Histological changes in patients who developed hepatocellular carcinoma after hepatitis C virus eradication by interferon-based therapy

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Abstract. Although the incidence of hepatocellular carcinoma (HCC) occurring after hepatitis C virus (HCV) eradication has decreased, there are still reports of hepatocarcinogenesis. The present study investigated the histological changes of non-cancerous liver tissue obtained prior to interferon (IFN) therapy and after HCC development. A total of 669 HCV-infected Japanese patients who achieved sustained virological response (SVR) by IFN-based therapy were retrospectively enrolled. Of these, the present study investigated 18 patients who developed HCC after IFN-based SVR. Specimens from 9 of 18 patients were available for histological comparisons prior to IFN therapy and following HCC development. Of these 9 patients, the specimens of 5 individuals were compared via immunohistochemical staining [CD3, CD4, CD8, CD20, forkhead box P3 (FOXP3), transforming growth factor-β1 and granzyme B]. The current study included 6 control patients with HCV-associated chronic liver disease who subsequently developed HCC (non-SVR-HCC group). Mann-Whitney and Wilcoxon tests were used to compare groups. Bonferroni correction was used for multiple comparisons. $P<0.05$ was used as a critical P-value, and following Bonferroni's correction, $P<0.017$ was considered to indicate a statistically significant difference. In the 9 patients examined, continuous inflammation and fibrosis were observed after HCC development. There was also a significant decrease in the positive rate of FOXP3 in all 5 patients at the time of HCC development compared with that prior to IFN therapy ($P=0.0084$). Additionally, there was a significant difference in the positive rate of FOXP3 between the 5 patients after HCC development and the control individuals ($P=0.0022$). In patients who developed HCC after IFN-based SVR, the frequency of FOXP3 decreased, but inflammation and fibrosis remained. The extent of the reduction of FOXP3 differed in patients who developed HCC in the presence of HCV. Inflammation and fibrosis remained for a long duration after SVR, which may be associated with hepatocarcinogenesis.

Introduction

Until the introduction of direct-acting antiviral (DAA) therapy, interferon (IFN)-based therapy for patients with chronic hepatitis C was the standard treatment. Although the incidence of hepatocellular carcinoma (HCC) has decreased after achieving a sustained virological response (SVR), some reports indicate that HCC develops in 0.5-8.8% of patients during an observation period of 3 to 5 years (1-9). In liver tissue, inflammation and fibrosis improve after achieving SVR. Shiratori *et al* reported that the activity grade improved in 89% of patients and fibrosis regressed at a rate of 0.282 U/year in SVR patients during an average observation period of 3.7 years (10).

On the other hand, Nirei *et al* (11) reported persistent hepatic inflammation in patients who developed HCC after IFN-based SVR. Motoyama *et al* (12) reported that lack of fibrosis improvement is a risk factor for HCC after SVR. However, there are no reports of immunohistochemistry for inflammatory cells in the portal area of patients who developed HCC after achieving SVR. Therefore, we examined pathological changes before IFN therapy and after HCC development with a focus on hepatic inflammation, fibrosis, and immunology.

Immunologically, eradication of hepatitis C virus can be achieved by vigorous antiviral T cell response. On the other

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Abbreviations: HCC, hepatocellular carcinoma; SVR, sustained virological response; DAA, direct-acting antiviral; FOXP3, forkhead box P3; TGF-β1, transforming growth factor β1; NAFLD, non-nalcoholic fatty liver disease; SASP, senescence-associated secretory phenotype

Key words: hepatocellular carcinoma, chronic hepatitis C, sustained virological response, histological inflammation, forkhead box P3

hand, a weak cellular immune response results in HCV persistence (13). In the immune response, $CD4^+$ T cells support $CD8^+$ T cells and B cells by secreting cytokines (14,15). To clarify changes after SVR in immunity, we investigated the immunological markers CD3, CD4, CD8 and CD20 (16,17).

We also investigated granzyme because it is a marker for CTL. We also investigated forkhead box P3 (FOXP3) because it is a specific marker for regulatory T cells (Tregs), which are immunosuppressive cells. In cancerous tissue, Tregs have a positive effect on tumor proliferation and thus are associated with a poor prognosis (18-20). Sakaki *et al* (21) reported that the frequency of FOXP3 in portal tracts in patients with chronic hepatitis C was significantly higher than that in normal controls. FOXP3 is also strongly correlated with the portal inflammation score (22). Transforming growth factor β1 (TGF-β1) was also examined because TGF-β1 suppresses liver regeneration and promotes tissue fibrosis in the liver (23).

In this study, we retrospectively examined the pathological changes before IFN therapy and after HCC development and used immunohistochemistry of infiltrating lymphocytes in the portal area to assess histological characteristics.

Materials and methods

Patients and controls. A total of 1,106 Japanese patients with type C chronic hepatitis or liver cirrhosis who visited Kurume University Hospital and were treated with IFN-based therapy between January 2003 and December 2016 were enrolled. Before IFN administration, baseline data were evaluated. All patients were positive for HCV antibody (by 2nd generation ELISA; Abbot, Tokyo, Japan). HCV RNA levels were measured using a Roche COBAS Taq Man test. Patients were considered to have achieved SVR if they remained negative for serum HCV RNA 24 weeks after the end of IFN therapy. We excluded patients who had hepatitis B surface antigen, a history of HCC before IFN-based therapy, and developed HCC within one year after the end of therapy. SVR was achieved in 669 patients by IFN-based therapy, and 19 patients who developed HCC were selected. We excluded one patient whose data were lacking. From among patients with HCV-related chronic liver disease who developed HCC and were treated at Kurume University in 2009 and 2010, we randomly selected 6 patients whose non-cancerous liver tissue was obtained as controls. Exclusion criteria for the control samples included previous IFN therapy. Four patients had chronic hepatitis, and 2 patients had cirrhosis. In control patients, laboratory data were obtained at the time when they were admitted to our hospital for initial HCC therapy. They were negative for hepatitis B surface antigen. All patients gave written informed consent according to a protocol approved by the Ethical Committee of Kurume University (approval No. 16244).

Histological examination. We examined 18 patients who developed HCC after IFN-based SVR. Specimens were obtained before IFN therapy by ultrasound-guided biopsy using 14-gauge needles. After developing HCC, non-cancerous liver tissues around the tumor were obtained by hepatic resection or by tumor biopsy before radiofrequency ablation. We compared 9 patients histologically before IFN therapy and after HCC development to evaluate histological changes. We also obtained non-cancerous liver specimens from six control patients before treatment for HCC.

We investigated specimens stained with haematoxylin and eosin (H&E). The degrees of hepatic inflammation and fibrosis were scored according to the classification of Desmet (24), Knodell (25), and Ishak (26) from A0 to A3 and from F0 to F4, respectively. When fat in the liver exceeded 5% histologically, the liver was defined as a liver steatosis. For the immunohistochemical examination, liver tissue was fixed with 10% formalin, embedded in paraffin, cut into 4-μm sections, examined on a coated slide glass, and then used for immunohistochemical analyses. The following primary antibodies were used: Mouse monoclonal anti-CD3 antibody (x300; Leica Biosystems, Nussloch, Germany), mouse monoclonal anti-CD4 antibody (x200; Leica Biosystems, Nussloch, Germany), mouse monoclonal anti-CD8 antibody (x200; Leica Biosystems, Nussloch, Germany), mouse monoclonal anti-CD20 antibody (x1,200; Dako, Glostrup, Denmark), mouse monoclonal anti-FOXP3 antibody (x600; Abcam, Cambridge, MA, USA), mouse polyclonal anti-TGF-β1 antibody (x300; Santa Cruz Biotechnology, Heidelberg, Germany), and mouse monoclonal anti-granzyme B antibody (x50; Leica Biosystems, Nussloch, Germany). Immunohistochemical examinations with CD3, CD4, CD8, CD20, TGF-β1, and granzyme B were performed on the same fully automated Bond-Max system (Leica Biosystems, Newcastle, UK) using onboard heat-induced antigen retrieval with ER2 for 10 min and the Refine polymer detection system. The chromogen 3,3'-diaminobenzidine-tetrachloride (DAB) was used for all immunostaining. FOXP3 immunostaining was performed using the Dako autostainer (Dako, Glostrup, Denmark). Briefly, specimens were boiled in a microwave oven for 30 min in 1 mmol/l ethylenediaminetetraacetic acid pH 9.0 and target retrieval solution for antigen recovery, and the specimens were then incubated with the antibody at 4˚C overnight. After washing with Tris-buffered saline (TBS), slides were incubated with labeled polymer-horseradish peroxidase secondary antibody for 30 min at room temperature. After washing with TBS, the slides were visualized using DAB.

Evaluation of histology (immunohistochemical staining). Immunohistochemical examination of $CD3^+$, $CD4^+$, $CD8^+$, and $CD20^+$ lymphocytes, and FOXP3, TGF-β1, and granzyme B-positive cells were performed in the portal area. Two relatively small-to-medium size portal tracts were investigated with a microscope to count positive cells. To investigate specimens, positive cells were counted in visual fields with clear inflammatory cell infiltration. The number of positive cells was counted twice. Because of the difference in the sizes of the portal tract, the positive rate of immunohistochemical staining was calculated as follows: Positive cell rate = (number of positive cells/number of total mononuclear cells) x 100.

Statistical analyses. Values were expressed as the median (IQR, interquartile range). Statistical analysis was performed using the JMP software package (release 13, SAS Institute, Cary, NC, USA). For comparison of variables, Mann-Whitney and Wilcoxon tests were performed as appropriate. Bonferroni's correction was used for multiple comparisons. $P<0.05$ was used as a critical P-value, and following Bonferroni's correction,

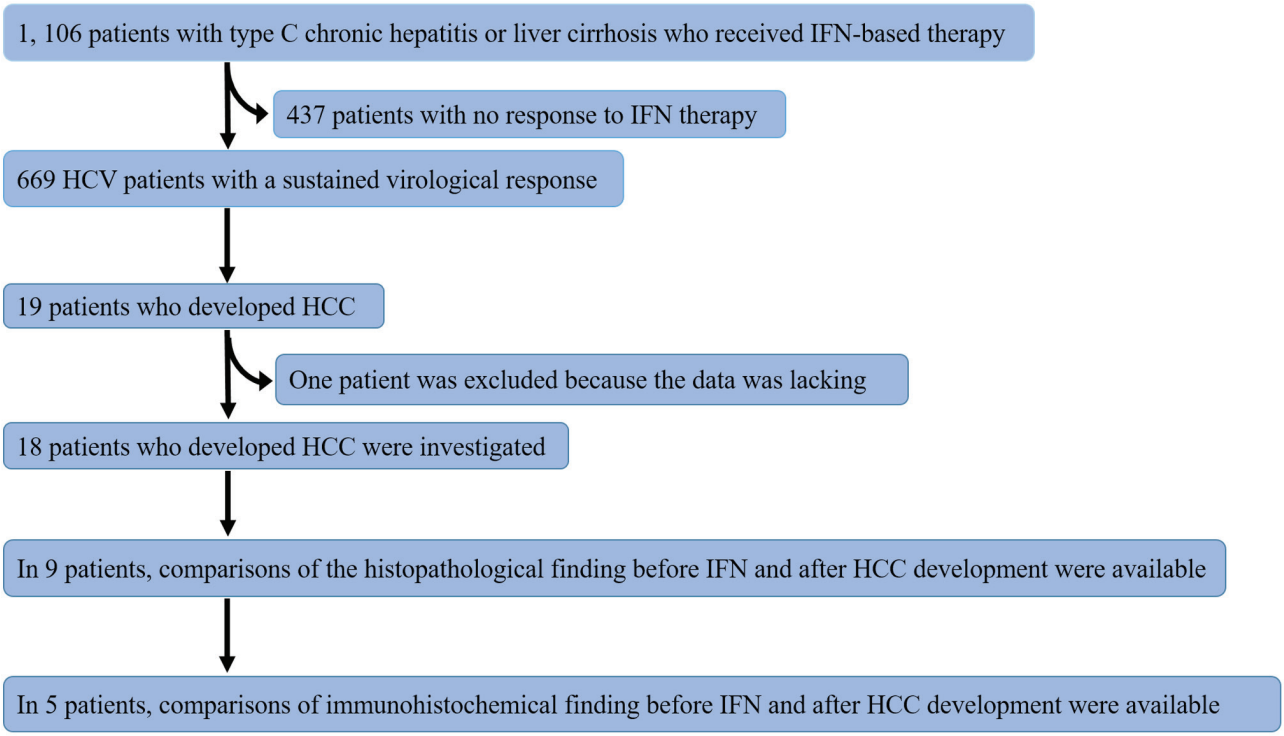

Figure 1. Flow chart of the subjects included in the present study. IFN, interferon; HCV, hepatitis C virus; HCC, hepatocellular carcinoma.

P<0.017 was considered to indicate a statistically significant difference.

Results

Clinical findings. A flow chart of the subjects is shown in Fig. 1. Tables I and II show the characteristics of 18 patients who developed HCC after IFN-based SVR. These tables show characteristics obtained before IFN treatment (Table I) and at the time HCC was diagnosed (Table II). To estimate the degree of liver fibrosis, the Fibrosis-4 index (27) was calculated. Two patients (cases 4 and 17) had a splenectomy before IFN-based therapy. Risk factors, such as age ≥65, male sex, or advanced fibrosis F≥3, were present in all cases except one (case 10). There were 11 of 18 patients (61%) that had a risk factor such as alcohol intake (≥60 mg/day), diabetes mellitus, or liver steatosis. In 9 patients (cases 1-9) whose specimens were available for comparison, 4 patients (cases 6-9) developed HCC 3 years after end of IFN therapy. In case 8, the duration between the end of IFN and HCC development was 112 months. That patient had risk factors for HCC including diabetes mellitus and alcohol intake 60 g/day.

Histological findings. Inflammatory cell infiltration consisting mostly of lymphocytes was observed histologically in the portal area of all 12 specimens after HCC development. In 9 patients, the degree of hepatic inflammation and fibrosis according to the classification of Desmet *et al* (24), Knodell *et al* (25), and Ishak *et al* (26) were comparable. Inflammation improved in 5 patients, worsened in 1 patient, and was unchanged in 3 patients. Fibrosis improved in 4 patients, worsened in 2 patients, and was unchanged in 3 patients. Fig. 2 shows representative specimens from 5 patients.

Immunohistochemical findings. From a total of 9 patients, we compared the specimens in 5 patients by immunohistochemical staining. We did not collect specimens before IFN and after HCC development in the remaining 4 patients because the specimens for immunohistochemical staining were lost or degraded. Fig. 3 shows the average frequency of $CD3^+$, $CD4^+$, $CD8^+$, and $CD20^+$ lymphocytes, and FOXP3, TGF-β1, and granzyme B-positive cells in the portal area from cases 1-5. In many specimens, the majority of infiltrating inflammatory cells were predominantly $CD3^+$ lymphocytes. The median positive rate of FOXP3 was 9.20% (7.23-12.93%) before IFN therapy and 2.28% (1.48-2.59%) after HCC development. Table III shows the ratio of positive cells after HCC development to that before IFN therapy for CD3, CD4, CD8, CD20, FOXP3, TGF-β1, and granzyme B. In all 5 cases, the ratio of FOXP3 was less than 1.0 (0.11-0.36). However, there was no change in $CD3^+$, $CD4^+$, $CD8^+$, and $CD20^+$ lymphocytes, and TGF-β1 and granzyme B-positive cells. Table IV shows the clinical characteristics and positive rate of FOXP3 in control patients. The median positive rate of FOXP3 was 8.58% (6.86-9.70%). Fig. 4 shows the positive rate of FOXP3 in cases 1-5 and control patients. For cases 1-5, there was a significant difference in the positive rate (P=0.0084) before IFN and after HCC development. In addition, there was a significant difference between after HCC development of cases 1-5 and control patients (P=0.0022). We examined liver specimens by immunohistochemical staining of CD4, CD8, FOXP3, and TGF-β1 in case 2 (Fig. 5).

Discussion

Previous studies have revealed improvement in liver tissue when SVR is achieved after IFN-based therapy. In 1991, Schvarcz *et al* (28) evaluated liver tissue before and 9 months

Table I. Characteristics of 18 patients before IFN therapy who developed hepatocellular carcinoma after an IFN-based SVR.

Case	Age (year)	Sex	Geno/Sero type	IFN therapy	BMI	AST (U/l)	ALT (U/l)	PLT ($10^4/\mu$l)	AFP (ng/ml)	FIB4 index	HBc Ab	Liver steatosis	DM	Alcohol) (g/day)	Activity/ fibrosis
1	67	M	1b	IFNα-2b+Rib	22.5	45	64	12.7	12.2	2.97	(+)	(+)	(-)	20	A1F2
2	49	M	1b	PEG-IFN+Rib	27.7	161	133	10.8	10.8	6.33	(-)	(-)	(-)	100	A3F3
3	67	F	1b	PEG-IFN +Rib+Simeprevir	24.2	24	37	14.2	3.2	1.86	(-)	(-)	(-)	(-)	A2F3
4	50	F	1b	PEG-IFN+Rib	19.7	78	79	6.7	19.1	6.55	(-)	(-)	(-)	(-)	A2F3
5	65	M	1b	PEG-IFN+Rib	17.7	94	133	13.1	10.0	4.04	(+-)	(-)	(+)	(-)	A2F2
6	49	F	1b	IFNβ	26.3	47	39	10.5	1.7	3.51	(-)	(-)	(+)	(-)	A1F4
7	69	F	1b	PEG-IFN+Rib	21.9	92	125	10.6	5.1	5.36	(-)	(-)	(-)	(-)	A2F2
8	46	M	2	IFNβ	29.4	44	62	18.3	8.3	1.40	(+)	(-)	(+)	60	A2F2
9	50	M	2a	PEG-IFN	23.1	61	59	19.3	6.6	2.06	(+)	(-)	(+)	60	A2F4
10	51	F	2	IFNα+Rib	20.4	18	18	14.4	4.0	1.50	(+)	(-)	(-)	200	A1F2
11	67	F	2b	PEG-IFN+Rib	22.9	24	24	12.3	2.4	2.67	n.d.	(-)	(-)	(-)	A1F1
12	56	M	1b	PEG-IFN+Rib	19.4	47	53	10.5	4.9	3.44	(+)	(-)	(+)	(-)	A2F3
13	55	M	1b	PEG-IFN+Rib	28.9	133	210	14.5	22.6	3.48	(-)	(+)	(-)	(-)	A2F2
14	72	F	1b	PEG-IFN+Rib	24.1	49	37	13.6	18.6	4.03	(-)	n.d.	(-)	(-)	n.d.
15	66	M	1b	PEG-IFN +Rib+Telaprevir	21.3	55	60	9.2	17.5	5.09	(-)	n.d.	(-)	(-)	n.d.
16	48	M	1b	PEG-IFN +Rib+Telaprevir	30.2	37	35	11.0	4.7	4.06	(-)	n.d.	(-)	(-)	n.d.
17	52	M	2a	IFNα-2b+Rib	33.0	104	90	13.5	6.7	4.22	(+)	n.d.	(+)	(-)	n.d.
18	58	M	2a	PEG-IFN	29.3	33	38	14.6	6.4	2.13	(-)	n.d.	(+)	(-)	n.d.

For cases 4 and 17, a splenectomy was performed before IFN-based therapy. When fat in the liver exceeded 5% histologically, the liver was defined as a liver steatosis. n.d., no data; A0, no necro-inflammatory reaction; A1, mild inflammatory reaction; A2, moderate inflammatory reaction; A3, severe inflammatory reaction; F0, no fibrosis in the portal tract; F1, portal fibrosis without septa; F2, portal fibrosis with a few septa; F3, numerous septa without cirrhosis; F4, cirrhosis; IFN, interferon; PEG-IFN, pegylated interferon; SVR, sustained virological response; Rib, ribavirin; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet; AFP, alpha-fetoprotein; HBcAb, hepatitis B core antibody; FIB4 index, Fibrosis-4 index; DM, diabetes mellitus; M, male; F, female.

Table II. Characteristics of 18 patients after HCC development.

Case	Age (years)	Sex	BMI	AST (U/l)	ALT (U/l)	PLT ($10^4/\mu$l)	AFP 6 months) end of IFN therapy (ng/ml	FIB4 index	Last HCVAb (COI)	Therapy for HCC	Duration between end of IFN and HCC development (months)	Liver steatosis	Activity/ fibrosis
1	69	M	21.8	17	12	14.3	n.d.	2.37	8.2	Resection	19	(-)	A2F1
2	53	M	28.2	69	52	12.1	5.3	4.19	10.0	Resection	29	(-)	A2F4
3	68	F	25.3	22	13	16.7	n.d.	2.48	11.6	Resection	13	(-)	A1F4
4	53	F	20.0	20	13	8.6	9.5	3.42	13.0	Resection	19	(-)	A2F3
5	67	M	21.8	20	19	18.5	2.6	1.71	7.9	RFA	18	(-)	A1F1
6	54	F	29.1	28	39	9.0	4.3	2.69	32.1	Resection	45	(+)	A1F4
7	75	F	22.3	21	12	15.7	2.0	2.90	n.d.	Resection	64	(-)	A1F1
8	56	M	27.7	26	27	21.5	n.d.	1.30	6.9	Resection	112	(-)	A2F2
9	56	M	25.2	28	22	16.8	3.6	1.99	17.1	RFA	65	(-)	A1F2
10	58	F	23.4	20	16	15.3	n.d.	1.90	32.8	RFA	88	n.d.	n.d.
11	73	F	21.9	337	407	11.4	2.0	10.7	n.d.	Chemotherapy	61	n.d.	n.d.
12	61	M	18.9	31	20	8.8	n.d.	4.81	14.6	Radiation, HAIC	51	n.d.	n.d.
13	61	M	29.4	60	56	25.0	4.4	1.96	12.5	TACE	59	n.d.	n.d.
14	77	F	23.2	25	13	12.6	13.8	4.24	n.d.	Resection	44	(-)	A1F1
15	69	M	20.7	23	16	10.6	4.6	3.74	15.6	Resection	26	(-)	A1F3
16	53	M	30.8	21	28	12.7	5.3	1.66	13.9	Resection	24	(+)	A1F4
17	56	M	32.6	39	36	15.1	4.8	2.41	33.6	RFA	24	n.d.	n.d.
18	64	M	26.9	12	13	10.4	6.3	2.05	8.3	RFA	66	n.d.	n.d.

HCVAb, most recent HCV antibody titer; HCC, hepatocellular carcinoma; IFN, interferon; RFA, radiofrequency ablation; HAIC, hepatic arterial infusion chemotherapy; TACE, transcatheter arterial chemoembolization; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet; n.d., no data; AFP, alpha-fetoprotein; A0, no necro-inflammatory response; A1, mild response; A2, moderate response; A3, severe response; F0, no portal tract fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with a few septa; F3, numerous septa without cirrhosis; F4, cirrhosis; M, male; F, female.

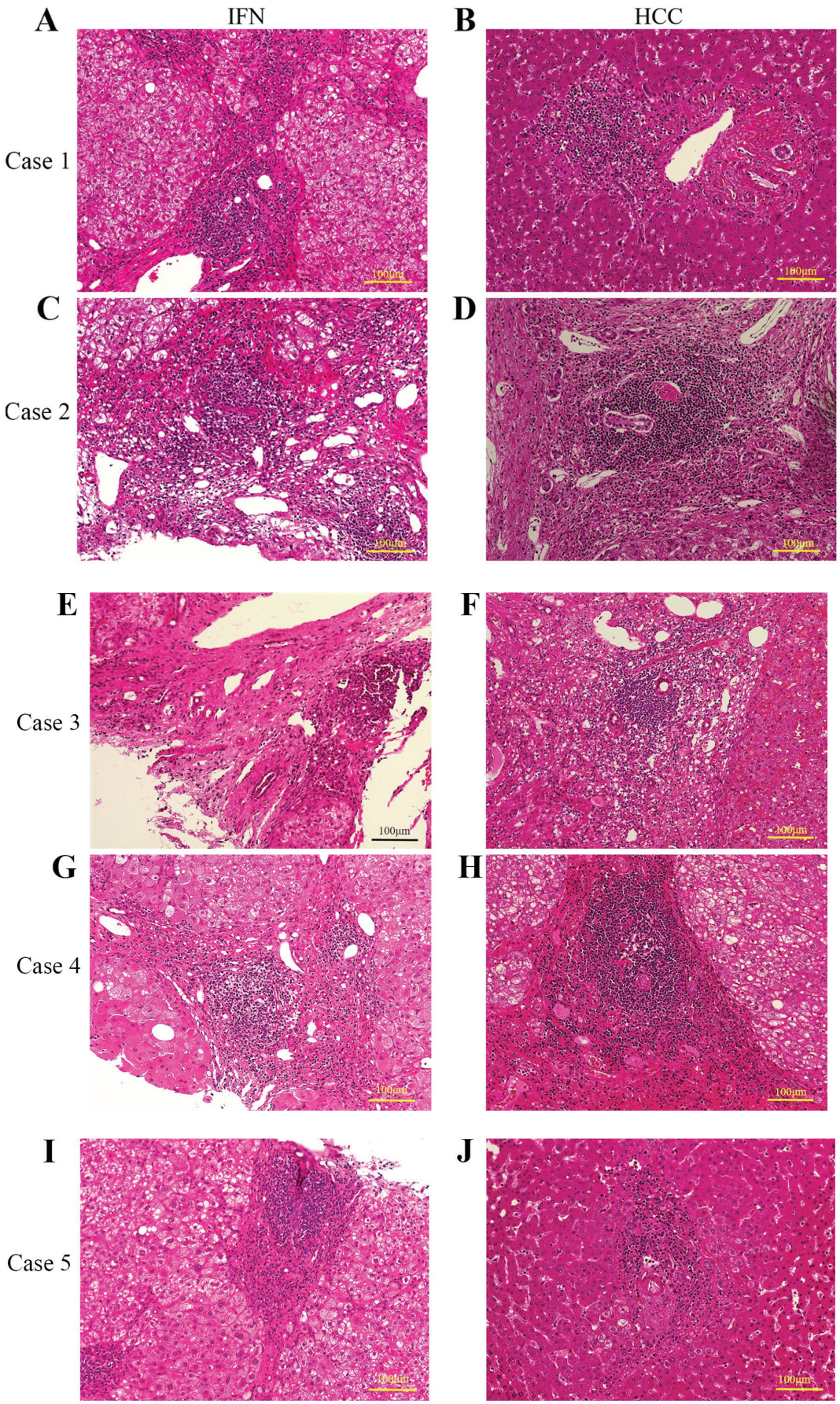

Figure 2. Representative H&E stained specimens of 5 patients that developed HCC after achieving an IFN-based SVR. Specimens are presented from case 1 (A) before IFN therapy and (B) after HCC development; case 2 (C) before IFN therapy and (D) after HCC development; case 3 (E) before IFN therapy and (F) after HCC development; case 4 (G) before IFN therapy and (H) after HCC development; and case 5 (I) before IFN therapy and (J) after HCC development. Inflammation and fibrosis remained in all specimens (cases 1-5). HCC, hepatocellular carcinoma; IFN, interferon. SVR, sustained virological response.

Table III. Ratio of positive staining after HCC development to that of staining before IFN therapy (Positive rate of staining after HCC development/that of staining before IFN therapy).

Case number	CD3	CD4	CD8	CD20	FOXP3	TGF-β1	Granzyme B
Case 1	2.73	3.25	1.21	2.90	0.11	3.09	0.78
Case 2	0.99	0.73	0.74	1.21	0.36	1.85	1.09
Case 3	0.47	0.22	0.85	0.79	0.16	0.96	0.23
Case 4	1.04	10.3	1.74	2.63	0.25	0.60	1.11
Case 5	1.57	1.09	2.43	0.80	0.35	3.01	0.28

FOX P3, forkhead box P3; TGF- β1, transforming growth factor-β1.

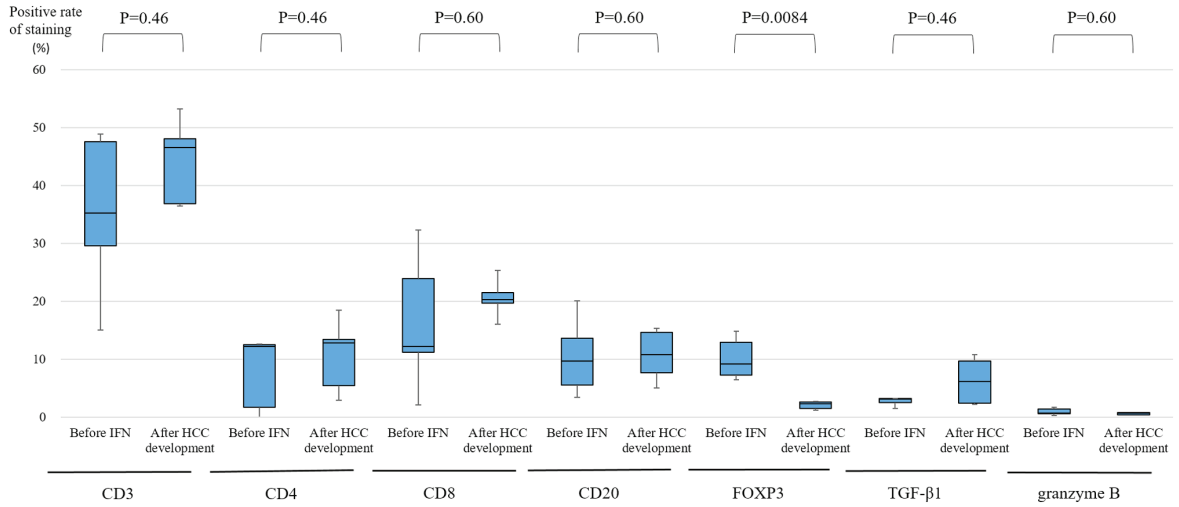

Figure 3. Average frequency of $CD3^+$, $CD4^+$, $CD8^+$ and $CD20^+$ lymphocytes, and FOXP3, TGF-β1, and granzyme B-positive cells in cases 1-5 within the portal area. For the enumeration of positive mononuclear cells, all mononuclear cells were counted in two microscopic fields of the portal tract, twice. For each sample, the mean percentage of positive cells was used. In FOXP3, there was a significant difference before IFN therapy and after HCC development (P=0.0084), as determined by the Wilcoxon test. No significant differences were identified in levels of CD3 (P=0.46), CD4 (P=0.46), CD8 (P=0.60), CD20 (P=0.60), TGF-β1 (P=0.46) and granzyme B (P=0.60) before IFN therapy compared with after HCC development. FOXP3, forkhead box P3; TGF-β1, transforming growth factor-β1; IFN, interferon; HCC, hepatocellular carcinoma; SVR, sustained virological response.

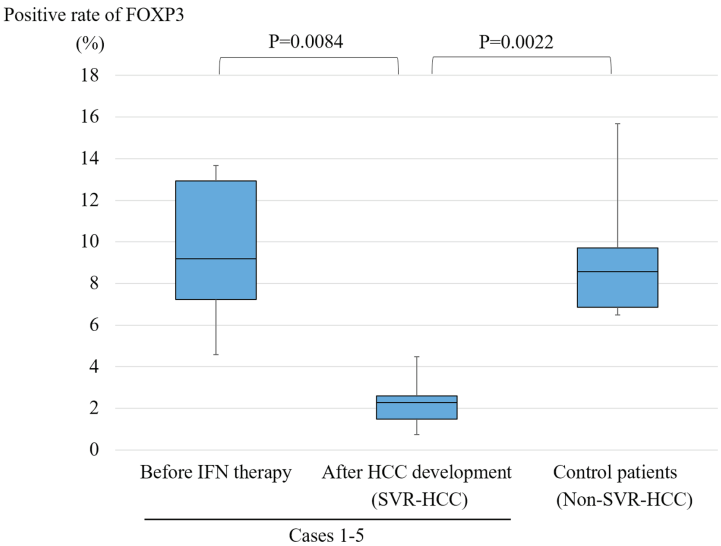

Figure 4. Positive rates of intrahepatic FOXP3 in cases 1-5 and in control individuals. There was a significant difference in positive rates before IFN therapy and after HCC development (SVR-HCC) in cases 1-5 (P=0.0084), as determined via a Wilcoxon test with Bonferroni correction. A significant difference was identified after HCC development in cases 1-5 (SVR-HCC) when compared with control patients (non-SVR-HCC; P=0.0022), as determined via a Mann-Whitney test with Bonferroni correction. The results present the median positive rate of FOXP3 (interquartile range). FOXP3, forkhead box P3; IFN, interferon; HCC, hepatocellular carcinoma; SVR, sustained virological response.

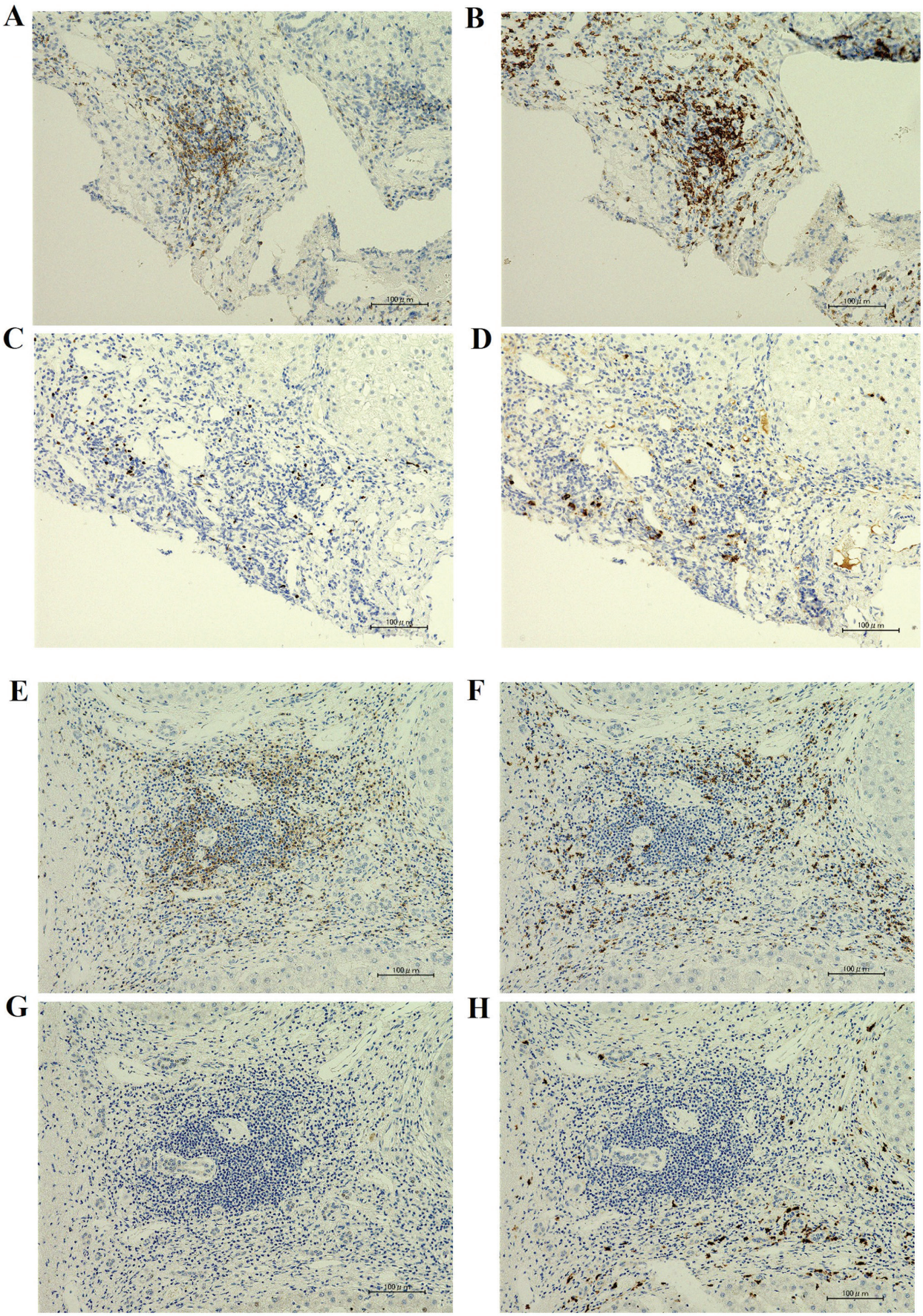

Figure 5. Representative of liver specimens immunohistochemically stained for CD4, CD8, FOXP3 and TGF-β1 (case 2). Sections were immunohistochemically stained before IFN therapy for (A) CD4, (B) CD8, (C) FOXP3, and (D) TGF-β1. Sections were then stained for (E) CD4, (F) CD8, (G) FOXP3 and (H) TGF-β1 after hepatocellular carcinoma development. CD4 and CD8 exhibited distinct membrane staining. FOXP3 exhibited distinct nuclear staining. The distribution of TGF-β1-positive cells were observed in the periportal area. FOXP3, forkhead box P3; TGF-β1, transforming growth factor-β1.

Table IV. Characteristics and the positive rates of FOXP3 in control patients.

Case	Age (years)	Sex	BMI	AST (U/l)	ALT (U/l)	PLT ($10^4/\mu l$)	FIB4 index	Therapy for HCC	Liver steatosis	DM	Alcohol (g/day)	Activity/ Fibrosis	Positive rate of FOXP3 (%)
1	65	F	24.1	78	52	4.7	14.96	RFA	(-)	(-)	(-)	A2F2	7.68
2	51	M	21.6	110	128	10.3	4.81	Resection, RFA	(-)	(-)	14	A2F2	9.47
3	73	M	22.8	70	45	4.9	15.55	Resection, RFA	(-)	(-)	(-)	A2F3	6.59
4	66	F	26.7	47	27	4.4	13.57	RFA	(-)	(-)	(-)	A2F4	6.50
5	75	F	24.1	83	39	7.0	14.24	HAIC	(-)	(-)	(-)	A2F3	9.78
6	53	F	21.7	52	48	3.7	10.75	Laparoscopic RFA	(-)	(-)	(-)	A2F4	15.69

Six patients with HCV-associated chronic liver disease who developed HCC were included in this analysis. For case 4, a splenectomy was performed 4 months before specimens were obtained. FOXP3, forkhead box P3; HCV, hepatitis C virus; M, male; F, female; AST, aspartate aminotransferase; ALT, aminotransferase; PLT, platelet; FIB4 index; fibrosis-4 index; RFA, radiofrequency ablation; HAIC, hepatic arterial infusion chemotherapy; DM, diabetes mellitus; A2, moderate necro-inflammation; F2, portal fibrosis with a few sepat; F3, numerous septa without cirrhosis; F4, cirrhosis.

after IFN monotherapy and observed improvement in fibrosis, necrosis, and inflammation in liver tissue after therapy. Terada *et al* (29) found improvement of staging and grading to A0 (or A1) and F0 (or F1) three years after achievement of SVR. Shiratori *et al* (10) followed patients for a mean of 3.7 years (1-10 years) and showed improvements of grading in 89% and fibrosis regression at a rate of 0.282 U/year. On the other hand, George *et al* (30). followed patients for 5 years on average and observed improvement of liver fibrosis in 82%, improvement of inflammation score in 92%, and improvement to a normal liver state in 20% of all patients. Thus, fibrosis and inflammation improved even with longer follow-up periods. In our study, although improvement of inflammation and fibrosis was observed in some of the 9 patients, inflammation and fibrosis still remained in these patients. Staging and grading improved after SVR in general, but some patients who developed HCC after SVR did not have improved staging and grading. These results differed from the general course of staging and grading after SVR. Nirei *et al* (11) reported that specimens in all 10 patients who developed HCC after SVR had persistent inflammation, which suggests an association with carcinogenesis. Motoyama *et al* (12) reported that hepatic stellate cell activation may inhibit improvement in fibrosis after SVR and potentially contribute to hepatocarcinogenesis. Ikeda *et al* (31) suggested that long-standing chronic liver inflammation and liver regeneration after SVR may trigger tumor development.

Clinical risk factors for carcinogenesis after SVR include advanced age, male sex, advanced fibrosis (3 major factors), alcohol abuse, diabetes mellitus, and liver steatosis (9). All 18 patients who developed HCC after SVR had at least one of these risk factors.

In immunohistochemical staining, Sakaki *et al* (21) evaluated FOXP3 expression in the portal tracts of patients with hepatitis C and normal controls and found significantly higher FOXP3 expression in the former group. Our immunohistological evaluation revealed a significant decrease in the positive rate of FOXP3 in all 5 patients at the time of HCC development, and the positive rate of FOXP3 in these patients was also significantly lower than that in the control group. HCV itself, especially the NS3 region, induces the infiltration of Tregs in liver tissues (32), which may be why the rate of Tregs decreased after achieving SVR. FOXP3 expression in the portal area before IFN therapy and after SVR (without HCC) has not been reported, but a previous report found that FOXP3 is strongly correlated with the portal inflammation score (22). Therefore, if portal inflammation improves after SVR, it is likely that FOXP3 expression has decreased. We found that FOXP3 was not related to HCC development because the expression levels of FOXP3 were higher in HCC patients with HCV-related chronic liver disease. Although FOXP3 expression decreased, there was continuous inflammation in patients who developed HCC after achieving SVR. Therefore, a different inflammatory mechanism may participate in hepatocarcinogenesis after SVR compared with that in the presence of HCV. Immunohistochemical examination of immune markers, such as CD3, 4, 8, 20, TGF-β, granzyme B, and FOXP3, of non-cancerous liver tissue of patients that achieved SVR with and without HCC is needed, but we could not collect non-cancerous liver tissue from patients who achieved SVR without HCC.

There was no change in $CD3^+$, $CD4^+$, $CD8^+$, and $CD20^+$ lymphocytes, and TGF-β1 and granzyme B-positive cells. Sakaki *et al* (21) also evaluated the portal tracts in patients with hepatitis C and normal controls (HCV-negative) and found there was no significant difference between the two groups in the frequency of $CD4^+$ and $CD8^+$. We also found no significant difference in the frequency of $CD4^+$ and $CD8^+$ before IFN therapy and after SVR (with HCC). This study was limited because the number of enrolled patients by immunohistochemical staining was small. This is mainly because the incidence of HCC development after achieving SVR is low in general (1-9). In addition, we did not collect many specimens before IFN therapy. After HCC development, non-cancerous liver tissues around the tumor were obtained in cases of hepatic resection or tumor biopsy before radiofrequency ablation was performed. Further cases are needed to draw more statistically robust conclusions.

Alcohol intake and liver steatosis may be involved in the inflammatory mechanism. Portal inflammation occurs in the livers of patients with alcoholic liver disease (ALD) or non-alcoholic fatty liver disease (NAFLD) (33-35), and portal inflammation is associated with advanced histological changes in fatty liver disease (both alcoholic and non-alcoholic) (36). In 9 patients (case 1-9) whose specimens were compared, 4 patients drank alcohol (≥20 mg/day). In 3 out of 4 patients, moderate inflammation (A2) was observed after HCC development in the portal area. Mild activity (A1) was observed in the portal area in the remaining 1 patient. In addition, we observed liver steatosis before IFN therapy in case 1. However, at the time of HCC development, liver steatosis was absent. Another possible reason why persistent hepatic inflammation remains after SVR may be cellular senescence. Cellular senescence is a state of irreversible cell cycle arrest caused by intrinsic and extrinsic stress (37). Senescent cells secrete senescence-associated secretory phenotype (SASP), which affects neighboring cells and causes chronic inflammation and tumorigenesis (38). SASP has an essential role in the pathological process of liver cirrhosis (39), and these factors may be involved in the inflammation mechanism.

In conclusion, we histopathologically evaluated patients who developed HCC after SVR and found continuous inflammation and fibrosis in the portal area after SVR. Immunohistological analysis indicated a different inflammatory mechanism may participate in hepatocarcinogenesis after SVR compared with that in the presence of HCV. These results suggest that factors other than hepatitis C, such as advanced age, male sex, advanced fibrosis, fatty liver (36), diabetes mellitus, or alcohol intake (33), are involved in the inflammation and fibrosis that remains. Further studies with additional cases are needed.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

TK, TI, RKo and YN conceived and designed the current study; TK, TI, RKo, YN, TAH, RKu, KA, TS, JA, KO, HY and TT acquired and analyzed the data. TT revised the manuscript. All authors read and approved the final manuscript.

Ethics and approval and consent to participate

The present study was reviewed and approved by the Ethics Committee of Kurume University School of Medicine (approval no. 16244). Informed consent was obtained from all the patients enrolled in the study.

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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