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The formation of capsule and septum in human hepatocellular carcinoma

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Abstract The formation of fibrous capsule around the cancer nodule and of the septum in the tumor is frequently observed with the development of hepatocellular carcinoma (HCC). We aimed to clarify how the capsule and septum were formed during the growth of HCC. Liver samples surgically resected from 25 patients with HCC were studied with in situ hybridization for type-I, -III, and -IV procollagen. Type-I and -III procollagen-expressing cells, mostly α-smooth muscle actin (SMA)-positive, were increased in the fibrous capsule and in the septum between HCC nodules. These cells were also found at the invasion front of HCC and around the necrotic cancer tissues. Type-IV procollagen gene expression was mainly observed in mesenchymal cells localized in both HCCs and non-cancerous liver. Cancer cells or hepatocytes did not express any of these procollagen genes. The present study reveals that the capsule and septum are mainly formed by α-SMA-positive mesenchymal cells at the interface between two different tissues (e.g., cancer nodule vs non-cancerous liver or another cancer nodule). The wound healing occurs even in HCC. The capsule formation may result from interaction between tumor and host liver and interfere the growth and invasion of HCC.

Keywords α-Smooth muscle actin · Collagens · Hepatocellular carcinoma · In situ hybridization · Stellate cell

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Introduction

Hepatocellular carcinoma (HCC) is one of the worst prognostic cancers, and the mortality from this disease in Japan is currently over 27,000 per year. Formation of fibrous capsule around the tumor has been frequently observed during the growth of HCC. The prognosis of patients with encapsulated HCCs has been significantly better than those with unencapsulated ones [8, 11, 14, 16]. A new HCC nodule with different histological features develops in many HCC nodules, particularly in the advanced stages of HCC. This is called “nodule in nodule”, and septum with extracellular matrix (ECM) commonly develops between cancer nodules.

The capsule around HCC has been shown to be composed of type-I and -III collagen according to an immunohistochemical study [22]. The major components of collagens in liver are type-I, -III, and -IV [19]. Type-I and -III collagens are abundant in fibrous stroma, whereas type-IV collagen is the major collagen type of basement membrane.

The stellate cell (also called Ito cell, fat-storing cell, or lipocyte) is regarded as the principle matrix-producing cell of liver and, thereby, plays a pivotal role in the development of liver fibrosis. Upon activation, the stellate cell becomes a myofibroblast-like cell and produces various ECM proteins [5]. Several studies in human liver have indicated that the stellate cell, when activated, expresses the α-isotype of smooth muscle actin (α-SMA) that is specific to the differentiation of smooth muscle cells [18]. It has been shown that some stromal cells in HCC are positive for α-SMA, and these cells might secrete some components of ECMs in the sinusoid-like blood vessels [3]. Myofibroblast-like cells have been reported as being a cellular source of capsular collagen [1, 17] and stroma [4] in HCCs.

Questions of how the capsule and septum are formed, what cells are responsible for the formation, and what the rationale of making these for host liver or HCC are

yet to be proven. The purpose of this study was to answer these questions and to test the involvement of activated stellate cells in the production of collagens in and around human HCCs.

Subjects and methods

Patients and tissue preparation

We studied 25 cases (21 males and 4 females, aged 45–78 years) who received surgical resection of HCC. The clinical background of the patients is shown in Table 1. This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki, and informed consent was obtained from each patient. A part of the liver tissue was routinely fixed in 10% neutral formalin immediately after resection and was embedded in paraffin. Sections (4 μm) were made and put onto silanized slides (Dako Japan, Tokyo, Japan) and were stained with hematoxylin and eosin for histological diagnosis. The histological types of the HCCs were diagnosed according to the general rules for the clinical and pathological study of primary liver cancer in Japan [10]. The serial sections were used for both in situ hybridization and immunohistochemistry. From the other part of the tissue, cancer, including capsule, was separated from non-cancerous liver, and these tissues were immediately frozen and stored at –80°C until extraction of RNA was performed.

Probes

Plasmid containing a 900-bp fragment of rat procollagen α1(I) was subcloned into pGEM3z (Promega, Madison, Wis.)[12]. The complementary (c)DNA for either the 811-bp fragment for human procollagen α1(III) cDNA (nucleotides 2891–4302) or the 1412-bp fragment for human procollagen α1(IV) cDNA (nucleotides 2662-3472) was subcloned into pBluescript SK(–) (Stratagene, La Jolla, Calif.). They were generous gifts of Drs. Yoshioka (Okayama, Japan) and Ramirez (New York City, N.Y.). Both constructs were confirmed using sequence analyses. The digoxigenin (DIG)-labeled antisense and sense (as a negative control) riboprobes were made by means of in vitro run-off transcription (Boehringer-Mannheim, Tokyo, Japan) after linearization of the plasmids either with *Hin*dIII or *Eco*RI for both procollagen α1(I) and α1(IV), and with *Xho*I or *Pst*I for procollagen α1(III). The riboprobes were confirmed through RNA gel electrophoresis, and the concentration was measured using a spectrophotometer.

In situ hybridization

In situ hybridization was performed as described previously [13]. Briefly, tissue sections were deparaffinized, rehydrated, and pretreated with 0.2 N HCl and 100 mg/ml pepsin, 0.3% Triton X-100 in phosphate-buffered saline (PBS), 20 μg/ml proteinase K (Boehringer-Mannheim, Tokyo, Japan) in 0.1 M Tris HCl with 50 mmol/l ethylene diamine tetraacetic acid (EDTA), 4% paraformaldehyde in PBS, 0.2% glycine, and 0.2 mol/l Tris (pH 7.5) and with 0.25% acetic anhydride. Sections were prehybridized in 2× standard saline citrate (SSC) with 30% formamide for procollagen α1(I) and 50% formamide for both procollagen α1(III) and α1(IV) for 2 h at 42°C. Hybridization was performed in hybridization buffer [0.01 mol/l Tris HCl; pH 7.5; 12.5% Denhardt's solution, 2×SSC, 0.5% sodium dodecyl sulfate (SDS), 250 μg/ml salmon sperm DNA, and 30% formamide for α1(I) or 50% for α1(III) and α1(IV)] with 2 μg/ml denatured riboprobe for at least 12 h at 42°C. After a rinse in 2×SSC, the slides were washed twice with 0.1×SSC for the probes of α1(III) and α1(IV) and 0.3×SSC for that of collagen α1(I), for 20 min at 42°C. The tissue was then treated with RNase A (100 μg/ml in 2×SSC) for 30 min at 37°C. The immunological detection of the DIG incorporated in riboprobe was performed by following the manufacturer's protocol [11]. Hematoxylin was used for counterstaining.

Table 1 Background of patients studied. *HBsAg* hepatitis B surface antigen; *HCV* hepatitis C virus; *Ab* antibody; *HCC* hepatocellular carcinoma; *CH* chronic hepatitis; *NA* not applicable; *+ or – of capsule* present or not present, respectively

	Age	Gender	Basal Liver disease	HBsAg	HCV-Ab	HCC		
						Size, (mm)	Differentiation grade	Capsule
1.	49	Male	CH	+	–	14×13	Well	–
2.	74	Female	Cirrhosis	–	+	15×11	Well	–
3.	64	Male	CH	–	+	15×15	Well	+
4.	60	Male	Cirrhosis	–	+	18×18	Well	–
5.	78	Male	CH	–	NA	19×17	Well	–
6.	55	Male	Cirrhosis	–	+	20×20	Well	+
7.	67	Male	CH	–	+	35×20	Well	+
8.	49	Male	CH	+	–	50×47	Well	+
9.	61	Male	Cirrhosis	+	+	18×16	Moderate	+
10.	71	Male	Cirrhosis	–	NA	19×17	Moderate	+
11.	63	Male	Cirrhosis	–	+	20×13	Moderate	+
12.	77	Male	Cirrhosis	–	+	20×15	Moderate	+
13.	61	Male	Cirrhosis	–	+	20×15	Moderate	+
14.	65	Male	Cirrhosis	–	+	22×20	Moderate	+
15.	67	Male	CH	–	NA	31×25	Moderate	+
16.	64	Male	CH	+	+	38×34	Moderate	+
17.	62	Male	CH	+	NA	40×35	Moderate	+
18.	67	Male	Cirrhosis	–	+	45×30	Moderate	+
19.	65	Male	Cirrhosis	–	+	50×47	Moderate	+
20.	76	Female	Cirrhosis	–	–	58×38	Moderate	+
21.	61	Female	CH	+	–	98×69	Moderate	+
22.	45	Male	CH	+	–	100×81	Moderate	+
23.	75	Male	CH	–	+	23×16	Poor	–
24.	71	Female	Cirrhosis	–	+	30×30	Poor	+
25.	64	Male	Cirrhosis	–	–	102×87	Poor	+

Northern-blot analyses

Northern-blot analyses using chemiluminescence was performed as was described previously [13]. Briefly, 20 µg of total RNA, extracted using the acid phenol-guanidinium thiocyanate-chloroform extraction method [20], was fractionated on a 1% agarose/formaldehyde gel and subsequently transferred onto a nylon membrane. The filters were prehybridized and then hybridized with 100 ng/ml of each probe in hybridization buffer containing 5×SSC, 50% formamide, 2% blocking reagent, 0.1% *N*-lauroylsarcosine, and 0.02% SDS at 70°C overnight. The membranes were washed once in 2×SSC and 0.1% SDS and twice in 0.1×SSC and 0.1% SDS at 70°C for 15 min. The visualization of the DIG using chemiluminescence was performed according to the manufacturer's protocol [13]. The luminescent light emission was recorded on X-ray films (Kodak, Tokyo, Japan).

Immunohistochemistry for α-smooth muscle actin

The direct immunoperoxidase method was used (Dako Japan, Japan). Tissue sections were deparaffinized with 100% xylene and rehydrated with a graded ethanol series. After abolishing endogenous peroxidase using 3% hydrogen peroxide for 5 min and rinsing with Tris-buffered saline for 5 min, the tissue sections were incubated for 45 min at room temperature with monoclonal anti-human α-smooth muscle actin antibody raised in mouse and horseradish peroxidase coupled to the antibody's inert polymer backbone (Dako, Japan). After rinsing in Tris-buffered saline for 5 min, the peroxidase activity was revealed with 30% 3,3-diaminobenzidine tetrahydrochloride or 3-amino-9-ethylcarbazole for serial immunohistochemistry or double staining, respectively.

Combination of in situ hybridization for α1(I) and immunohistochemistry for α-SMA

Immunohistochemistry for α-SMA was performed after in situ hybridization for α1(I). However, pepsin treatment in the in situ hybridization and the counterstaining of nuclei were avoided.

Results

The transcripts for procollagen α1(I), α1(III), and α1(IV) genes were observed in mesenchymal cells of both HCC nodules and non-cancerous liver of all the cases. Cancer cells or hepatocytes did not express any of these procollagen genes. The distribution and intensity of the gene expression differed according to the types of procollagen. α-SMA-positive cells mostly co-localized along with the expression of procollagen α1(I) and α1(III).

Capsule formation around cancer nodules

In the thick capsule around the HCC, numerous cells expressing procollagen α1(I) and α1(III) genes were observed (Fig. 1A), but those cells were barely found around the tumor without a capsule (data not shown). Along the extracapsular invasion of HCC toward the non-cancerous liver, some α-SMA-positive cells were aligned (Fig. 1B), and expression of procollagen α1(I) and α1(III) genes was detected (Fig. 1C). The capsule around HCC with non-cirrhotic liver appeared to be formed in the same manner as that with cirrhotic liver. The expression of the procollagen α1(IV) gene in the capsule and the septum of HCC nodules was weaker than that of procollagen α1(I) or α1(III) (data not shown). Hybridization with sense probes for procollagen α1(I), α1(III), and α1(IV) genes as negative controls showed no significant signals (Fig. 1D).

Inside of cancer nodules and septum between nodules

In the septum between two different nodules and in the "nodule in nodule", where stromal cells were accumulated and aligned, increased expression of procollagen α1(I) and α1(III) genes was observed (Fig. 2A). Localization of the expression was similar to the expression of α-SMA-positive cells, which was examined with serial sections (Fig. 2B). Expression of procollagen α1(IV) was primarily observed in non-parenchymal cells along sinusoid-like blood vessels in HCC nodules (Fig. 3).

Around the necrosis in HCC

Accumulation of fibrous tissue was observed around the necrosis in cancer nodules. Stromal cells that express procollagen α1(I) and α1(III) genes were increased around the necrosis (Fig. 2C), and α-SMA-positive cells were also localized in the same area.

Combination of in situ hybridization and immunohistochemistry

In order to identify the cell type(s) that express(es) procollagen genes, in situ hybridization for procollagen α1(I) combined with immunohistochemistry for α-SMA was performed. Procollagen α1(I) gene transcripts were mainly demonstrated in α-SMA-positive cells (Fig. 4), but not all α-SMA-positive cells expressed procollagen α1(I).

Northern-blot analyses

Expression of procollagen α1(I), α1(III), and α1(IV) genes was demonstrated in both HCC and non-cancerous liver. The intensity of the procollagen gene expression of HCCs, including capsule, was stronger than that of the surrounding non-cancerous tissues (Fig. 5).

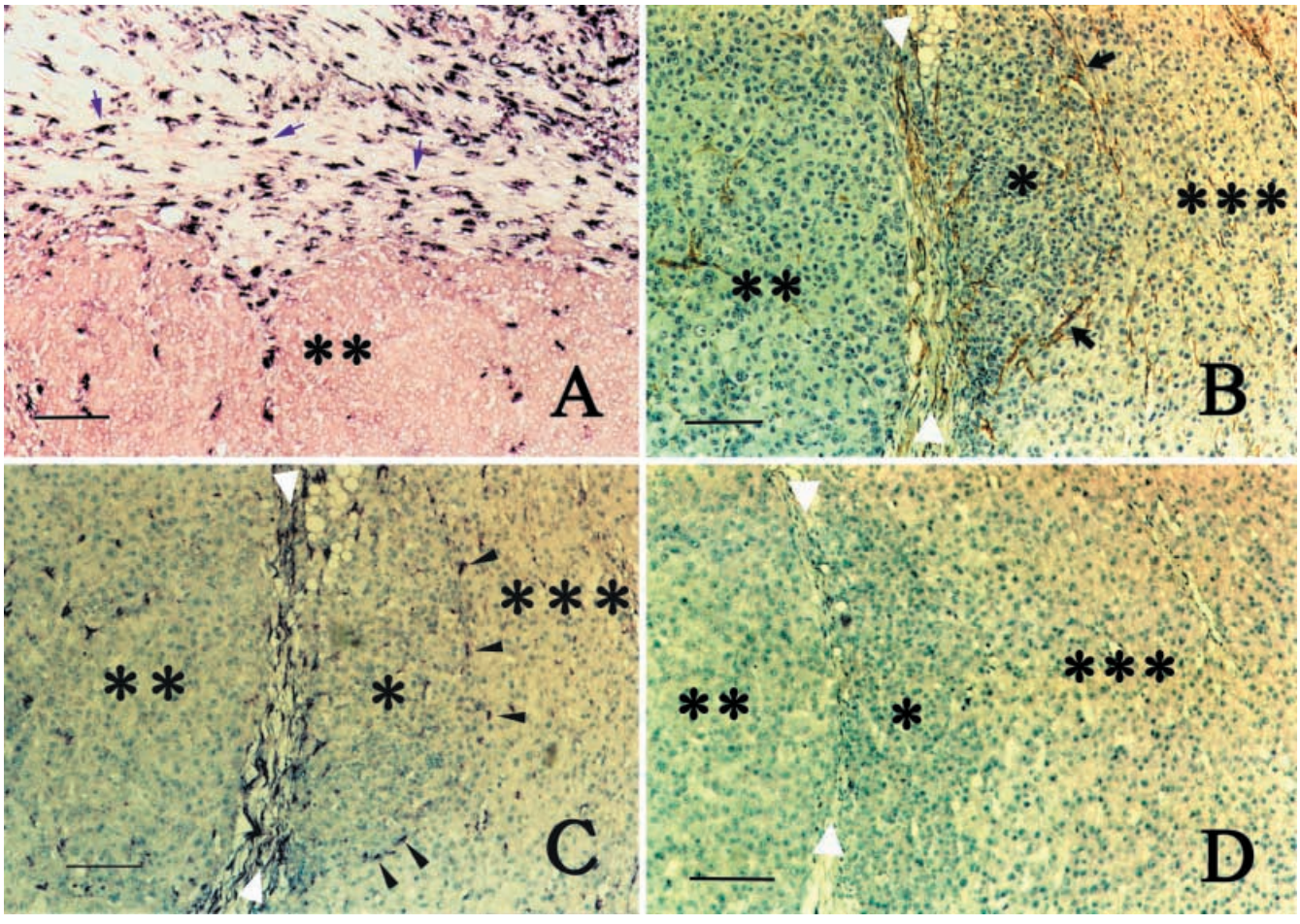

Fig. 1 Localization of procollagen α1(III) or α1(I) transcripts and α-smooth muscle actin protein (α-SMA) around the hepatocellular carcinoma (HCC)-liver interface. Note that the transcripts are revealed as *dark brown* (*or blue*) *staining* in the cells using in situ hybridization. * Extracapsular invasion of HCC; ** main HCC nodule; *** non-cancerous counterpart; *white arrows* capsule around HCC nodule. *Bars* 100 μm; original magnification ×100. **A** In situ hybridization of procollagen α1(III). In a capsule (*upper half of the picture*) around HCC (** *lower half of the picture*), strong expression of procollagen α1(III) messenger (m)RNA (*arrows*) is observed in many stromal cells. The expression is also found in some stromal cells in the HCC nodule (counterstaining was not added). **B** Immunohistochemistry of α-SMA demonstrates the localization of α-SMA-positive cells around invasion of HCC (counterstained with hematoxylin). Extracapsular invasion (*) of HCC is shown in between main HCC nodule (**) and non-cancerous liver (***).α-SMA-positive cells (*black arrows*) exist along the interface between extracapsular invasion of HCC and non-cancerous liver. **C** In situ hybridization demonstrates the localization of procollagen α1(I) transcripts around invasion of HCC (serial section of **B** and counterstained with hematoxylin). Procollagen α1(I)-expressing cells (*arrowheads*) are detected along the interface between extracapsular invasion (*) of HCC and non-cancerous liver (***). Procollagen α1(I) expression is also observed in the septum (*white arrows*) and in both cancer nodule and non-cancerous liver. **D** In situ hybridization with sense probe as a negative control disclosed no significant staining (serial section of **B** and **C** and counterstained with hematoxylin)

Discussion

The present study reveals that type-I and -III procollagen genes are expressed in mesenchymal cells in the capsule formed around the cancer nodules, along the invasion front of HCC, in the septum between different HCC nodules, and around necrotic cancer tissues. These procollagen-expressing cells are mostly α-SMA-positive stellate cells but neither hepatocytes nor HCC cells. The type-IV procollagen gene is primarily expressed in mesenchymal cells in both HCC (e.g., endothelial cells along the sinusoid-like blood vessels) and non-cancerous tumor-bearing liver. The fibrogenesis for the formation of the capsule and septum occurs at the interface between two different tissues (e.g., capsule between cancer nodule and non-cancerous liver and septum between cancer nodule and another cancer nodule). Ischemic necrosis of HCC-surrounding tissues followed by a repair process with ECM deposition has been speculated as part of the capsule-forming sequence [6]. However, it may not be necessarily required, because the ischemic necrosis is barely observed in the present study.

The fibrogenesis in the capsule and septum of the HCC could be regulated by many factors, including various enzymes, growth factors, and cytokines. Accumulation of ECM results from the exceeding synthesis over degradation of ECM proteins. Under this condition, the

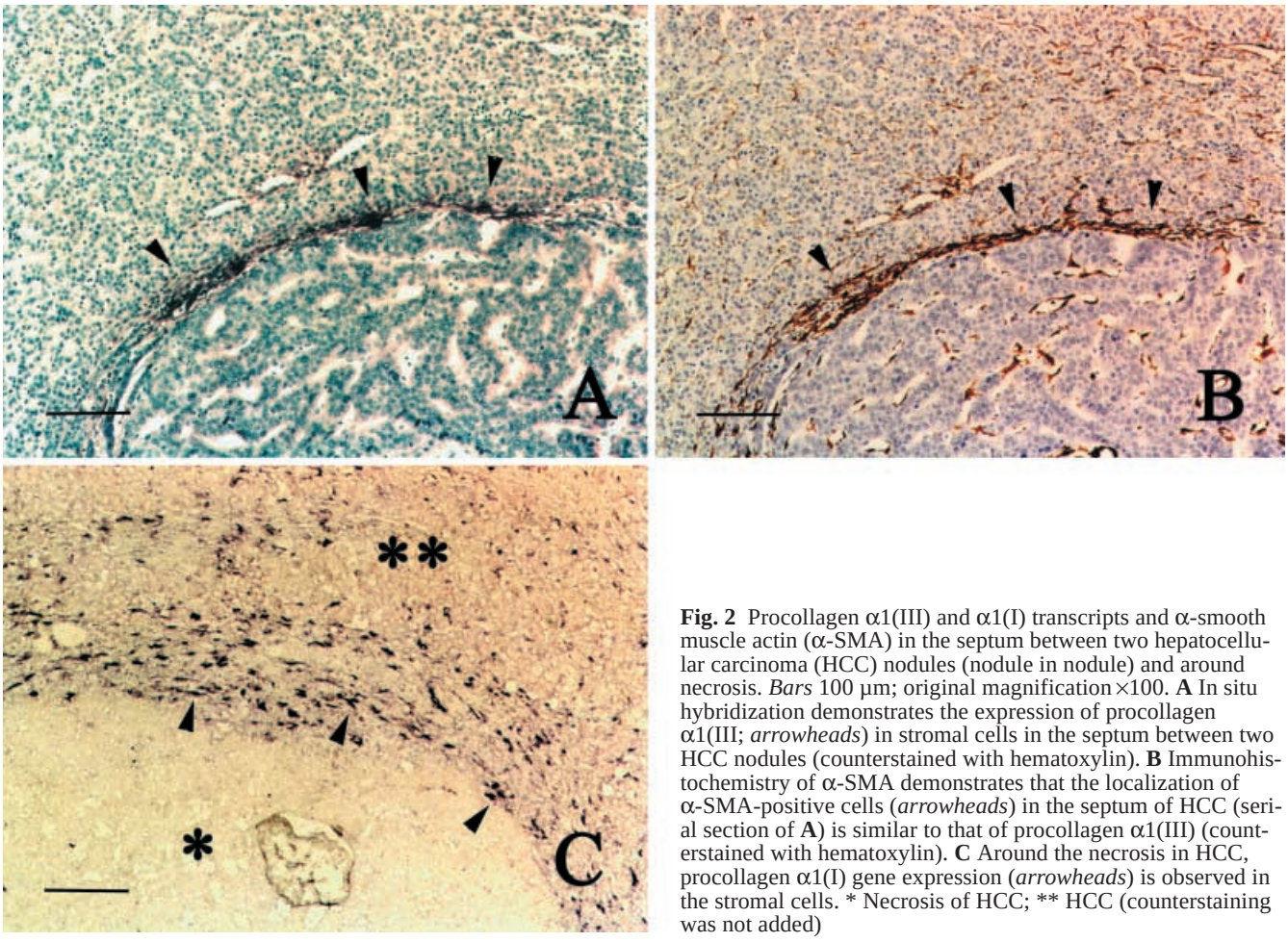

Fig. 2 Procollagen α1(III) and α1(I) transcripts and α-smooth muscle actin (α-SMA) in the septum between two hepatocellular carcinoma (HCC) nodules (nodule in nodule) and around necrosis. *Bars* 100 μm; original magnification ×100. **A** In situ hybridization demonstrates the expression of procollagen α1(III; *arrowheads*) in stromal cells in the septum between two HCC nodules (counterstained with hematoxylin). **B** Immunohistochemistry of α-SMA demonstrates that the localization of α-SMA-positive cells (*arrowheads*) in the septum of HCC (serial section of **A**) is similar to that of procollagen α1(III) (counterstained with hematoxylin). **C** Around the necrosis in HCC, procollagen α1(I) gene expression (*arrowheads*) is observed in the stromal cells. * Necrosis of HCC; ** HCC (counterstaining was not added)

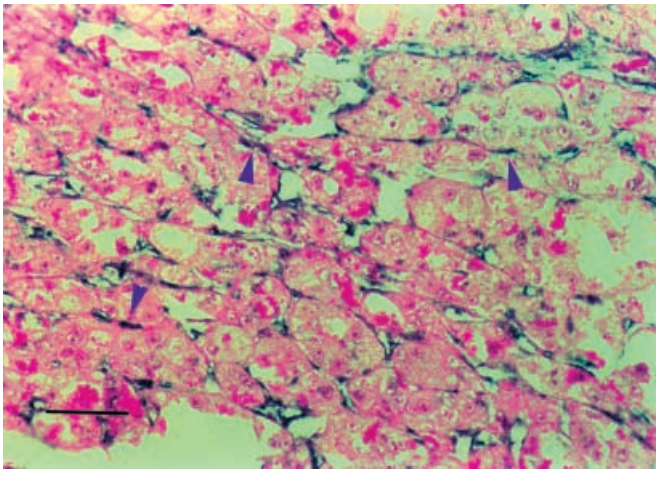

Fig. 3 Localization of procollagen α1(IV) gene transcripts in HCC (counterstained with eosin but not with hematoxylin). Procollagen α1(IV) transcripts are mainly demonstrated in the cells (*arrowheads*) along sinusoid-like blood vessels of HCC nodule. *Bar* 50 μm; original magnification ×200

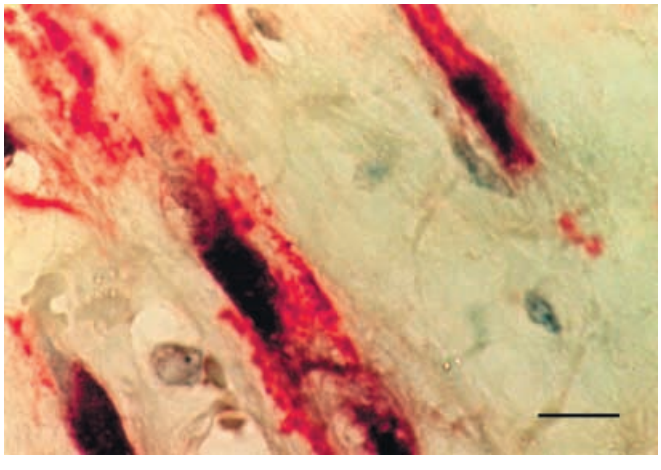

Fig. 4 Co-localization of α-smooth muscle actin (α-SMA) and procollagen α1(I) transcripts revealed using a combination of in situ hybridizatioin of procollagen α1(I) and immunohistochemistry of α-SMA. *Bar* 17 μm; original magnification ×600. The expression of procollagen α1(I) transcripts is localized mainly in α-SMA-positive cells. Procollagen α1(I) transcripts are visualized with *dark brown*, and α-SMA is visualized with *red color*

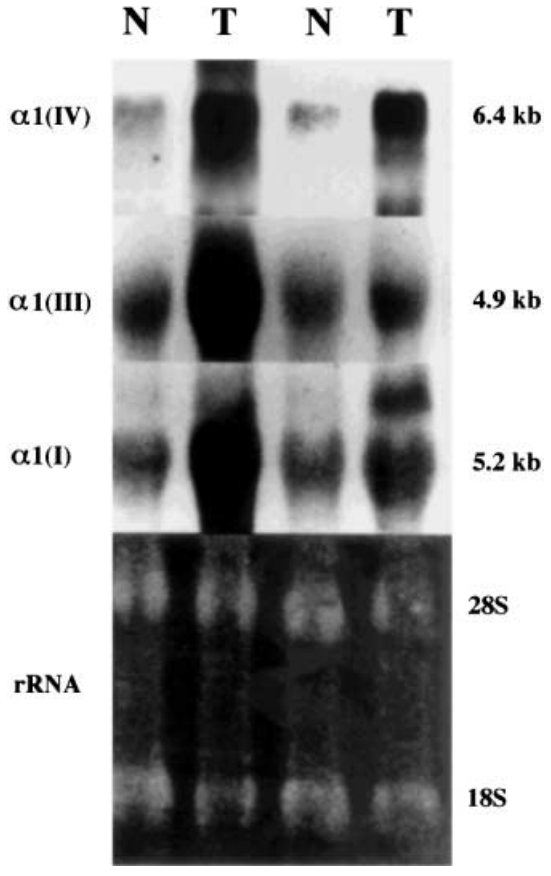

Fig. 5 Northern-blot analyses for procollagen α1(I), α1(III), and α1(IV) genes in hepatocellular carcinoma (HCCs) and non-cancerous liver. Expression of procollagen α1(I), α1(III), and α1(IV) genes are observed in both HCC and non-cancerous liver. The intensity of the procollagen gene expression in HCC, which includes capsule, is stronger than that in the surrounding liver. Ribosomal RNA stained with ethidium bromide is shown as a control

production of ECMs, particularly collagens, is essential. Matrix metalloproteinases [2, 15] are the major degrading enzymes and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), also play an important role [13]. In this regulation network, the activation of α-SMA-positive cells (activated stellate cells) appears to be a pivotal step. Kupffer cell (or macrophage) has been suggested to be closely related to stellate cell activation by secreting various cytokines [5]. Transforming growth factor (TGF)-β, one of the most potent fibrogenic mediators, is overexpressed in human HCC nodules [7], perisinusoidal cells of rat HCCs [12], and also in Kupffer cells, suggesting the strong involvement of TGF-β in the capsule and in septum formation. HCC and host liver may also interact with each other. It has been suggested that soluble mitogenic factor(s) released by cancer cells stimulate(s) the proliferation of human myofibroblasts [23]. Therefore, the formation of capsule and septum is due to a result of tumor–host interaction and affects the growth and invasion of HCC.

In tumor invasion, one of the characteristic features of malignancy, the degradation of ECMs surrounding tumors, has been considered critical [9]. The matrix-degrading metalloproteinases play an important role at the front of HCC invasion [2]. The present study reveals the existence of α-SMA-positive stromal cells and their expression of type-I and -III procollagen genes in the host liver adjacent to the invasion of HCC. The capsule is formed by host mesenchymal cells, not by HCC cells, and may prevent free invasion of HCC to the host liver. This is supported by the clinical evidence that the prognosis of HCC patients with capsule is better than those without [8, 11, 14, 16]. These lines of evidence suggest that the capsule formation may be one of the protective mechanisms of the host against tumor invasion.

In malignant tumors, particularly in low differentiation stages, not many non-malignant cells exist. However, non-parenchymal stromal cells, including myofibroblasts [4], inside of HCC, exist among cancer cells even in moderately differentiated HCCs, as was reported in a previous study [3, 21]. These cells gather and perhaps proliferate both in the septum between cancer nodules and around the necrosis of cancer tissue and express procollagens. The latter is similar to wound healing commonly observed in injured non-malignant tissues. It is intriguing that the wound-healing mechanism occurs even inside the malignant tumor. These procollagen-expressing stromal cells may contribute to the repair of cancer tissues upon necrosis and possibly influence the growth of HCC.

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