Int J Clin Exp Pathol 2018;11(10):4806-4816
www.ijcep.com /ISSN:1936-2625/IJCEP0083313

Original Article

The clinicopathological characterization of small hepatocellular carcinoma with fibrous stroma

Toshihiro Yagi[1,2], Yasuni Nakanuma[3], Yasunori Sato[1], Takeo Nitta[1,4], Motoko Sasaki[1], Toshifumi Gabata[2], Kenichi Harada[1]

Departments of [1]Human Pathology, [2]Radiology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; [3]Department of Diagnostic Pathology, Fukui-ken Saiseikai Hospital, Fukui, Japan; [4]Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Received July 30, 2018; Accepted August 28, 2018; Epub October 1, 2018; Published October 15, 2018

Abstract: Recent studies emphasized the significance of fibrous stroma affecting tumor biology in hepatocellular carcinoma (HCC). To further clarify fibrous stroma's significance, this study investigated the clinicopathological characteristics of HCC with fibrous stroma. A total of 214 nodules of HCC smaller than 3 cm in diameter were analyzed, and 22 (10%) were regarded as HCC with fibrous stroma. Most cases of HCC with fibrous stroma were the simple nodular type without a fibrous capsule, and histologically well- or moderately-differentiated. A subset of the scirrhous variant of HCC was included in this category, and steatohepatitic features, such as Mallory-Denk body formation and lymphoid infiltrates, were also frequently found. Foci with a histological appearance corresponding to dysplastic nodules and/or early HCC were rarely observed in HCC with fibrous stroma, suggesting that some cases occurred via a *de novo* carcinogenic process. The immunohistochemical expression of cytokeratin 7 and the epithelial cell adhesion molecule was more significantly increased in HCC with fibrous stroma than in conventional HCC. Furthermore, the expression of C reactive protein and serum amyloid A, indicative of the activation of the IL-6/STAT pathway, was increased in HCC with fibrous stroma. Radiologically, HCC with fibrous stroma exhibited hyperdense nodules on computed tomography and did not show a nodule-in-nodule appearance. Overall survival and disease-free survival were not significantly different between cases of HCC with fibrous stroma and conventional HCC. This study elucidated the clinicopathological features of HCC with fibrous stroma, which may represent a biologically different process occurring in HCC.

Keywords: Hepatocellular carcinoma, fibrous stroma, scirrhous HCC, steatohepatitic HCC, hepatocarcinogenesis

Introduction

Hepatocellular carcinoma (HCC) is the most common histological subtype of primary liver cancer, and HCC rates are the highest in East Asia (China and Japan) [1]. Geographical variations in the incidence and mortality of HCC have been attributed to different levels of exposure to the following HCC-associated risk factors: chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholic cirrhosis, diabetes, and possibly nonalcoholic fatty liver disease (NAFLD) [2]. Approximately 70% of Japanese HCC cases are derived from HCV-associated liver cirrhosis and chronic hepatitis, with less than 20% of patients being positive for HBV [3].

The concept of multistep hepatocarcinogenesis and related small hepatocellular nodules in patients with chronic liver diseases, particularly those with cirrhosis or chronic hepatitis caused by HBV or HCV, was mainly developed in Japan [4]. These nodules include low-grade dysplastic nodules (DN), high-grade DN, and HCC [5]. In addition, small HCC (less than 2 cm in diameter) are divided into early and advanced HCC. Early HCC has a vaguely nodular appearance and is well differentiated, while advanced HCC has a distinctly nodular pattern and is mostly moderately differentiated.

Most cases of HCC contain no or small amounts of fibrous stroma. However, some cases of HCC without a history of preoperative treatment have various amounts of fibrous stroma between tumor nests. The scirrhous variant of HCC (scirrhous HCC) and fibrolamellar HCC are less common subtypes of HCC that are characterized by abundant fibrous stroma [1].

Table 1. Primary antibodies used for immunostaining

Primary antibody	Clone	Source	Dilution
Alpha-1-fetoprotein	Polyclonal	Dako, Tokyo, Japan	1:500
Cytokeratin 7	OV-TL 12/30	Dako	1:50*
Cytokeratin 19	RCK108	Dako	1:50*
C reactive protein	Polyclonal	Abcam, Tokyo, Japan	1:400†
Epithelial cell adhesion molecule	HEA125	Abcam	1:5*
Glypican 3	1G12	Nichirei, Tokyo, Japan	Prediluted†
Glutamine synthetase	GS-6	Millipore; Merck, Darmstadt, Germany	1:200*
Human amyloid A	mcl	Dako	1:100*
Human hepatocyte	OCH1E5	Dako	1:100*
Ubiquitin	Polyclonal	Dako	1:100

In immunohistochemical staining, antigen retrieval was performed by microwaving in 10 mmol/L citrate buffer pH 6.0 (*), and by heating in Tris-ethylenediaminetetraacetic acid buffer (pH 9.0) with a pressure cooker (†).

Table 2. Summary of patients

	HCC with fibrous stroma	Conventional HCC
N	22	177
Age (years)	66 ± 12	64 ± 10
Sex		
Male	3 (14%)	45 (25%)
Female	19 (86%)	132 (75%)
Viral marker		
HBsAg positive	9 (41%)	51 (29%)
HCV Ab positive	8 (36%)	90 (51%)
Background liver		
Normal/subnormal	0 (0%)	7 (4%)
Chronic hepatitis	7 (32%)	70 (40%)
Liver cirrhosis	15 (68%)	100 (56%)

Abbreviations: HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV Ab, hepatitis C virus antibody.

Furthermore, interstitial fibrosis represents one of the histological hallmarks of HCC with steatohepatitic features (steatohepatitic HCC) [6, 7]. The role of the fibrous stromal component in HCC has been recently investigated, and the findings obtained suggest that tumor biology and behavior are significantly affected by the presence of the fibrous stromal component in the tumor microenvironment [8-10].

The biological nature of a tumor and its microenvironment, including fibrous stroma, may markedly change during the progression of HCC. In this context, small-sized lesions may maintain and reflect their original nature. For example, in small-sized lesions, a subnodule with features diagnostic for HCC may be observed in DN, and these lesions can be recognized as HCC arising in DN. Another example is our previous study showing two cases of minute scirrhous HCC undergoing different carcinogenic processes [11]; one small-sized nodule (1.8 cm in diameter) of scirrhous HCC was speculated to occur via a multistep carcinogenic process because of the presence of dysplastic foci at the periphery of the nodule, while the other nodule (1.2 cm in diameter) was attributed to *de novo* development in the cirrhotic liver.

Thus, the analysis of small-sized HCC appears to be important for understanding the development and progression of HCC. In the present study, the clinicopathological characteristics of small HCC were investigated with a focus on the fibrous stromal component in order to further clarify its significance in tumor biology.

Materials and methods

This study using human materials was performed with the approval of the Ethics Committee of Kanazawa University Graduate School of Medicine (Permit Number: 1985-3).

Case selection

From among 199 patients who underwent hepatectomy at Kanazawa University Hospital and its affiliated hospitals between 2003 and 2013, 214 nodules smaller than 3 cm in diameter were analyzed. Postoperative pathological

Table 3. Histopathological characteristics of HCC with fibrous stroma

	HCC with fibrous stroma	Conventional HCC	*P* value
*N**	22	192	
Size (mm)	19 ± 6	22 ± 6	NS
Gross appearance			NS
Simple nodular	18 (82%)	118 (61%)	
Simple nodular with extranodular growth	4 (18%)	62 (32%)	
Confluent multinodular	0 (0%)	12 (6%)	
Fibrous capsule			< 0.01
Present	4 (18%)	181 (94%)	
Absent	18 (82%)	11 (6%)	
Differentiation			NS
Well differentiated	9 (41%)	66 (34%)	
Moderately differentiated	13 (59%)	123 (64%)	
Poorly differentiated	0 (0%)	3 (2%)	
Portal vein invasion			NS
Present	5 (23%)	39 (20%)	
Absent	17 (77%)	153 (80%)	
Hepatic artery invasion			NS
Present	0 (0%)	1 (1%)	
Absent	22 (100%)	191 (99%)	
Dysplastic and/or early HCC foci**			< 0.05
Present (extensive)	0 (0%)	8 (4%)	
Present (focal)	1 (5%)	25 (13%)	
Absent	21 (95%)	159 (83%)	

*A total of 214 nodular lesions obtained from 199 patients were analyzed. **The peripheral portion of the nodular lesion was evaluated for the presence or absence of dysplastic and/or early HCC foci, as described in the Materials and Methods section. Abbreviations: HCC, hepatocellular carcinoma; NS, not significant.

examinations confirmed that they were HCC. Cases in which treatments such as transcatheter arterial embolization and radiofrequency ablation had been performed prior to surgery were excluded.

Formalin-fixed, paraffin-embedded liver sections were used. One to four sections representing the entire area of a nodular lesion were prepared for each case. Sections were stained with picrosirius red to evaluate the volume of fibrous stroma. In the present study, cases in which fibrous stroma occupied > 10% of the tumor area were regarded as HCC with fibrous stroma, whereas cases of HCC that lacked fibrous stroma or had fibrous stroma accounting for less than 10% of the tumor area were regarded as conventional HCC.

Immunohistochemistry

After deparaffinization, antigen retrieval was performed. To block the activity of endogenous peroxidase, sections were immersed in 0.3% hydrogen peroxidase in methanol at room temperature for 10 minutes. After a pretreatment with a blocking serum (Dako, Tokyo, Japan), the sections were incubated at room temperature for 1 hour with the individual primary antibodies listed in **Table 1**. Sections were then incubated with a secondary antibody using Histofine Simple Stain MAX PO (Nichirei, Tokyo, Japan). Color development was performed using 3, 3'-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin. Negative controls were obtained by substituting the primary antibodies with non-immunized serum, resulting in no signal detection.

Histological assessment

The presence or absence of the foci that had histological features corresponding to DN or early HCC was surveyed on the sections stained with hematoxylin and eosin. Silver staining was also used for the analysis. DN and early

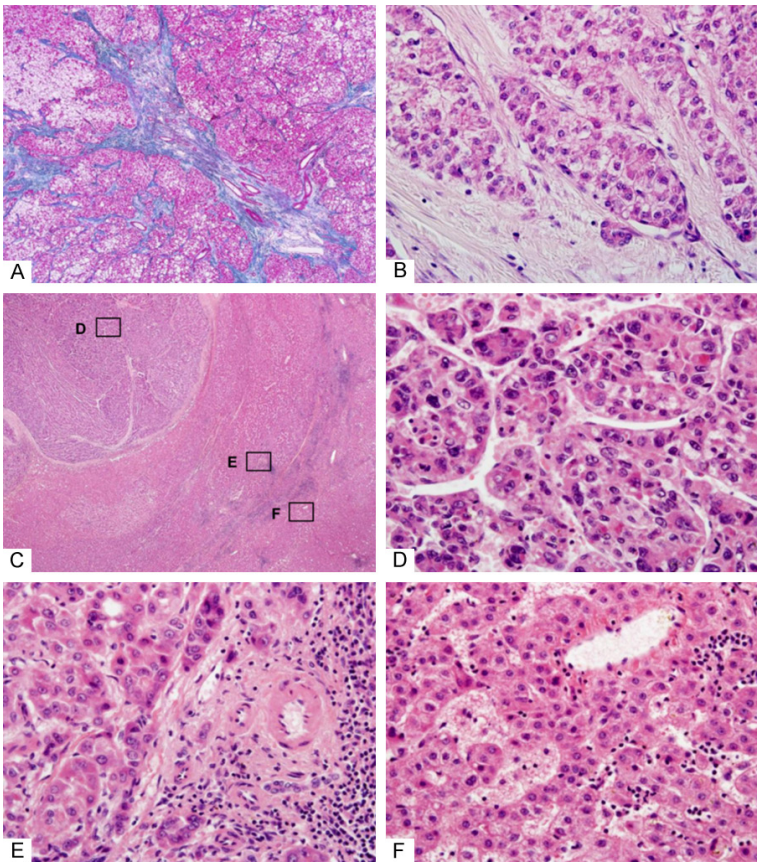

Figure 1. Representative histology of hepatocellular carcinoma (HCC) with fibrous stroma and conventional HCC. (A, B) HCC with fibrous stroma. Stellate fibrosis was observed in the central part of the lesion. (C-F) Conventional HCC in which moderately differentiated HCC (D) was surrounded by the foci of well-differentiated HCC with stromal invasion to the portal tract (E) and hypercellular dysplastic foci (F). The squared areas of (C) are magnified in (D-F). (A) Original magnification: (A) (Azan-Mallory staining), × 40; (B, D-F), × 400; (C), × 20.

HCC both have the following histological features: increased cell density, fatty changes, and pseudoglandular structures. Stromal invasion to the portal tracts further qualified the lesion as early HCC, although a histological distinction between the two types of lesion may sometimes be impossible [1]. The histological features of DN and early HCC were carefully reviewed by two independent expert pathologists (Y.N. and M.S.). The peripheral portions of the nodular lesions were evaluated. When the foci of DN or early HCC was present in less than half of the area surrounding the entire lesion, the area of the foci of DN or early HCC was regarded as 'focal'. If the foci of DN or early HCC was present in half or more of the area surrounding the entire lesion, the area was regarded as 'extensive'.

The frequencies of the cytological changes of the HCC, such as clear cell change, Mallory-Denk body formation, and lymphoid cell infiltration in tumor stroma, were assessed by a semiquantitative analysis based on the following grades: 0, negligible; 1+, occasional; 2+, many. The fatty changes of the HCC were assessed based on the following grades: 0, no fatty change; 1+, 1% to 10% of cells in the lesion showed fatty change; 2+, more that 10% of cells in the lesion showed fatty change.

A semiquantitative analysis of the immunohistochemical results was performed as follows: expression was evaluated according to the percentage of positively stained cells: 0, negative; 1+, 1% to 25% of cells in the lesion stained positive; 2+, 26% to 75% of cells in the lesion stained positive; and 3+, more than 75% of cells in the lesion stained positive.

Statistical analysis

The significance of differences was assessed using the χ^2 test and Mann-Whitney *U*-test. In the univariate analysis, the probability of postoperative survival was calculated by the Kaplan-Meier method using IBM SPSS Statistics software (version 19; IBM Japan, Ltd., Tokyo, Japan). A *P* value less than 0.05 was considered to be significant.

Results

Frequency of HCC with fibrous stroma in small-sized nodules

In the present study, 214 small-sized nodules, each less than 3 cm in diameter, were analyzed, with cases in which fibrous stroma occupied > 10% of the tumor area being regarded as HCC with fibrous stroma. Twenty-two out of 214 nodules (10%) corresponded to HCC with fibrous stroma. No significant differences were observed in age or sex distribution between the

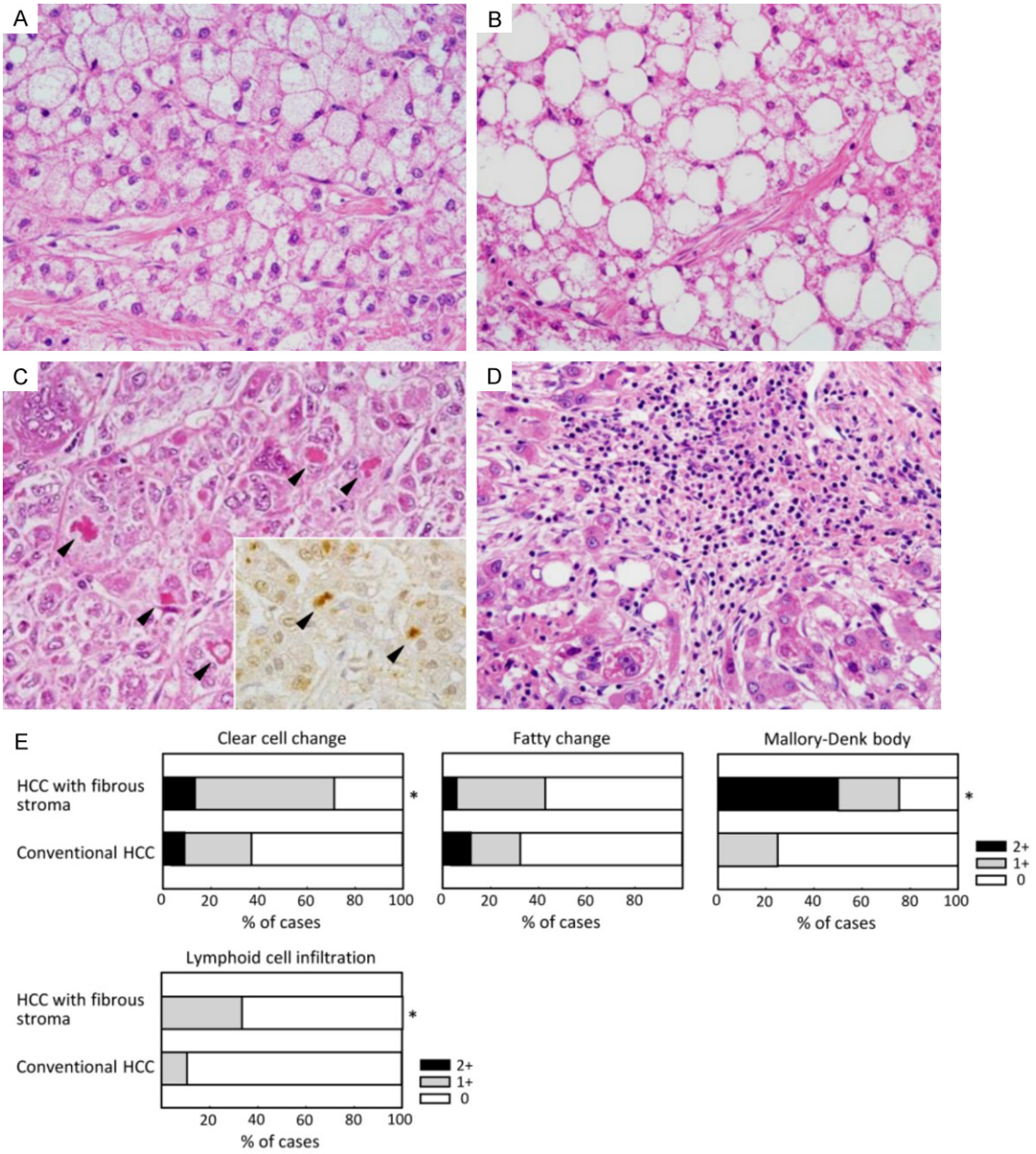

Figure 2. Histological characteristics of hepatocellular carcinoma (HCC) with fibrous stroma. HCC with fibrous stroma showing the histological features of (A) clear cell change, (B) fatty change, (C) Mallory-Denk body formation (arrowheads; Inset is the result of immunostaining for ubiquitin), and (D) lymphoid cell infiltration. (E) The results of a semiquantitative analysis, showing that the frequencies of the occurrence of clear cell change, Mallory-Denk body formation, and lymphoid cell infiltration were significantly higher in cases of HCC with fibrous stroma than in those of conventional HCC. *, $P < 0.05$ (vs. conventional HCC). Original magnification; (A-D), × 400.

cases of HCC with fibrous stroma and those of conventional HCC (**Table 2**). Approximately 80% of the cases in the two groups were associated with hepatitis virus infection showing serological positivity for the hepatitis B surface antigen or the HCV antibody (**Table 2**).

Pathological characteristics of HCC with fibrous stroma

The pathological characteristics of HCC with fibrous stroma are summarized in **Table 3**. The gross appearance of HCC with fibrous stroma

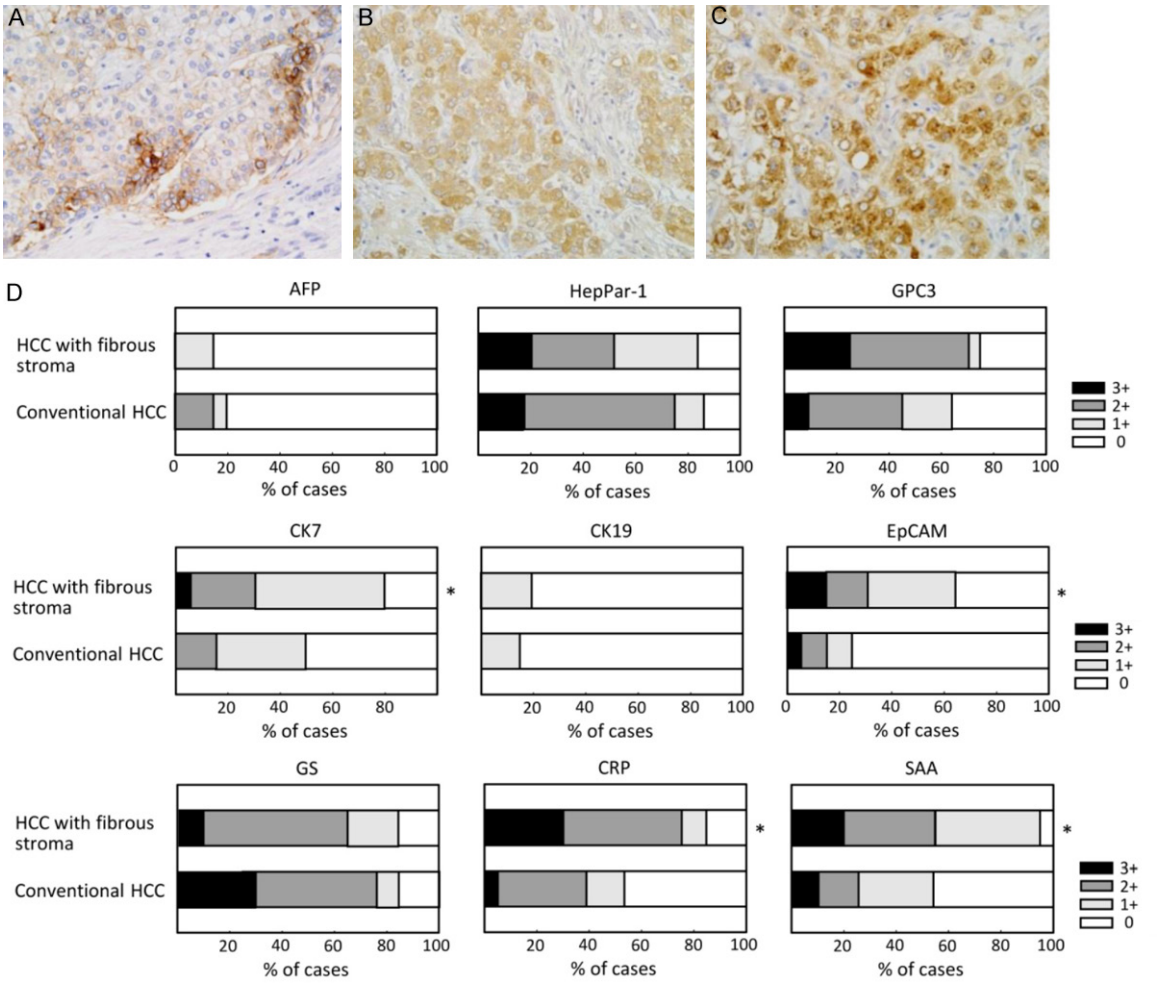

Figure 3. Immunohistochemical analysis of hepatocellular carcinoma (HCC) with fibrous stroma. The results of immunostaining for (A) epithelial cell adhesion molecule (EpCAM), (B) C reactive protein (CRP), and (C) serum amyloid A (SAA) in cases of HCC with fibrous stroma. (D) Semiquantitative analysis of immunohistochemical results, showing that the expression of cytokeratin (CK)7, EpCAM, CRP, and SAA was significantly stronger in the cases of HCC with fibrous stroma than in those of conventional HCC. The expression status of alpha-fetoprotein (AFP), Hepatocyte Paraffin 1 (HepPar-1), glypican 3 (GPC3), CK19, and glutamine synthetase (GS) was not significantly different between the groups. *, $P < 0.05$ (vs. conventional HCC). Original magnification; (A-C), × 400.

was mostly the simple nodular type, followed by the simple nodular type with extranodular growth. The confluent nodular type was not observed. More than 80% of cases were not associated with a fibrous capsule, whereas a partial or complete fibrous capsule was observed in most cases of conventional HCC, with a significant difference being observed between them.

Stellate fibrosis was occasionally observed in the central part of HCC with fibrous stroma, exhibiting the appearance of a central scar (**Figure 1A**). Other patterns of fibrous stroma included multiple fibrotic foci surrounding tumor nests in the nodule. Cases of fibrolamellar HCC with dense lamellated collagenous bands and a sparse cellular component were not observed.

HCC with fibrous stroma typically had cytologic atypia abundantly diagnostic for carcinoma (**Figure 1B**). They corresponded to well- or moderately-differentiated HCC, and poorly differentiated HCC was not included. The frequency of portal vein invasion and hepatic artery invasion

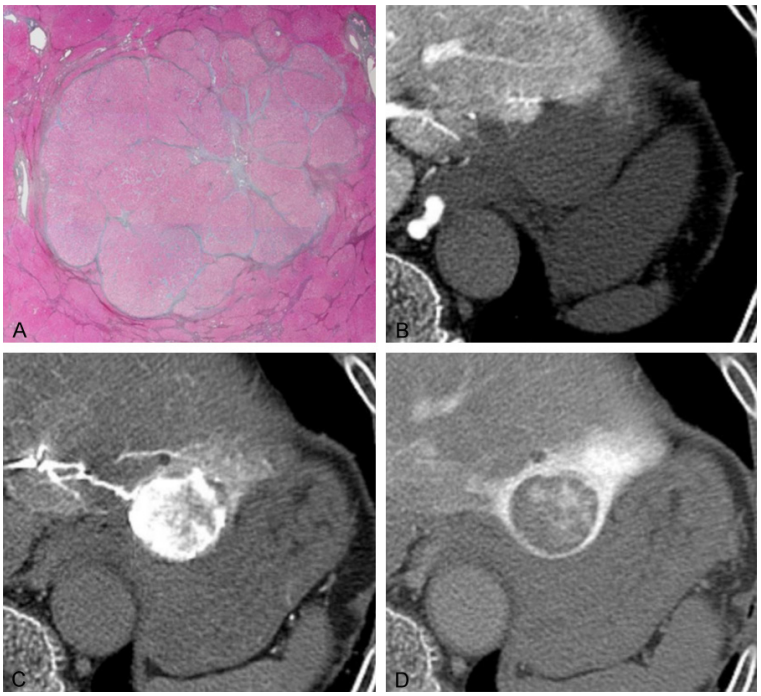

Figure 4. Radiological findings of hepatocellular carcinoma (HCC) with fibrous stroma. (A) Whole mount histological view of HCC with a central scar (Azan-Mallory staining). Radiological images of (B) computed tomography during the arterial phase (CTAP), and (C) CT during hepatic arteriography (CTHA) in the early phase, showing a hyperdense nodule, for the same case shown in (A). (D) The central scar was recognized as faintly hyperdense foci within the nodule on CTHA in the late phase.

was not significantly different between HCC with fibrous stroma and conventional HCC.

In 33 out of 192 nodules (17%) of conventional HCC, foci that had a histological appearance corresponding to DN and/or early HCC were observed at the peripheral portion of the nodule (**Table 3**; **Figure 1C-F**). In contrast, dysplastic and/or early HCC foci were rare in HCC with fibrous stroma, and were only observed in a single case (1/22 cases, 5%).

The frequent histological findings of HCC with fibrous stroma included clear cell change, fatty change, Mallory-Denk body formation, and lymphoid cell infiltration (**Figure 2A-D**). The presence of Mallory-Denk bodies was further confirmed by immunostaining for ubiquitin (**Figure 2C**, inset). A semiquantitative analysis revealed that clear cell change, Mallory-Denk body formation, and lymphoid cell infiltration occurred more frequently in HCC with fibrous stroma than in conventional HCC (**Figure 2E**).

Immunohistochemical analysis of HCC with fibrous stroma

Immunostaining for alpha-fetoprotein (AFP), Hepatocyte Paraffin 1 (HepPar-1), glypican 3 (GPC3), cytokeratin (CK)7, CK19, the epithelial cell adhesion molecule (EpCAM), glutamine synthetase (GS), C reactive protein (CRP), and serum amyloid A (SAA) was performed for the cases of HCC with fibrous stroma and conventional HCC. Among these molecules, a semiquantitative analysis revealed that the expression of CK7, EpCAM, CRP, and SAA was significantly more increased in HCC with fibrous stroma than in conventional HCC (**Figure 3D**). In cases of HCC with fibrous stroma, carcinoma cells positive for CK7, CRP, and SAA were more likely to be irregularly distributed within the nodules, but there were cases in which carcinoma cells in the periphery of the tumor nests facing the fibrous stroma showed the expression of EpCAM (**Figure 3A-C**).

When the results of immunostaining were compared with the presence of clear cell change, fatty change, Mallory-Denk bodies, and lymphoid cell infiltration in HCC with fibrous stroma, the expression of CRP positively correlated with the presence of Mallory-Denk bodies.

Radiological findings of HCC with fibrous stroma

Radiological images from 15 cases of HCC with fibrous stroma, in which computed tomography during hepatic arteriography (CTHA) was performed prior to the surgical resection, were examined. All cases exhibited a hyperdense nodule on CTHA in the early phase (**Figure 4**). In three out of 15 cases, the fibrous stroma was recognized as faintly hyperdense foci within the nodule on CTHA in the late phase (**Figure 4D**). There were no cases of HCC with fibrous

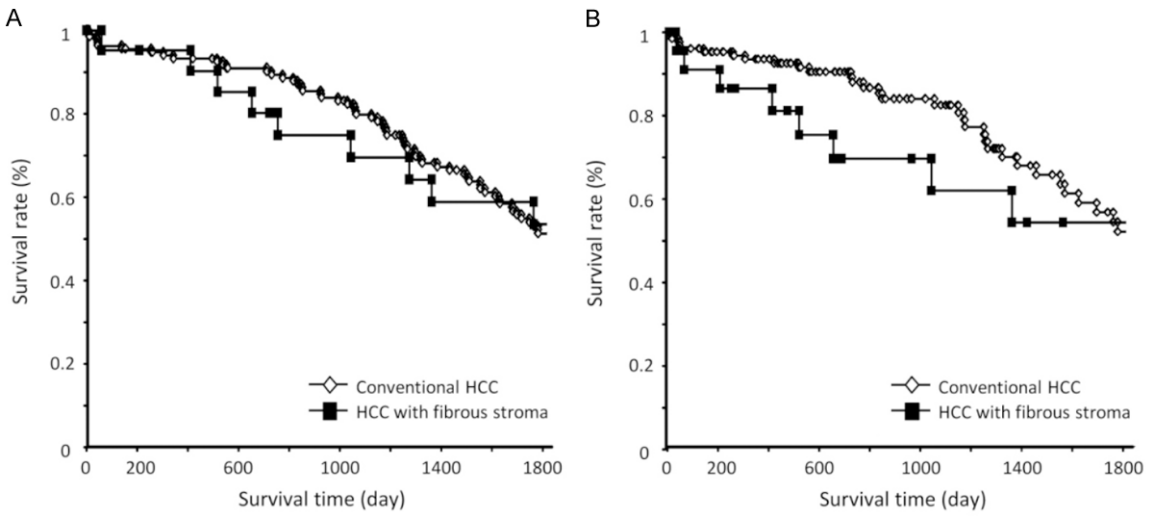

Figure 5. Survival curve of hepatocellular carcinoma (HCC) in relation to histological subtypes. (A) Overall survival and (B) disease-free survival were analyzed in 21 patients with HCC with fibrous stroma and 142 patients with conventional HCC. Overall and disease-free survival were not significantly different between the groups.

stroma that radiologically showed a nodule-in-nodule appearance.

Relationship between histological subtypes and patient survival

Postoperative follow-up data were available for 21 cases of HCC with fibrous stroma and 142 cases of conventional HCC. No significant differences were observed in overall survival (**Figure 5A**) or disease-free survival (**Figure 5B**) between the cases of HCC with fibrous stroma and conventional HCC.

Discussion

In the present study, the analysis of small HCC revealed that 10% of the cases were accompanied by abundant fibrous stroma. Cases of HCC with fibrous stroma were occasionally associated with histological findings such as fatty change, Mallory-Denk bodies, and/or inflammatory cell infiltration, sharing these features with steatohepatitic HCC. Clear cell change was also a frequent histological finding of HCC with fibrous stroma. A subset of scirrhous HCC also appeared to be included in the category of HCC with fibrous stroma. Furthermore, the presence of foci corresponding to DN and/or early HCC, indicative of the occurrence of a multistep carcinogenic process, was rarely observed in the peripheral portion of HCC with fibrous stroma.

In previous studies, the diagnosis of steatohepatitic HCC was made if the tumor fulfilled four of the following criteria: steatosis (> 5% tumor cells), ballooning or Mallory-Denk body formation, interstitial fibrosis, and inflammatory infiltrates [12, 13]. Steatohepatitic HCC arises in the background liver of NAFLD, and also in patients with hepatitis virus infection, particularly HCV infection, which was consistent with the results of the present study [6, 12, 14]. A previous study demonstrated that steatohepatitic features in HCC did not affect overall or disease-free survival [12].

Immunohistochemically, conventional HCC is reported to express GS, CRP, and SAA to various degrees [15]. Among these proteins, the present study showed that the expression of CRP and SAA was stronger in HCC with fibrous stroma than in conventional HCC. CRP and SAA are both known to be induced by IL-6/STAT activation [16, 17]. Recent studies showed that the IL-6/STAT signaling pathway is frequently activated in steatohepatitic HCC [18, 19]. These findings indicate that the overexpression of CRP and SAA in HCC with fibrous stroma is associated with the activation of the IL-6/STAT signaling pathway, which, in turn, causes steatohepatitic features. A positive correlation between CPR expression and Mallory-Denk body formation may also support this consideration.

Scirrhous HCC is a histological variant of HCC that shows a scirrhous growth pattern characterized by marked fibrosis along sinusoid-like blood spaces, and accounts for approximately 5% of HCC cases [1]. A better prognosis has been reported in some, but not all studies [20]. Regarding the diagnosis of scirrhous HCC, some studies have used the criteria of HCC with fibrous stroma comprising at least 50% of one low-power field, and other definitions, such as > 25% fibrous stroma and/or a combination of gross and microscopic features, have also been employed [21-24]. Some cases of HCC with fibrous stroma in the present study complied with these criteria of scirrhous HCC. However, the distinct classification of scirrhous HCC and steatohepatitic HCC was sometimes difficult. For example, cases such as scirrhous HCC that focally had the histological features of steatohepatitic HCC were identified.

The immunohistochemical characteristics of scirrhous HCC in previous studies included the stronger expression of CK7, CK19 and EpCAM and weaker expression of HepPar-1 [22, 24, 25]. Consistent with these findings, the expression of CK7 and EpCAM was stronger in cases of HCC with fibrous stroma in the present study. However, the stronger expression of CK19 and weaker expression of HepPar-1 were not observed. The expression of EpCAM observed in cases of HCC with fibrous stroma at the periphery of tumor nests facing fibrous stroma resembled the histological picture of combined hepatocellular-cholangiocarcinoma with stem-cell features, a typical subtype in the current WHO classification [26, 27]. Thus, HCC with fibrous stroma may comprise a number of histological subgroups.

A recent study examined early HCC in which cases were subclassified into low-grade and high-grade early HCC according to their histological appearance [28]. In that study, cases of HCC with stromal fibrosis with the scirrhous HCC-like pattern (scirrhous component) were classified into high-grade early HCC. Cases of early HCC with the scirrhous component had an increased malignant potential, and this may be a transitional stage to advanced HCC. Although there appear to cases of HCC with fibrous stroma that undergo a multistep carcinogenic process, we consider a *de novo* carcinogenic process to also exist because foci corresponding to DN and/or early HCC were rarely observed at the periphery of HCC with fibrous stoma in the present study. Radiologically, HCC with fibrous stroma did not show a nodule-in-nodule appearance, and *de novo* cases are predicted to show this appearance less frequently. Furthermore, the stronger expression of the stemness marker EpCAM in HCC with fibrous stroma may also be related to a *de novo* carcinogenic process.

In conclusion, cases of HCC with fibrous stroma are composed of histologically heterogenous subgroups, and a subset of steatohepatitic HCC and scirrhous HCC appear to be included in this category. They are characterized by the stronger immunohistochemical expression of CK7, EpCAM, CRP, and SAA. HCC with fibrous stroma appears to have developed not only through a multistep carcinogenic process, but also via a *de novo* process. These morphological varieties of small HCC may reflect biologically different processes occurring in the development and progression of HCC.

Acknowledgements

The authors would like to thank to Dr. Hiroshi Demachi (Department of Radiology, Toyama Prefectural Central Hospital, Toyama, Japan), Dr. Shin Ishizawa (Department of Pathology, Toyama Prefectural Central Hospital, Toyama, Japan), and Dr. Atsuhiro Kawashima (Department of Pathology, National Hospital Organization, Kanazawa Medical Center, Kanazawa, Japan) for providing experimental materials.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Kenichi Harada, Department of Human Pathology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan. Tel: +81-76-265-2195; Fax: +81-76-234-4229; E-mail: kenichih@med.kanazawa-u.ac.jp

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