP rs2596538. DNAs precipitated without antibody are served as a negative control. PCR primers flanking the 3′ UTR region of *MICA* are served as a negative control. (**E**) Genotype distribution at SNP rs2596538 in PCR fragment amplified from the input genomic DNA and DNA-protein complex immunopurified from HepG2 cells by using anit-SP1 antibody. *$P<0.05$ by Student's *t*-test.
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SNP rs2596538 regulates the binding of SP1

Since *in silico* analysis identified a putative GC box in a protective G allele but not in a risk A allele (Fig. 2a), the transcription factor SP1 might preferentially bind to the G allele. Base on this information, we further performed competitor assay using non-labeled oligonucleotides (Table S2) and found that among seven tested oligonucleotides, only SP1-consensus oligonucleotides could effectively inhibit the binding of the nuclear protein(s) to the labeled G allele (Fig. 2b). In addition, we identified that the addition of anti-SP1 antibody caused a supershift of a band corresponding to the DNA-protein complex while control IgG did not cause the band shift (Fig. 2c). This result clearly indicated that the SP1 protein is very likely to be a component of the DNA-protein complex.

Furthermore, we performed chromatin immunoprecipitation (ChIP) assay to confirm the binding of SP1 to this genomic region *in vivo*. We had used two cell lines with different genetic backgrounds at SNP rs2596538 locus: HLE cells carrying the only G allele, while HepG2 cells harboring both A and G alleles. After the introduction of SP1 expression vector (pCAGGS-SP1) into these cell lines, the cell extracts were subjected to ChIP assay using anti-SP1 antibody (Fig. 2d). Subsequent PCR experiments indicated that SP1 bound to a genomic fragment containing the G allele of SNP rs2596538 *in vivo*, while 3′ UTR region of *MICA* (negative control) was not immunoprecipitated with anti-SP1 antibody. To further evaluate the binding ability of SP1 to each allele *in vivo*, we sub-cloned the DNA fragment that amplified from genomic DNA of HepG2 cells before and after immunoprecipitation by anti-SP1 antibody. The subsequent sequencing results showed that 26 out of 29 tested clones contained the G allele, demonstrating the preferential binding of SP1 to the G allele (Fig. 2e).

SP1 over-expression preferentially up-regulates MICA expression at G allele

To further investigate the physiological role of the interaction between SP1 and this genomic region, we performed reporter gene assay. Three copies of 31-bp DNA fragments flanking the candidate functional SNP rs2596538 were subcloned into the multiple cloning sites of the pGL3 promoter vector. The relative luciferase activity of the plasmid including the G allele was significantly higher than that including the A allele (Fig. 3a). Furthermore, over-expression of SP1 in the cells could significantly enhance the luciferase activity of the G-allele vector, while the enhancement of the A-allele vector was relatively modest (Fig. 3a).