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Effect of Heavy Alcohol Intake on Long-term Results after Curative Resection of Hepatitis C Virus-related Hepatocellular Carcinoma

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We studied the effect of heavy alcohol intake (ethanol intake ≥80 g/day for ≥5 yr) on long-term results in 53 patients with hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC) who had undergone curative hepatic resection. Cell proliferative activity in the tumor and non-tumorous liver was also assessed by counting argyrophilic nucleolar organizer region-associated proteins (Ag-NOR) in the resected specimens. Twenty patients (20 males, 0 females) were positive for heavy alcohol intake [AI(+)] and 33 (28 males, 5 females) were not [AI(−)]. All patients were positive for HCV antibody and negative for hepatitis B surface antigen. Carcinoma recurred within 3 to 51 postoperative months in 42 (79.2%) of the 53 patients. The median disease-free survival time was 12.6 mo in the AI(+) group and 25.4 mo in the AI(−) group ($P<0.01$). The AI(+) group also had significantly poorer survival than the AI(−) group ($P<0.05$, 3-year survival rate: 66.7% vs. 93.5%). HCC tumor in the AI(+) group showed significantly increased proliferative activity compared with that in the AI(−) group ($P<0.05$, Ag-NOR number: 2.3±0.8 vs. 1.9±0.4). However, there was no significant difference between the numbers of Ag-NORs in non-tumorous liver from these two groups (1.5±0.2 vs. 1.5±0.2). Patients with heavy alcohol intake should be followed particularly closely, even if they have received curative surgery, since heavy alcohol intake is closely related to a poor postoperative prognosis.

Key words: Alcohol intake — Hepatitis C virus — Hepatocellular carcinoma — Nucleolar organizer region

HCC is one of the most common tumors throughout the world and the major cause of its high incidence is chronic viral hepatitis progressing to cirrhosis and HCC. The infection of HCV in particular plays an important etiologic role in HCC development in areas (including Japan) where the hepatitis B virus infection rate is low, although the exact mechanism whereby HCC is induced by HCV is yet to be elucidated.[1–4] A large increase in the frequency of HCC in Japan has been caused by a recent increase in HCV-related, but not hepatitis B virus-related HCC.[2] On the other hand, AI increases the relative risk of developing HCC in patients with HCV, suggesting that AI enhances the development of HCC related to HCV infection.[3,5] However, only a few reports have addressed the relationship between AI and the prognosis in HCC, and as yet, no definite conclusion has been established.

We studied the effect of AI on postoperative prognosis in patients with HCV-related HCC. We also assessed cell proliferative activity in the tumor and non-tumorous liver by counting Ag-NORs in resected specimens. This single-institution study was undertaken using uniform methods of diagnosis and treatment of HCC and identical follow-up examinations, thus facilitating confirmation of the effect of AI on postoperative prognosis.

Abbreviations: AI, alcohol intake; Ag-NOR, argyrophilic nucleolar organizer region; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

MATERIALS AND METHODS

Patients The participants were 53 patients who had undergone curative hepatectomy to treat HCV-related HCC between September 1988 and November 1990 at the National Cancer Center Hospital, Tokyo. All patients were positive for HCV antibody by first-and/or second-generation enzyme-linked immunosorbent assays and negative for hepatitis B surface antigen by the reverse passive haemagglutination assay. The term curative operation as used herein indicates that no visible tumor and/or tumor demonstrable by intraoperative ultrasonography was evident in the remnant liver at laparotomy. Of the 53 patients, 3 underwent lobular resection, 7 segmentectomy, 8 sub-segmentectomy, and 35 partial wedge resection. The diagnosis was confirmed by postoperative histological examination of all patients. None of the patients received any postoperative adjuvant anti-cancer treatment, or died within 1 mo after surgery.

Influence of alcohol intake The patients were divided into two groups according to their levels of alcohol intake and the postoperative prognosis in each group was ana-

lyzed by using univariate analysis and a multiple regression model. Patients who had an ethanol intake of 80 g or more per day for a period of 5 or more years were classified as AI-positive [AI(+)]. Patients who did not meet these criteria were classified as AI-negative [AI(−)]. Information on alcohol intake was obtained before operation through interviews by a doctor and/or a nurse. Patients were asked three questions, including the type of alcoholic drink consumed, the quantity of each drink habitually consumed per day and the duration of drinking. Analysis of clinicopathological factors included microscopic examination to identify associated liver cirrhosis and vascular invasion, as well as tumor cell differentiation. In the assessment of tumor differentiation, the predominant histologic grade of each nodule was assigned according to the scale of Edmondson and Steiner.[6] The patient group with vascular invasion also included those with intrahepatic metastasis, since the latter is thought to be mainly a product of the former. We regarded "multiple" intrahepatic tumor nodules as those other than the metastatic HCC type. Metastatic HCC nodules were defined according to the pathological criteria as tumors apparently growing from portal vein tumor thrombi or multiple satellite nodules surrounding a large main tumor.[7] The DNA content was measured by means of flow cytometry (FACScan; Becton Dickinson, San Jose, CA) using fresh or frozen samples, as described.[8] The DNA ploidy pattern was classified as DNA diploidy or DNA aneuploidy. When different G0/G1 peaks were present in the tumors of one patient, the patient was considered to have DNA aneuploid tumors.

Follow-up The patients were closely followed-up at our out-patient clinic. Serum alpha-fetoprotein levels were measured every 3 months, as we also conducted ultrasonography and/or dynamic computed tomography with contrast medium and chest X-rays. Hepatic angiography was performed when recurrence of intrahepatic tumor was suspected. Intrahepatic recurrence was diagnosed on the basis of either histological examination, or markedly elevated serum alpha-fetoprotein levels (>400 ng/ml) along with lesions that were demonstrable by various imaging procedures, and typical computed tomographic and/or angiographic findings. Extrahepatic recurrence was diagnosed based on a complete clinical assessment including chest X-ray examination, ultrasonography and computed tomography of the abdomen, as well as radionuclide bone scanning.

Survival was measured from the date of hepatectomy until death from cancer or to the last day of follow-up. None of the patients died due to causes other than cancer during the follow-up period.

Postoperative recurrence All tumors detected after curative surgery were regarded as recurrent, because it is difficult to distinguish nonexcised cancer from *de novo* primary tumors. The disease-free survival time was defined as the period from the date of surgery until detection of the first recurrence of HCC. Patients who remained free of documented disease recurrence up to the time of analysis were included in the calculations of disease-free survival rates. Recurrence was classified according to the hepatographic findings as follows: single-nodular (intrahepatic single nodule), multi-nodular (intrahepatic multiple nodules), whole liver (diffuse or widespread multi-nodular recurrence over the whole remnant liver) and extrahepatic (extrahepatic recurrence alone).

Cell proliferative activity Thirty resected specimens of HCC from patients with a solitary nodule were examined, as described.[9] Sections were cut at a thickness of 3 μm from routinely processed paraffin blocks. These were dewaxed in xylene and rehydrated through a graded ethanol series to deionized water. The Ag-NOR stain was prepared by dissolving gelatin in 1% aqueous formic acid at a concentration of 2%. This solution was mixed at a 1:2 volume ratio with 150% (g/dl) silver nitrate to give the final working solution. This was poured over the tissue sections and left for 20–30 min at room temperature in the dark. The silver colloid was washed off with deionized water, then the sections were dehydrated to xylene and mounted in synthetic medium. The number of Ag-NOR nuclear dots was counted in 200 hepatocytes in each HCC nodule and in the surrounding non-tumorous parenchyma, using a ×100 oil-immersion objective. In these HCC lesions, 100 hepatocytes were randomly selected from the central and peripheral portions. Bile ductular, vascular, inflammatory and Kupffer cells were not included in the enumeration procedure, and in binucleate or multinucleate cells, the number of Ag-NORs was counted in each nucleus. The Ag-NORs were visualized as black dots. All intranuclear silver-stained structures were counted. However, clustered tiny black dots within silver-stained nucleoli were treated as one entity. The mean (± standard deviation) numbers of Ag-NORs for 200 hepatocytes of each lesion (Ag-NOR numbers) were determined.

Statistical methods Survival curves were calculated by the Kaplan-Meier method, and the significance of the difference between curves was evaluated using log-rank tests. The Cox proportional hazards model was used to determine the variables most significantly related to disease recurrence. Forward and backward stepwise regression procedures based on the partial likelihood ratio were used to determine the major independent predictive factors for recurrence. The χ^2 test was used to examine the significance of the difference between groups in the frequency of various attributes. The significance of differences in quantitative variables between groups was assessed by using Wilcoxon's test. The criterion of signifi-

cance was taken as a P value of 0.05 or less. All P values in this report were of the two-tailed type.

RESULTS

Of the 53 patients with HCC, 20 and 33 were in the AI(+) and AI(−) groups, respectively. Among the 20 patients with AI, 4 were ex-drinkers who had stopped drinking 3 or more years prior to hepatic surgery and the remaining 16 were current drinkers. The follow-up period from surgery until death or until follow-up was terminated, ranged from 17 to 79 mo with a median of 46 mo. During the follow-up period, 12 of the 16 current drinkers ceased alcohol intake after hepatectomy, but the remaining 4 occasionally drank a small amount of alcohol.

Clinicopathological data Table I shows the clinicopathological data for the AI(+) and AI(−) groups. Patients in the AI(+) group tended to be younger than those in the AI(−) group, and males tended to be more common in the former. However, there was no significant difference between the groups. The ratio of HCC nodules classified as Edmondson III was significantly higher in the AI(+), than in the AI(−) group. However, there was no significant difference between these two groups in terms of any other clinicopathological data, including the number of intrahepatic tumor nodules, largest tumor size, hepatic reserve assessed by Child's classification and the ratio of patients receiving preoperative transcatheter arterial embolization.

Table I. Clinicopathological Data for 20 and 33 Patients with and without Heavy Alcohol Intake, Respectively

Characteristics		No. of patients (%)		
		Patients with alcohol intake	Patients without alcohol intake	P value
Age	<60 yr	9 (45)[a]	10 (30)	NS[b]
	≥60 yr	11 (55)	23 (70)	
Sex	Men	20 (100)	28 (85)	NS
	Women	0 (0)	5 (15)	
History of BTF[c] (+)		8 (40)	16 (49)	NS
Associated LC[d] (+)		9 (45)	20 (61)	NS
Child's classification				NS
A		13 (65)	20 (61)	
B		7 (35)	13 (39)	
No. of intrahepatic nodules				NS
Single		12 (60)	18 (55)	
Multiple		8 (40)	15 (45)	
Maximal tumor size				NS
<50 mm		17 (85)	30 (91)	
≥50 mm		3 (15)	3 (9)	
Vascular invasion (+)		11 (55)	13 (39)	NS
Tumor cell differentiation				$P<0.05$
Edmondson I, II		13 (65)	29 (88)	
Edmondson III		7 (35)	4 (12)	
Nuclear DNA content				NS
DNA diploidy		7 (35)	15 (45)	
DNA aneuploidy		13 (65)	18 (55)	
Alpha-fetoprotein				NS
<200 ng/ml		12 (60)	25 (76)	
≥200 ng/ml		8 (40)	8 (24)	
Preoperative TAE[e] (+)		6 (30)	8 (24)	NS

a) Numbers in parentheses are percentages.
b) Not significant.
c) Blood transfusion.
d) Liver cirrhosis.
e) Transcatheter arterial embolization.

Recurrence HCC recurred within 3 to 51 postoperative months in 42 (79.2%) of the 53 patients. Recurrent tumors were detected within 1 yr, between 1 and 2 yr, and over 2 yr after the operation in 14 (33.3%), 14 (33.3%), and 14 patients (33.3%), respectively. The median disease-free survival time and 1-, 3-, and 5-yr disease-free survival rates were 21.6 mo, 72.5%, 24.0%, and 8.4%, respectively.

Recurrence was evident in 17 (85.0%) of the 20 patients with AI and in 25 (75.8%) of the 33 patients without AI. The median disease-free survival time and 1- and 3-yr disease-free survival rates were 12.6 mo, 51.0% and 14.6%, respectively, in the AI(+) group and 25.4 mo, 83.9%, and 28.7%, respectively, in the AI(−) group. There was a significant difference between the groups in the disease-free survival time ($P<0.01$, Fig. 1). Multivariate regression analysis showed that heavy alcohol intake had independent predictive significance for recurrence (Table II).

Since all the patients in the AI(+) group were male, the effect of AI on recurrence was analyzed in sex-matched patients. Among the 48 male patients, the disease-free survival for the AI(+) group was significantly

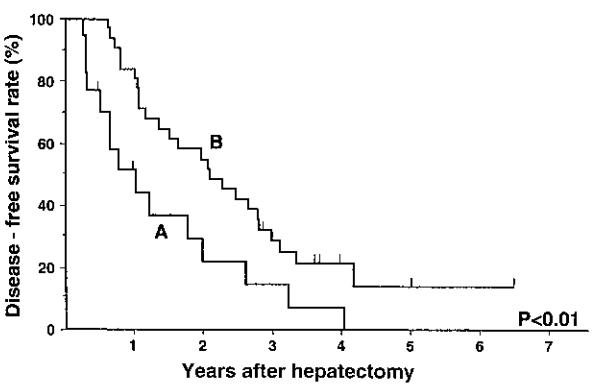

Fig. 1. Disease-free survival curves for 20 patients with (A) and 33 patients without alcohol intake (B). There was a significant difference between the two groups ($P<0.01$).

Table II. Significant Predictive Factors Associated with Recurrence for 53 Patients who Underwent Curative Resection for HCV-related HCC as Determined by the Cox Proportional Hazards Model

Variable	Coefficient	Recurrence rate ratio (95% confidence interval)	P
Nuclear DNA content	1.05	2.86 (1.43–5.70)	$P<0.01$
Alcohol intake	0.93	2.53 (1.39–4.62)	$P<0.01$
Vascular invasion	0.64	1.90 (1.06–3.39)	$P<0.05$

Table III. Types of Postoperative HCC Recurrence in 42 Patients

Recurrence type	Patients with alcohol intake	Patients without alcohol intake
Single-nodular	5 (29)[a)]	10 (40)
Multi-nodular	7 (41)	12 (48)
Whole liver	5 (29)	1 (4)
Extrahepatic	0 (0)	2 (8)
Total	17 (100)	25 (100)

a) Numbers in parentheses are percentages.

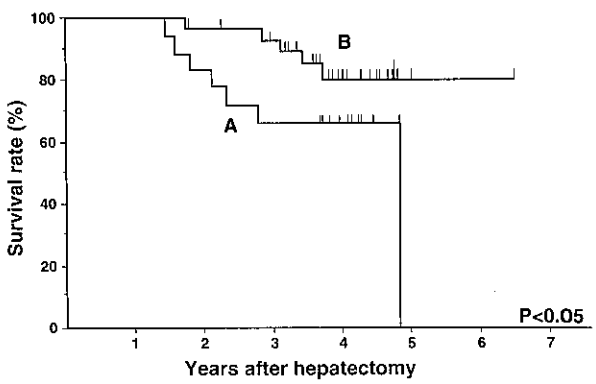

Fig. 2. Survival curves for 20 patients with (A) and 33 patients without alcohol intake (B). There was a significant difference between the two groups ($P<0.05$).

poorer than that for the AI(−) group, with the median disease-free survival time for the former group being 12.6 mo and the value for the latter, 25.4 mo ($P<0.05$).

In order to examine the dose-response relationship between alcohol intake and postoperative recurrence, the 20 patients with AI were divided into 2 groups, i.e., heavy drinkers (ethanol intake ≥ 80 g/day for ≥ 5 yr: 12 patients) and very heavy drinkers (ethanol intake ≥ 130 g/day for ≥ 10 yr: 8 patients). The disease-free survival time for each of these two groups was significantly shorter than that for those without AI ($P<0.05$), with the median disease-free survival time for the heavy drinkers being 9.5 mo and the value for the very heavy drinkers, 12.6 mo. However, there was no significant difference between these two groups in the disease-free survival time.

The pattern of postoperative recurrence is shown in Table III. The first recurrent tumor was located in the residual liver of 40 patients and in the lung in the remaining 2 patients. Five (29.4%) of the 17 patients with recurrence in the AI(+) group had the whole liver type, compared with only 1 (4.0%) of the 25 patients with recurrent tumors in the AI(−) group. There was a significant difference between the two groups in the incidence of the postoperative recurrence pattern ($P<0.05$, 2×4 χ^2 test with Fisher's exact method).

Survival Of the 53 patients, 41 (77.4%) were still alive at the time of analysis. The 1-, 3- and 5-yr survival rates were 100%, 83.6% and 50.9%, respectively.

Seven (35.0%) of the 20 patients with AI and 5 (15.2%) of the 33 patients without AI died. The 1-, 2- and 3-yr survival rates were 100%, 83.3% and 66.7%, respectively, in the AI(+) group and 100%, 96.9% and 93.5%, respectively, in the AI(−) group. There was a significant difference between the groups in the survival time ($P<0.05$, Fig. 2).

The effect of AI on survival was also analyzed in the 48 male patients, because all the patients in the AI(+) group were male. The survival for the AI(+) group was significantly poorer than that for the AI(−) group, with the 3-yr survival rate for the former group being 66.7% and the value for the latter, 94.7% ($P<0.05$).

The survival time for each of the 12 heavy drinkers and the 8 very heavy drinkers tended to be shorter than that for those without AI, with the 3-yr survival rate for the heavy drinkers being 60.0% and the value for the very heavy drinkers, 75.0%. However, there was no significant difference between either of these groups and the group without AI in the survival time.

Proliferative activity Ag-NORs were intensely stained as black nuclear dots of about 1–2 μm in diameter (Fig. 3). Fig. 4 summarizes the mean number of Ag-NORs per cell nucleus in both groups. The mean numbers of Ag-NORs in HCC lesions of the AI(+) and (−) groups were 2.3 $\pm$0.8 and 1.9$\pm$0.4, respectively, and the values in surrounding non-tumorous parenchyma were 1.5$\pm$0.2 and

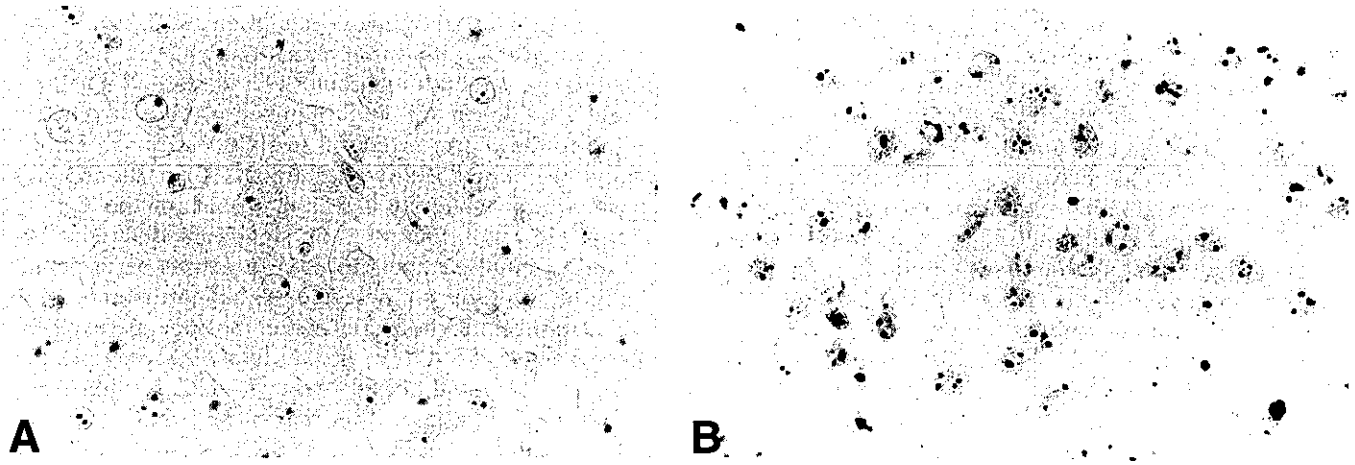

Fig. 3. A: Non-tumorous parenchyma. Only one or two dots are seen in each nucleus (Ag-NOR staining, original magnification ×1000). B: HCC nodule. Most nuclei contain several dots (Ag-NOR staining, original magnification ×1000).

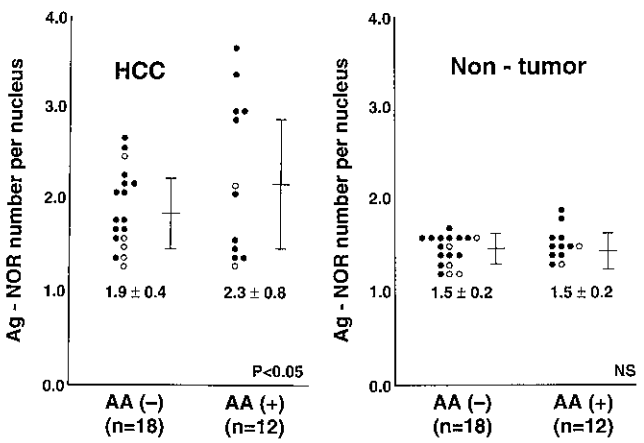

Fig. 4. Scattergram showing the Ag-NOR numbers (means ± standard deviation) of HCC nodules and non-tumorous parenchyma with reference to alcohol intake. Filled (●) and unfilled circles (○) indicate patients with and without recurrence, respectively.

1.5±0.2, respectively. The Ag-NOR number for HCC in the AI(+) group varied widely with a maximum of 3.7, and it was significantly higher than that for the AI(−) group ($P<0.05$). No significant difference was recognized between the Ag-NOR numbers for non-tumorous parenchyma in the two groups.

DISCUSSION

HCV and alcohol are major causes of liver diseases, including HCC. Some studies indicate that HCV and alcohol cooperate in the development of HCC, although the mechanism of such interaction has not yet been demonstrated.[3, 5, 10] The odds ratio for HCC development in alcoholics with HCV-related cirrhosis is significantly higher than that for corresponding non-alcoholics.[5] On the other hand, HCV infection plays an important role in the pathogenesis of HCC in patients with chronic liver disease related to alcohol. Among patients with AI, HCC developed more frequently in HCV-positive patients.[10] Furthermore, HCV RNA levels in the serum of alcoholics with chronic hepatitis C are higher than those of non-alcoholics.[11] This enhancing effect of alcohol intake on HCV replication may modify the development of HCV-related HCC, although the mechanism whereby HCC is induced by HCV and alcohol is yet to be elucidated. Thus, an etiological relationship between HCV infection and AI on hepatocarcinogenesis has been recognized. However, the effect of AI on the prognosis of HCV-related HCC has not been clarified.

In this study, the disease-free survival of patients with AI was significantly shorter than that of patients without AI. With respect to recurrence, there was a significant difference between the two groups in the postoperative recurrence pattern. In particular, the whole liver type, which is generally refractory to treatment, was seen more commonly in the AI(+) than in the AI(−) group. The higher rate and the different type of postoperative recurrence in the patients with AI can be considered the major reasons for their lower survival. To the best of our knowledge, the interaction between AI and the postoperative prognosis of HCV-related HCC has never previously been reported, although alcohol intake worsens the prognosis in patients with chronic hepatitis

C.[12,13] The precise mechanism by which AI affects the recurrence of HCV-related HCC remains to be elucidated. However, there are at least two possible reasons for the higher postoperative recurrence rate in patients with AI. These patients may be more susceptible to new primary HCC after hepatectomy, because alcohol enhances HCV-related hepatocarcinogenesis.[3,5] They may also have a greater incidence of unrecognized metastatic tumors at the time of the first operation, since alcohol may be related to the malignancy of HCV-related HCC, including the rate of metastasis.

With regard to pathological findings, the AI(+) group had a significantly higher ratio of HCC nodules classified as Edmondson III. Moreover, vascular invasion and DNA aneuploidy, which were closely related to intrahepatic metastasis, tended to be more frequent in HCC nodules of the AI(+) group.[14,15] On the other hand, associated liver cirrhosis, which has a higher carcinogenic potential than chronic hepatitis, tended to occur less frequently in the AI(+) group.[4] These findings suggest that the postoperative recurrence in the AI (+) group is caused mainly by metastasis of the primary HCC rather than by multicentric carcinogenesis based on associated chronic liver disease.

Nucleolar organizer regions, which are easily demonstrated by Ag-NOR staining because of the argyrophilia of their associated protein, are loops of DNA that encode rRNA production.[16] The number of Ag-NORs reflects the level of nuclear and cellular activity, and provides an index of the cell proliferative activity and biological malignancy.[17,18] In fact, with respect to HCC, a higher Ag-NOR number in hepatocytes in biopsied liver specimens correlates with an increased risk of HCC development in patients with chronic liver disease.[19] Furthermore, the higher Ag-NOR number in HCC is closely linked to the poorer prognosis in HCC patients.[20] In our study, the Ag-NOR number for HCC in the AI(+) group was significantly higher than that for the AI(−) group. However, there was no significant difference between Ag-NOR numbers for non-tumorous parenchyma from the two groups. These findings support the etiologic speculation concerning the higher postoperative recurrence rates in the AI(+) group, that AI accelerates the malignant potential of HCV-related HCC, resulting in a high rate of metastasis from the original HCC.

In conclusion, HCV-related HCC patients with AI should be closely followed-up to detect recurrent HCC nodules early enough for additional radical treatment, regardless of curative surgery, since AI is closely associated with a poor postoperative prognosis. In addition, education concerning the adverse effects of AI or the discontinuation of AI by patients with HCV-related chronic liver disease is also considered to be important.

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