High expression of liver stem/progenitor cell markers, transforming growth factor-β signal and epithelial mesenchymal transition-related genes in scirrhous hepatocellular carcinoma

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Directed by Professor Young Nyun Park

The Doctoral Dissertation submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy of Medical Science

Jae Yeon Seok

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This certifies that the Doctoral Dissertation
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Finally, I would like to dedicate this work to the Lord of the whole creation with deep humility.

Jae Yeon Seok

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ABSTRACT

High expression of liver stem/progenitor cell markers, transforming growth factor-β signal and epithelial mesenchymal transition-related genes in scirrhous hepatocellular carcinoma

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Recently cancer stem cell has been reported to be involved in the chemo-resistance and poor prognosis of cancer patients. Epithelial mesenchymal transition (EMT), which is important in vascular invasion and metastasis of cancer, has been reported to induce cancer stem cell and a fibrogenic cytokine transforming growth factor-β (TGF-β) has been reported to induce EMT during tumorigenesis. Scirrhous hepatocellular carcinoma (HCC) is characterized by abundant fibrous stroma between tumor trabeculae, whereas the fibrous stroma is usually very little in classical HCC. Scirrhous HCC has been considered as one of histological patterns of HCC and its clinicopathological significance remains unclear. In this study, we selected 19 cases of resected scirrhous HCC (>60% of fibrous stroma of tumor volume) without pre-operative treatment and 24 cases of classical HCCs as a control group. The clinicopathological features, the expression of liver stem/progenitor cell markers [epithelial cell adhesion molecule (EpCAM), keratin (K) 19, K7, CD56, CD133, Oct3/4, cMET and alpha fetoprotein (AFP)], hepatocyte marker (Hep Par 1), TGF-β signal pathway [TGF-β, TGF-β receptor I (TGFβRI), TGF-β receptor II (TGFβRII) and Smad4], and EMT-related genes (Snail and Twist) were investigated in scirrhous HCCs and classical HCCs by real time quantitative PCR and immunohistochemical

stain and compared between two groups.

Scirrhous HCCs showed significantly higher incidence of microvascular invasion ($p=0.004$), portal vein invasion ($p=0.047$) and intrahepatic metastasis ($p=0.044$), and significantly lower incidence of tumor capsule formation ($p<0.0001$) compared to classical HCCs. The incidence of positive expression of liver stem/progenitor cell markers (EpCAM, K19, K7, CD56, and AFP), detected by immunohistochemical stain, was 26.3~94.7% in scirrhous HCCs in contrast to 4.2~50% in classical HCCs, and the mRNA levels of EpCAM, K19, CD133, Oct3/4, and cMET were significantly up-regulated in scirrhous HCCs than in classical HCCs ($p<0.05$). The expression rate of hepatocyte marker (Hep Par 1) was low in scirrhous HCCs (68.4%) compared to classical HCCs (100%) ($p<0.05$). The mRNA levels of TGF-β signal pathway (TGF-β, TGFβRI, TGFβRII, Smad4) and EMT related genes (Snail and Twist) were significantly up-regulated in scirrhous HCCs compared to classical HCCs ($p<0.05$). The tophographical expression pattern of liver stem/progenitor cell markers (EpCAM and K19) and Snail was evaluated by double immunohistochemical stain. The expression of liver stem/progenitor cell markers was mainly detected in the periphery of tumor cell nests, facing the fibrous stroma and most of them (69%) showed co-expression of Snail. The tumor recurrence was significantly higher in scirrhous HCCs (52.6%) than in classical HCCs (20.8%) during the mean follow up of 18 months ($p=0.030$). In conclusion, scirrhous HCC is suggested to be a distinctive subtype showing invasive and aggressive clinicopathological characteristics, in which EMT, up-regulated TGF-β pathway and induction of liver stem/progenitor cell markers are involved.

Key words: scirrhous HCC, liver stem/progenitor cell markers, TGF-β signal, EMT

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I. INTRODUCTION

Absence of fibrous stroma or desmoplasia is one of the typical histologic feature of ordinary hepatocellular carcinoma (HCC)[1]. On the other hand, cholangiocarcinoma (CC), which is a primary hepatic carcinoma of bile duct origin, has abundant fibrous stroma like any adenocarcinomas of other organs. Scirrhous HCC, characterized by abundant fibrosis between tumor trabeculae without any preoperative treatment, is one of the histological or architectural patterns of HCC according to World Health Organization (WHO) classification[1]. The incidence of scirrhous HCC is 0.2% to 4.6%[2-5]. Recently a few studies demonstrated that liver stem/progenitor cell markers such as keratin (K) 7, K19 and epithelial cell adhesion molecule (EpCAM) are frequently expressed in scirrhous HCCs[4, 6].

The clinical course of HCC is extremely variable from patient to patient[7, 8] and many investigators are immersed in finding out the distinct subgroup of

HCC by searching the underlying molecular heterogeneity, which is responsible for the highly variable clinical outcome. A remarkable study showed HCCs with liver stem/progenitor cell molecular signature were biologically distinct aggressive group in which the molecules associated with invasion and metastasis were activated[9].

Liver stem/progenitor cell markers include various molecules such as K7, K19, EpCAM, neural cell adhesion molecule (NCAM or CD56), CD133, Oct3/4 and cMET, normally expressed in the liver stem/progenitor cells, which are located in canal of Hering and have potential to differentiate into both hepatocyte and cholangiocyte[10-13].

K7 and K19, which have been well-known as biliary differentiation markers, have long been studied as a stratification marker of HCC[14-17]. Wu et al. demonstrated that HCCs with K19 immunoexpression showed faster recurrence and poor survival rate[14]. Other studies reported that HCCs with K19 expression were associated with lower disease free survival rate, metastasis, high recurrence after transplantation[15, 16, 18]. Aishima et al. reported that K19-positive and mucin-positive small HCCs were related to poor survival rate, intermediate tumor cell morphology, intratumoral inflammatory cell infiltration and desmoplastic stroma[17].

EpCAM is a glycoprotein of 40 kd and it has diverse functions including cell-cell adhesion, migration, differentiation, proliferation in normal epithelium[19]. In liver, adult hepatocytes are EpCAM-negative and in hepatoblasts or regenerating process such as cirrhosis and hepatitis the EpCAM expression is activated[20]. EpCAM is expressed in about 20% of HCCs[20]. Recently it has been noticed as a novel bad prognostic marker of HCC[21, 22].

CD56 is a cell surface molecule that is related to morphogenesis, migration and cell-cell interaction[23] and some of proliferating bile ductular cells are CD56-positive[24]. Zhou et al reported that CD56 immuno-reactivity was

observed in the bipotent liver stem/progenitor cells of ductular reaction and the intermediate cells showed loss of CD56 immuno-reactivity when they differentiate into hepatocytic or cholangiocytic lineage[25]. A small portion of CD56-positive ductular cells co-expressed K19 but not hepatocytic differentiation marker Hep Par 1. About 10% of HCCs[26] and 25% of HCCs with biliary differentiation marker expression express CD56[17]. But the clinical implication of CD56 expression of HCC has not been fully studied.

CD133 has been known as a hematopoietic and neuronal stem cell marker and it is expressed in the proliferating ductule of damaged liver[26]. A small population of CD133-positive cells exists in HCC cell lines and primary HCC tissues[27]. CD133 were expressed in about 40% of HCCs and patients with increased CD133 levels showed shorter overall survival and higher recurrence rates compared with patients with low CD133 expression[28, 29]. CD133-positive and CD44-positive tumor cells of HCC showed stem cell properties, including extensive proliferation, self-renewal and differentiation and CD133-positive cells of HCC showed up-regulation of ATP-binding cassette superfamily transporters and activation of Akt/PKB and Bcl-2 cell survival response, resulting in chemotherapeutic resistance[30-32].

The hepatocyte growth factor receptor, MET has physiological functions such as cell proliferation, motility, differentiation and angiogenesis and pathological role as a proto-oncogene and invasion and metastasis formation of tumor[33]. It has been known as a phenotypic expression protein of putative liver stem cells[12, 34]. In HCCs, the presence of MET signature revealed significant correlation with vascular invasion, microvessel density and decreased mean survival time[35].

Transforming growth factor β (TGF-β) has pleiotropic effects on diverse aspects of inflammation, immune regulation, angiogenesis and fibrosis[36]. TGF-β binds two receptor types, the TGF-β receptor I (TGFβRI) and TGF-β receptor II (TGFβRII) to form active signaling complex. Phosphorylated

TGFβRI transmits the signal intracellularly by phosphorylating the transcription factor, Smad2 and Smad3, and then they form a complex with Smad4. The Smad complex moves into nucleus where it regulates the expression of target genes[37, 38]. TGF-β plays a major role in cancer by suppressing tumor growth in the early phase, while promoting tumor progression and metastasis in later phases[36]. The suppression of tumor cell growth depends on its ability to up-regulate cyclin kinase inhibitors. In late phase, TGF-β becomes a tumor promoter by inducing epithelial mesenchymal transition (EMT), which is associated with tumor invasion and metastasis[39-42]. Because of the two conflicting effect of TGF-β on tumors, the studies about the expression of TGF-β and its downstream molecules in HCCs showed heterogeneous results[43-47]. Recently Coulouarn et al. demonstrated that HCC with early and late TGF-β signature exhibited different outcome and the late TGF-β signature showed shortened mean survival time, more invasive phenotype and increased tumor recurrence[48]. This finding has drove molecular classification according to the early and late TGF-β signature as well as liver stem/progenitor cell marker phenotype[49].

EMT is the differentiation switch between polarized epithelial cells and contractile and motile mesenchymal cells[42]. EMT is a fundamental process governing morphogenesis during embryogenesis, reactivated in fibrogenic diseases and associated with progression of carcinoma[50 41]. The maintainer of epithelial integrity, E-cadherin repression is a crucial step of the EMT and Snail and Twist are the direct repressors of E-cadherin[51]. Like other malignancies, E-cadherin and Snail expression showed inverse correlation in HCC cells[52, 53]. They are associated with invasion and metastasis of HCC[54, 55].

In this study, it was that in scirrhous HCCs the liver stem/progenitor cell markers were frequently expressed and the clinical outcome would be more aggressive than the classical HCCs according to the previous data related to the subclass of HCCs with liver stem/progenitor cell markers expression. And

the scirrhous HCCs which produce abundant fibrous stroma are closely related to TGF-β signal. If the clinical outcome of scirrhous HCCs was more aggressive than classical HCCs, the possible mechanism would be the EMT, the late signature of TGF-β signaling. In this study the expression of liver stem/progenitor cell markers, TGF-β signal and EMT-related genes were investigated in scirrhous HCCs compared to classical HCCs.

II. Material and Methods

1. Materials

A. Patient selection

We selected surgically resected primary hepatic carcinomas diagnosed at the Department of Pathology of Yonsei University from 2000 to 2008. The histologic diagnosis was performed according to the criteria of the classification of the WHO. Firstly we selected 19 cases of preoperatively untreated HCCs with fibrous or scirrhous stroma involving more than 60% of the tumor area. Then 24 cases of HCCs without fibrous stroma showing typical histologic features of HCC were selected. Finally, 19 cases of typical CCs of peripheral type were selected for the control group of prognostic evaluation. The clinical data and the histologic features were reviewed and evaluated. The tumor capsule formation was categorized as complete if more than 50% of tumor circumference, partial if less than 50% of tumor circumference showed capsule formation and none if there was no capsule formation. The vascular invasion (portal vein invasion and microvessel invasion respectively) was evaluated as frequent if more than 5 foci, present if 1~5 foci of vascular invasion were observed and absent if no invasive foci was detected in 100~400 microscopic magnification of more than 3 slides per case.

2. Methods

A. Immunohistochemistry and special staining

Formalin-fixed paraffin-embedded tissues were sliced into 4 μm-thick sections, and immunohistochemistry was performed using the DAKO Envision Kit (Dako, Glostrup, Denmark). In brief, sections were deparaffinized in xylene, rehydrated in graded alcohol and quenched in 3% hydrogen peroxidase. Antigen retrieval was performed in citrate buffer (pH 6.0) in a 700W microwave oven for 15 minutes for K7, K19, EpCAM, CD56, alpha fetoprotein (AFP), Hep Par 1, Smad4 and E-cadherin. The following primary antibodies were applied to the slides: K7 (OV-TL 12/30, Dako, Glostrup, Denmark; 1:150), K19 (BA17, Dako, Glostrup, Denmark; 1:200), EpCAM (OP187, Calbiochem, Darmstadt, Germany; 1:3000), CD56 (123C3, Zymed Laboratoies Inc., San Francisco, CA, USA; 1:100), AFP (RB-9064-R7, Thermo Scientific, Fremont, CA, USA; 1:1000), Hep Par 1 (OCH1E5, Dako, Glostrup, Denmark; 1:50), Smad4 (sc-7966, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:50), Snail (ab17732, Abcam, Cambridge, MA, USA; 1:100) and E-cadherin (36B5, Novocastra, Newcastle Upon Tyne, UK; 1:80). For Snail antibody, antigen retrieval was performed in Proteinase K (S3020, Dako, Glostrup, Denmark) for 5 minutes. Incubation was performed for 1 hour at room temperature. After rinsing, incubation with a secondary antibody was carried out using the DAKO EnVision Rabbit/Mouse kit, and then developed with 3,3-diaminobenzidine (Dako). Slides were then counterstained with hematoxylin, dehydrated, cleared and mounted. The cytoplasmic expression of K7, K19, AFP and Hep Par 1 over 5% of tumor cells was evaluated as positive. The membranous and/or cytoplasmic expression of EpCAM and CD56 over 5% of tumor cells was evaluated as positive. The expression of each marker was scored as 1+ if 5% to 10%, 2+ if 11% to 50% and 3+ if over 50% of tumor cells were stained. The E-cadherin was scored as reduced if less than 75% and preserved if more than 75% of tumor cells were

stained.

For double immunohistochemical staining, tissue slides were incubated with the first primary antibody and the EnVision AP system (Dako) for 30 minutes and developed with Vector Blue Alkaline Phosphatase Substrate Kit1 (Vector Laboratories, Burlingame, CA, USA) for the first dye and Vector Red Alkaline Phosphatase Substrate Kit1 (Vector Laboratories) for the second dye.

The scirrhous stroma area within tumor was analyzed by blue-stained portion per area with Masson Trichrome stain (x200 magnification, two scirrhous foci) using Image Pro Plus 5.0 software (MediaCybernetics, Silver Spring, MD, USA).

B. Total RNA extraction, cDNA synthesis and Real-time quantitative RT-PCR

Total RNA was extracted from human HCC tissues using an RNeasy total RNA isolation kit (Axygen Scientific Inc., Union city, CA, USA) according to the manufacturer's instructions. The integrity and quantity of total RNA were determined by electrophoresis and NanoDrop (Thermo Scientific, Wilmington, DE, USA), respectively, and absence of genomic DNA contamination was then confirmed by PCR. Primer sets for specific reverse transcription including K19, EpCAM, CD133, Oct3/4, c-Met, TGF-β, TGFβRI, TGFβRII, Smad4, Snail, Twist, Gapdh and the high-capacity RNA to cDNA Kit were utilized following the manufacturer's protocol. All reagents and instruments were from Applied Biosystems Inc (Applied Biosystems Inc., Foster City, CA, USA). Briefly, the reaction master mix containing 2× RT Buffer, 20× Enzyme Mix, and nuclease-free water was mixed with 20 ng of each total RNA sample. Mixtures were incubated for 60 minutes at 37℃, 5 minutes at 95℃, and then kept at 4℃. Real-time quantitative RT-PCR was carried out using Applied Biosystems 7500 Real-Time PCR System. The PCR master mix containing TaqMan 2× Universal PCR Master Mix, 20× TaqMan assay, and RT products

in 20 μl volume were processed as follows: 95℃ for 10 minutes and then 40 cycles of 95℃ for 15 seconds and 60℃ for 60 seconds (n = 3). The signal was collected at the endpoint of every cycle. The fresh sample also was processed at the same time.

C. Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software. We assessed the immunohistochemical stain results using Chi-square test and the student T test were used to compare the results of the real-time quantitative RT-PCR. The survival analysis was done by the Kaplan-Meier method and the differences were analyzed with the log-rank test. A p-value of less than 0.05 was considered statistically significant for all analyses.

III. Results

1. Clinical Features

The clinical features are summarized in Table 1. The age of the patients ranged from 27 to 81 years. For the sex, tumor size, serum AFP level and etiology, there were no significant differences between scirrhous HCC and classical HCC.

Table 1. Clinical features

		Scirrhous HCC (n=19)	Classical HCC (n=24)	P-value
Age (years)		51 ± 12 (27~68)	58 ± 10 (40~81)	0.058
Sex (M:F)		14:5	17:7	0.556
Tumor size (cm)		4.6 ± 3.51 (1.4~17)	4.4 ± 1.65 (1.8~9)	0.262
Serum AFP (ng/ml)		514.8 ± 974.32 (3.7~3879.0)	1832.0 ± 6301.43 (1.3~30676.8)	0.076
Etiology	HBsAg (+)	13 (68.4%)	18 (75%)	0.118
	HCV Ab (+)	0 (0%)	4 (16.7%)	
	Alcohol	2 (10.5%)	0 (0%)	
	Unknown	4 (21%)	2 (8.4%)	

2. Pathological features

Scirrhous HCCs showed abundant fibrous stroma between trabeculae of tumor cells or central scar-like fibrosis embedding the solid tumor cell nests (Fig. 1). The tumor cells were arranged in various patterns including round solid nests, thin trabeculae, anastomosing chain or cord-like pattern within fibrous stroma. Tumor cells of round solid nests showed characteristic small oval cells at the periphery of the nests in face of fibrous stroma, which had

small amount of dark cytoplasm. The anastomosing chain or cord-like pattern resembled the proliferating bile ductular structures architecturally and it was predominantly composed of small tumor cells with occasional hepatocytic differentiation of polygonal abundant cytoplasm within the cord.

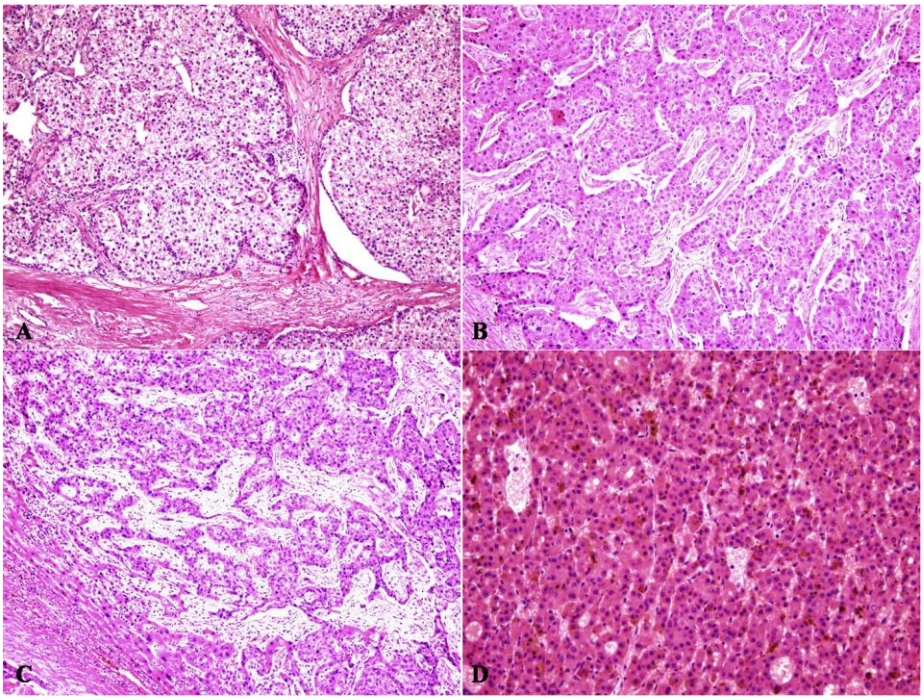


Figure 1. Histological features of scirrhous HCCs. The pattern of fibrous stroma is divided into three patterns. (A) The fibrous stroma separates the tumor cells with nesting pattern and small dark tumor cells are arranged at the periphery of the nests facing the fibrous stroma (HE, x100). (B) The tumor cells show trabecular pattern and the fibrous stroma is intervening the trabeculae (HE, x100). (C) The anastomosing cords are embedded in fibrous stroma and the cords are composed of small dark tumor cells in thin cords and large polygonal tumor cells having abundant granular cytoplasm in thick cords (HE, x100). (D) The classical HCC shows trabecular pattern and sinusoid-like spaces between tumor cells without fibrous stroma (HE, x200).

The pathological features are summarized in Table 2. The scirrhous stroma area within tumor was analyzed by blue-stained portion per area with Masson Trichrome stain (x200 magnification). In scirrhous HCCs, the fibrous portion occupied 20.3% in average. The classical HCCs showed low proportion of fibrous stroma area with 5.9% in average. The difference was statistically significant between scirrhous HCC and classical HCC ($p<0.001$) (Fig. 2). The capsule formation was significantly different between scirrhous HCC and classical HCC. In scirrhous HCCs 15 out of 19 cases (78.9%) had no tumor capsule and 4 out of 19 cases (21.1%) formed partial capsule. The complete capsule formation was observed in classical HCCs only with 41.7% of prevalence (Fig. 3). The portal vein invasion and microvessel invasion were observed more frequently in scirrhous HCCs than classical HCCs (Fig. 4). The intrahepatic metastasis was more frequent in scirrhous HCCs with statistical significance ($p=0.044$). The lymph node metastasis was not statistically different between scirrhous HCC and classical HCC.

Table 2. Pathological features

		Scirrhous HCC (n=19)	Classical HCC (n=24)	P-value
Scirrhous stroma within tumor (%)		20 ± 11.6 (2.5~53.7)	6 ± 5.6 (0.4~18.2)	<0.0001*
Capsule formation[1]	Complete	0 (0%)	10 (41.7%)	<0.0001*
	Partial	4 (21.1%)	13 (54.2%)	
	None	15 (78.9%)	1 (4.2%)	
Portal vein invasion[2]	Absent	12 (63.2%)	22 (91.7%)	0.047*
	Present	5 (26.3%)	2 (8.3%)	
	Frequent	2 (10.5%)	0 (0%)	
Microvessel invasion[2]	Absent	7 (36.8%)	17 (70.8%)	0.004*
	Present	5 (26.3%)	7 (29.2%)	
	Frequent	7 (36.8%)	0 (0%)	
Intrahepatic metastasis	Absent	16 (84.2%)	24 (100%)	0.044*
	Present	3 (15.8%)	0 (0%)	
Lymph node metastasis	Absent	18 (94.7%)	23 (95.8%)	0.694
	Present	1 (5.3%)	1 (4.2%)	

1: Complete capsule indicates that tumor capsule circumscribes more than 50% of tumor and partial capsule less than 50%. 2: More than 5 foci of vascular invasion are observed in tumors of “frequent” category and from 1 to 4 foci of vascular invasion in tumors of “present” category. *P<0.05.

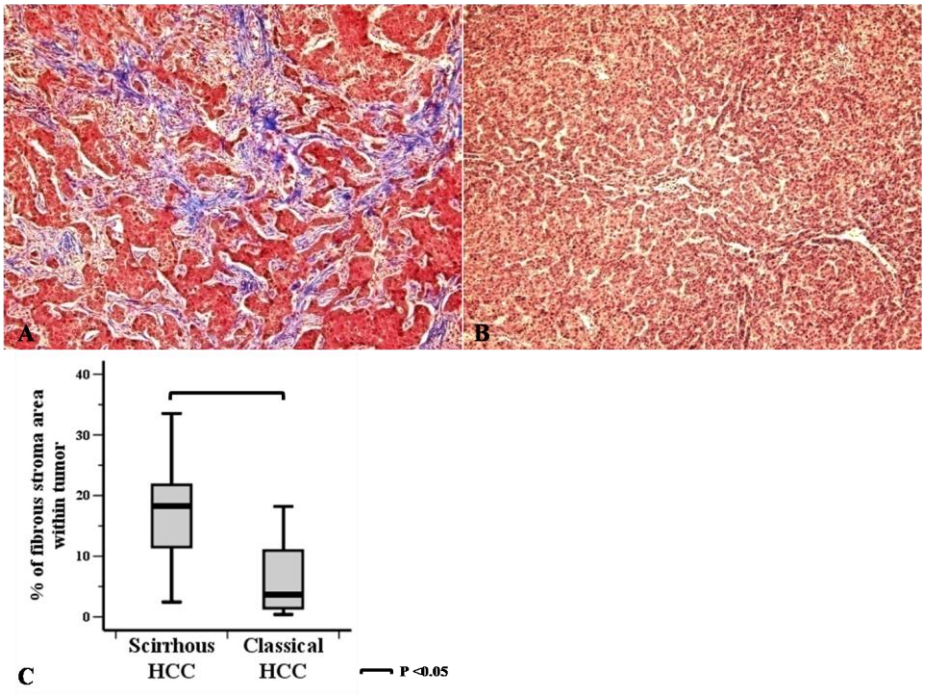


Figure 2. Proportion of fibrous stroma area within tumor. The fibrous stroma area (stained in blue color) is abundant in scirrhous HCCs (A) in comparison to classical HCCs (B) (Trichrome stain, x100). (C) The difference of the proportion of scirrhous stroma area is statistically significant between scirrhous HCC and classical HCC.

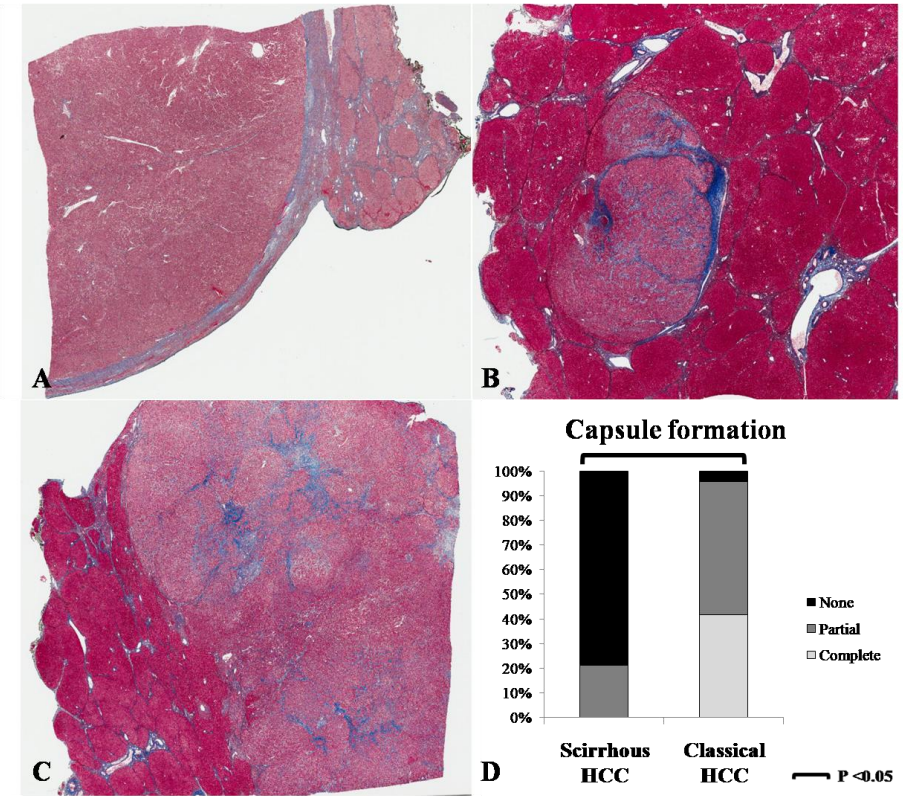


Figure 3. Tumor capsule formation. The tumor capsule formation is categorized as complete (A), partial (B) and none (C) (Trichrome stain, scan power). (D) The difference of capsule formation is statistically significant between scirrhous HCC and classical HCC.

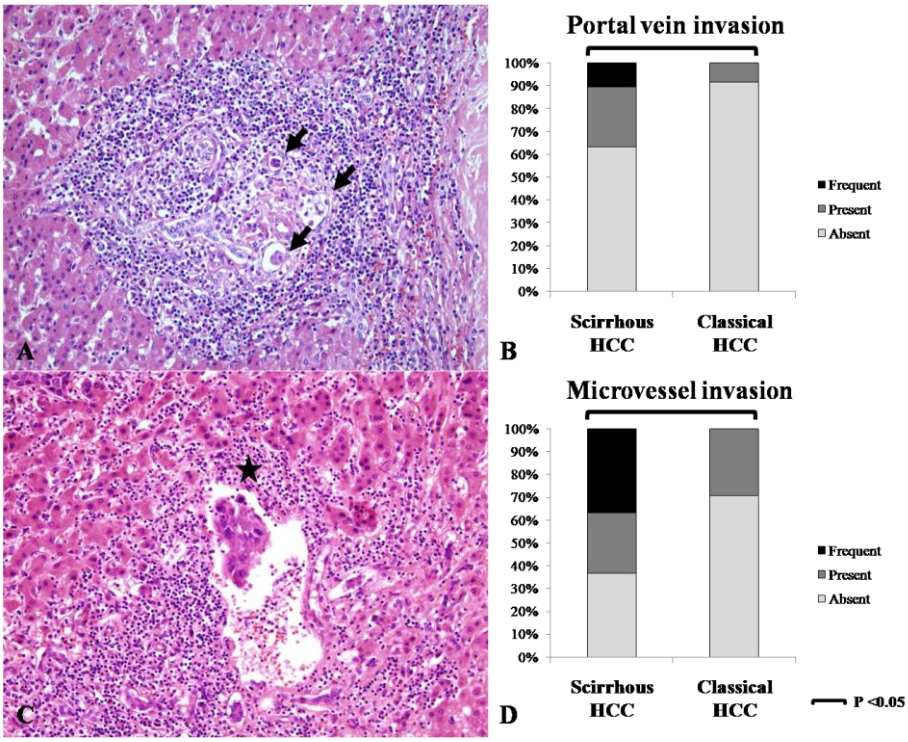


Figure 4. Vascular invasion. (A) Inside the portal tract small portal veins contain tumor cell clusters (arrow) (HE, x200). (B) The portal vein invasion is more frequently observed in scirrhous HCCs than in classical HCCs. (C) The tumor cells are observed in the microvessel (astrix) (HE, x200). (D) The microvessel invasion is more frequently observed in scirrhous HCCs than in classical HCCs.

3. Survival analysis

The disease free survival of patients was gradually worse from classical HCCs, scirrhous HCCs to CCs (p=0.001) (Fig. 5). The mean follow up period was 18 ± 19 (1~88) months. The recurrence rate of scirrhous HCCs was 52.6% and 20.8% in classical HCCs, which showed statistical difference (P=0.030).

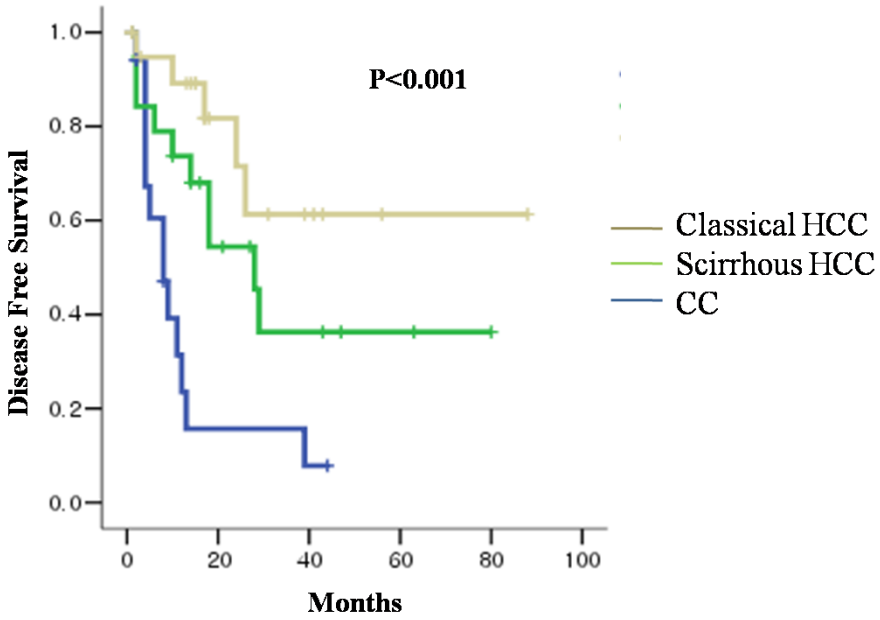


Figure 5. Disease free survival curve. The disease free survival curve indicates that the scirrhous HCCs have a significantly worse outcome than do the classical HCCs (Kaplan-Meier analysis).

4. Liver stem/progenitor cell marker expression

A. The mRNA expression levels of liver stem/progenitor cell markers

The mRNA expression levels of liver stem/progenitor cell markers including K19 (p=0.003), EpCAM (p<0.0001), CD133 (p=0.001), Oct3/4 (p=0.001) and cMET (p=0.004) were significantly high in scirrhous HCCs than in classical HCCs (Fig. 6).

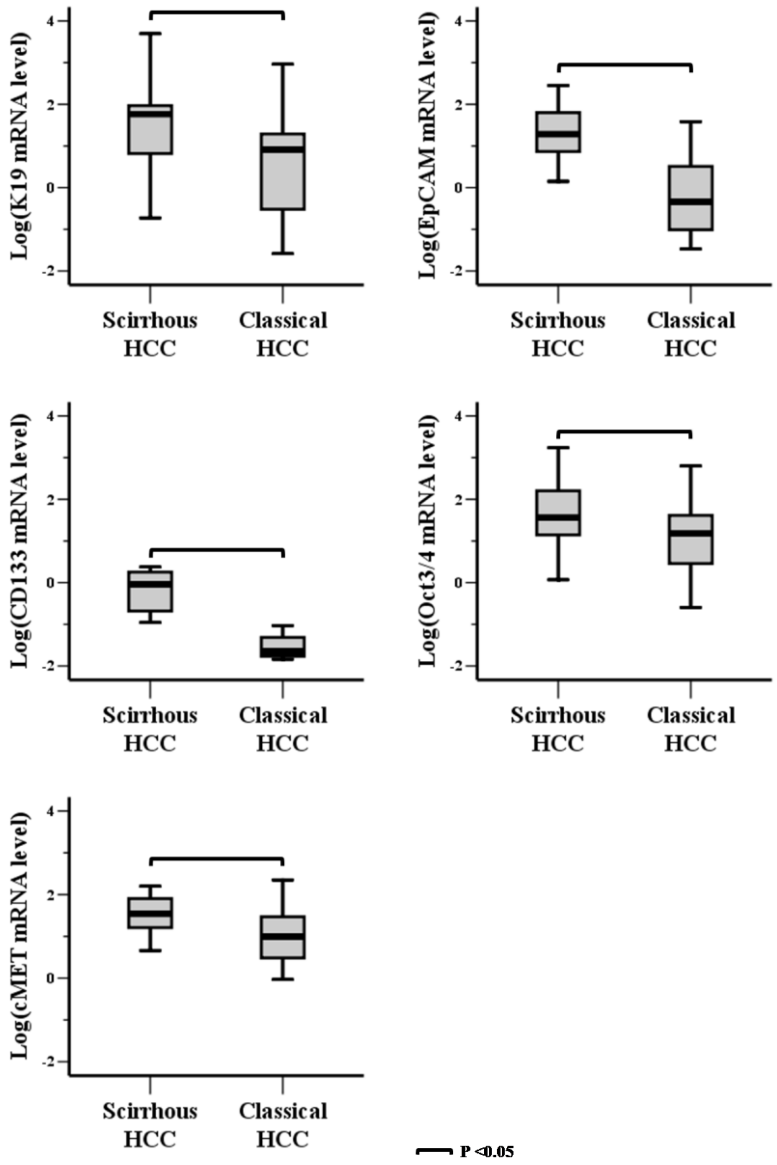


Figure 6. mRNA expression levels of liver stem/progenitor cell markers. The mRNA of liver stem/progenitor cell markers (K19, EpCAM, CD133, Oct3/4 and cMET) are significantly up-regulated in scirrhous HCCs than in classical HCCs.

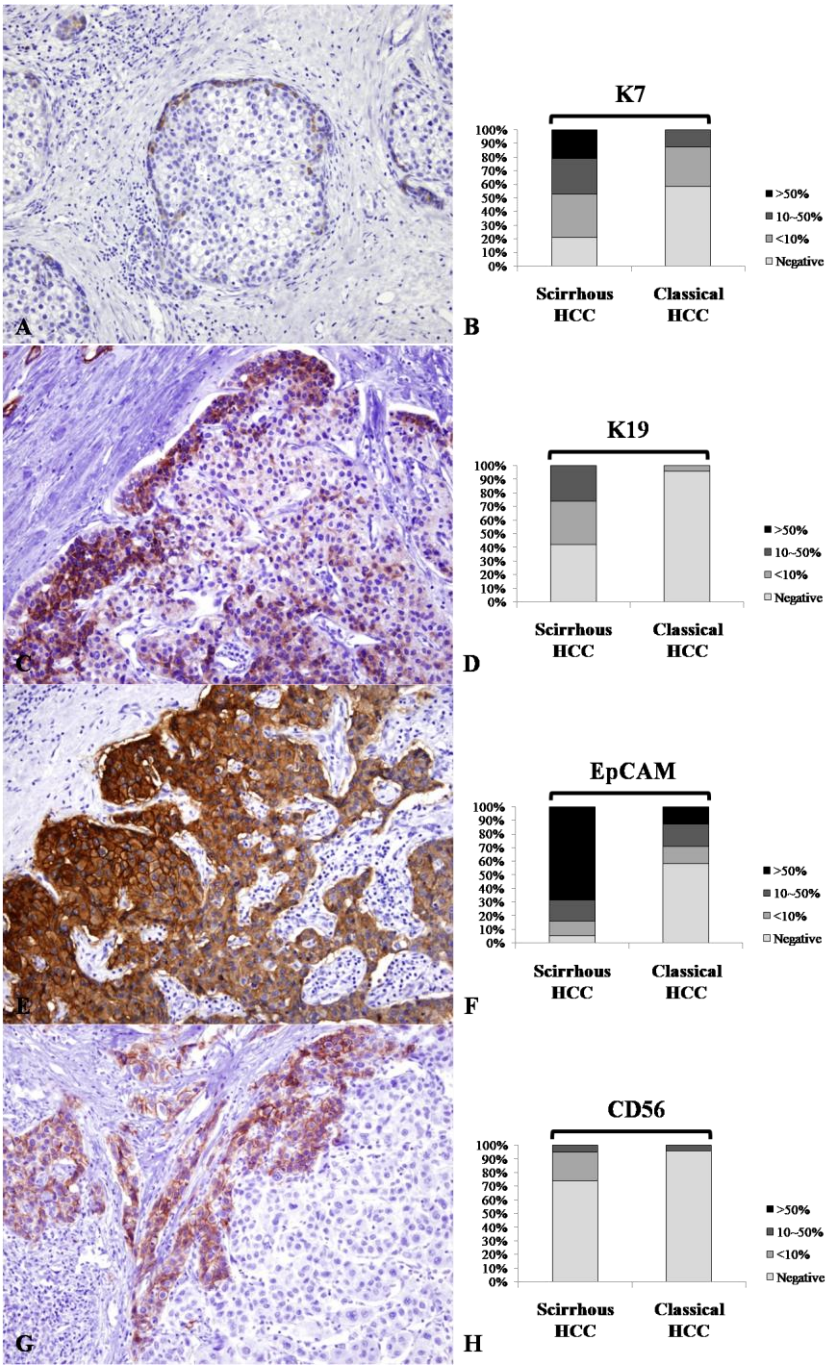
B. The protein expression levels of liver stem/progenitor cell markers

The protein expression rates of liver stem/progenitor cell markers (K7, K19, EpCAM and CD56) were significantly higher in scirrhous HCCs than in classical HCCs (Table 3 and Fig. 7).

Table 3. Protein expression rates of liver stem/progenitor cell markers

		Scirrhous HCC (n=19)	Classical HCC (n=24)	P-value
K7	Negative	4 (21.1%)	14 (58.3%)	0.007*
	<10%	6 (31.6%)	7 (29.2%)	
	10~50%	5 (26.3%)	3 (12.5%)	
	>50%	4 (21.1%)	0 (0%)	
K19	Negative	8 (42.1%)	23 (95.8%)	<0.0001*
	<10%	6 (31.6%)	1 (4.2%)	
	10~50%	5 (26.3%)	0 (0%)	
	>50%	0 (0%)	0 (0%)	
EpCAM	Negative	1 (5.3%)	14 (58.3%)	<0.0001*
	<10%	2 (10.5%)	3 (12.5%)	
	10~50%	3 (15.8%)	4 (16.7%)	
	>50%	13 (68.4%)	3 (12.5%)	
CD56	Negative	14 (73.7%)	23 (95.8%)	0.031*
	<10%	4 (21.1%)	0 (0%)	
	10~50%	1 (5.3%)	1 (4.2%)	
	>50%	0 (0%)	0 (0%)	
AFP	Negative	5 (26.3%)	12 (50%)	0.194
	<10%	8 (42.1%)	7 (29.2%)	
	10~50%	5 (26.3%)	2 (8.3%)	
	>50%	1 (5.3%)	3 (12.5%)	

*P<0.05


A
B
K7
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Scirrhous HCC
Classical HCC
>50%
10~50%
<10%
Negative
C
D
K19
E
F
EpCAM
G
H
CD56

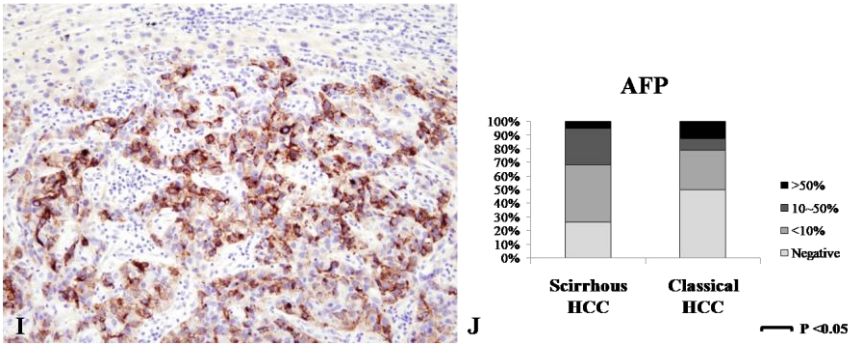


Figure 7. Protein expression of liver stem/progenitor cell markers (K7, K19, EpCAM, CD56 and AFP). (A) The K7 is expressed in cytoplasm of tumor cells characteristically at the periphery of the tumor cell nests of scirrhous HCC. (B) The expression rates of K7 are higher in scirrhous HCCs than in classical HCCs. (C) The tumor cells show strong expression of K19 in cytoplasm. (D) The expression rates of K19 are higher in scirrhous HCCs than in classical HCCs. (E) The EpCAM is strongly expressed in cytoplasm and membrane. (F) The expression rates of EpCAM are higher in scirrhous HCCs than in classical HCCs. (G) The CD56 is expressed in the cytoplasmic membrane. (H) The expression rates of CD56 are higher in scirrhous HCCs than in classical HCCs. (I) The AFP is expressed in cytoplasm of tumor cells with granular pattern. (J) The expression rates of AFP showed no significant difference between scirrhous HCC and classical HCC.

Among the liver stem/progenitor cell markers the EpCAM was most frequently expressed in both scirrhous and classical HCCs. All of the K19-positive tumors and the CD56-positive tumors were immuno-reactive for EpCAM (Fig. 8).

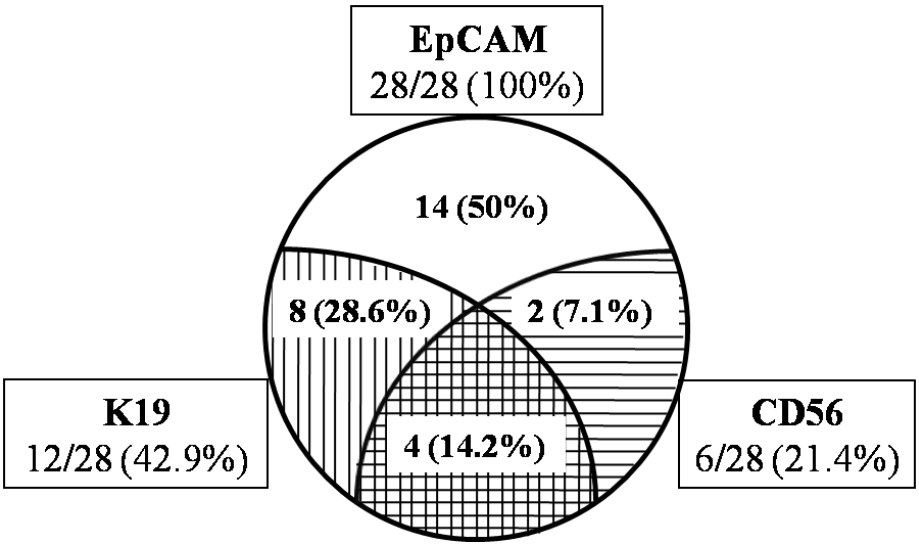


Figure 8. Distribution of liver stem/progenitor cell markers (EpCAM, K19 and CD56) expression in HCCs. In both scirrhous and classical HCCs, the K19-positive tumors and the CD56-positive tumors were immune-reactive for EpCAM.

The mRNA expression levels of liver stem/progenitor cell markers were highly correlated with each other (Fig. 9). The mRNA expression levels of K19 were correlated with EpCAM ($P<0.0001$, $R=0.678$), CD133 ($P<0.0001$, $R=0.856$), Oct3/4 ($P<0.0001$, $R=0.820$). The mRNA expression levels of EpCAM were correlated with CD133 ($P<0.0001$, $R=0.809$) and Oct3/4 ($P<0.0001$, $R=0.581$). The mRNA expression levels of CD133 were correlated with Oct3/4 ($P=0.002$, $R=0.676$). The fibrous stroma area within tumor was correlated with liver stem/progenitor cell markers including K19 ($P=0.003$, $R=0.395$), EpCAM ($P=0.042$, $R=0.280$) and cMET ($P=0.043$, $R=0.293$).

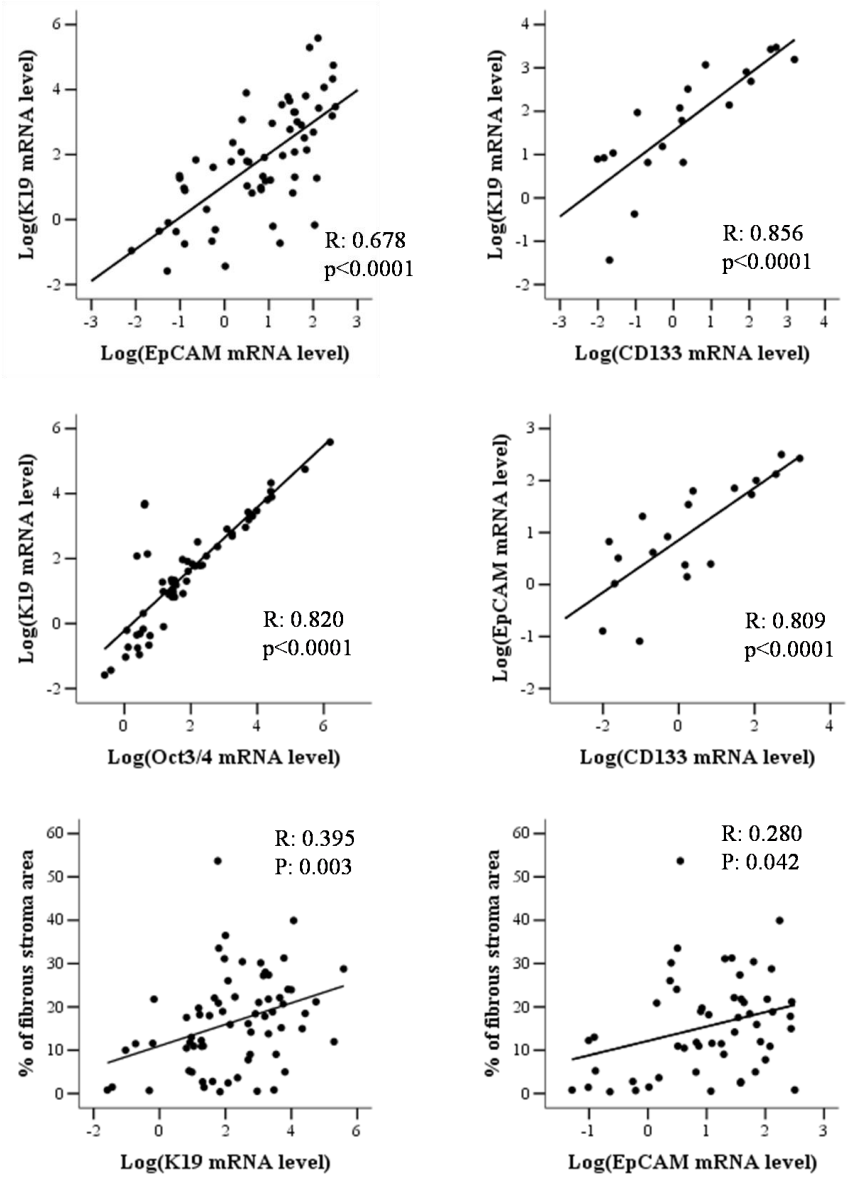


Figure 9. Scatter plots of liver stem/progenitor cells markers and fibrous stroma area within tumor. The liver stem/progenitor cell markers were highly correlated with each other and the fibrous stroma area within tumor was correlated with liver stem/progenitor cell markers.

The expression rate of Hep Par 1, the hepatocytic differentiation marker, was lower in scirrhous HCCs than in classical HCCs ($p<0.0001$) (Table 4 and Fig. 10).

Table 4. Protein expression rates of hepatocytic differentiation marker

		Scirrhous HCC (n=19)	Classical HCC (n=24)	P-value
Hep Par 1	Negative	6 (31.6%)	0 (0%)	<0.0001*
	<10%	7 (36.8%)	0 (0%)	
	10~50%	5 (26.3%)	8 (33.3%)	
	>50%	1 (5.3%)	16 (66.7%)	

*P<0.05

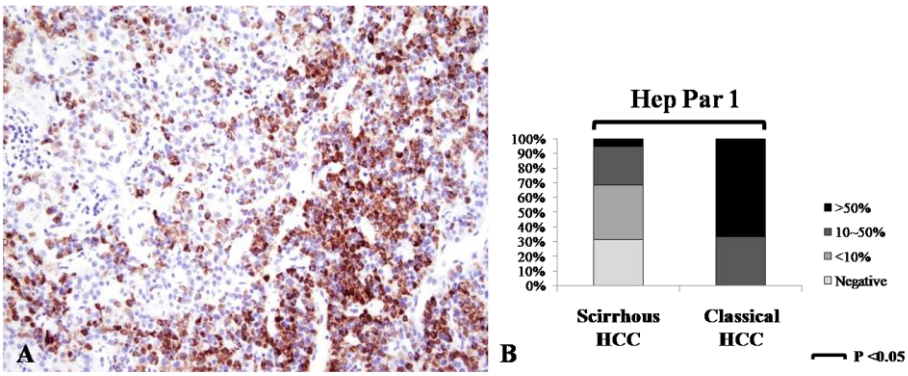


Figure 10. Protein expression of hepatocytic differentiation marker, Hep Par 1. (A) The hepatocytic differentiation marker, Hep Par 1 is expressed in cytoplasm of tumor cells with granular pattern. (B) The expression rates of hepatocytic differentiation marker (Hep Par 1) are significantly lower in scirrhous HCCs than in classical HCCs.

C. The topographical analysis of liver stem/progenitor cell markers and hepatocytic differentiation marker expression using double immunohistochemical staining

The tumor cells were immuno-reactive for liver stem/progenitor cell markers (K7, K19, EpCAM and CD56) and hepatocytic differentiation marker (Hep Par 1) with three distinct patterns (stroma-facing, pseudoglandular and random patterns), demonstrated by double immunohistochemical staining (Fig. 11). The stroma-facing (peripheral) pattern was seen in round solid tumor cell nests. Liver stem/progenitor cell marker-positive tumor cells were arranged along the periphery of the tumor cell nests, facing the scirrhous stroma and Hep Par 1-positive tumor cells were located inside the tumor cell nests. This pattern resembled ductular cells at the periphery of the regenerating hepatic nodule with maturation toward inside of the hepatic lobule. This pattern showed anastomosing cord or chain-like architecture embedded in fibrous stroma. The tumor cell cord end is sharp with acute angle and the end side of the cord was composed of small tumor cells having dark scanty cytoplasm which were immuno-reactive for liver stem/progenitor cell markers including K7, K19, EpCAM and CD56. In the center of the cord, Hep Par 1-positive tumor cells were seen with more mature hepatocytic feature. In the pseudoglandular pattern, liver stem/progenitor cell marker-positive tumor cells were arranged along the pseudoglandular structure, resembling dilated canaliculi. In the random pattern, liver stem/progenitor cell marker-positive tumor cells were admixed with Hep Par 1-positive tumor cells without specific pattern. The stroma-facing (peripheral) pattern is characteristically observed in scirrhous HCCs and the pseudoglandular pattern is mainly observed in classical HCCs (Table 5).

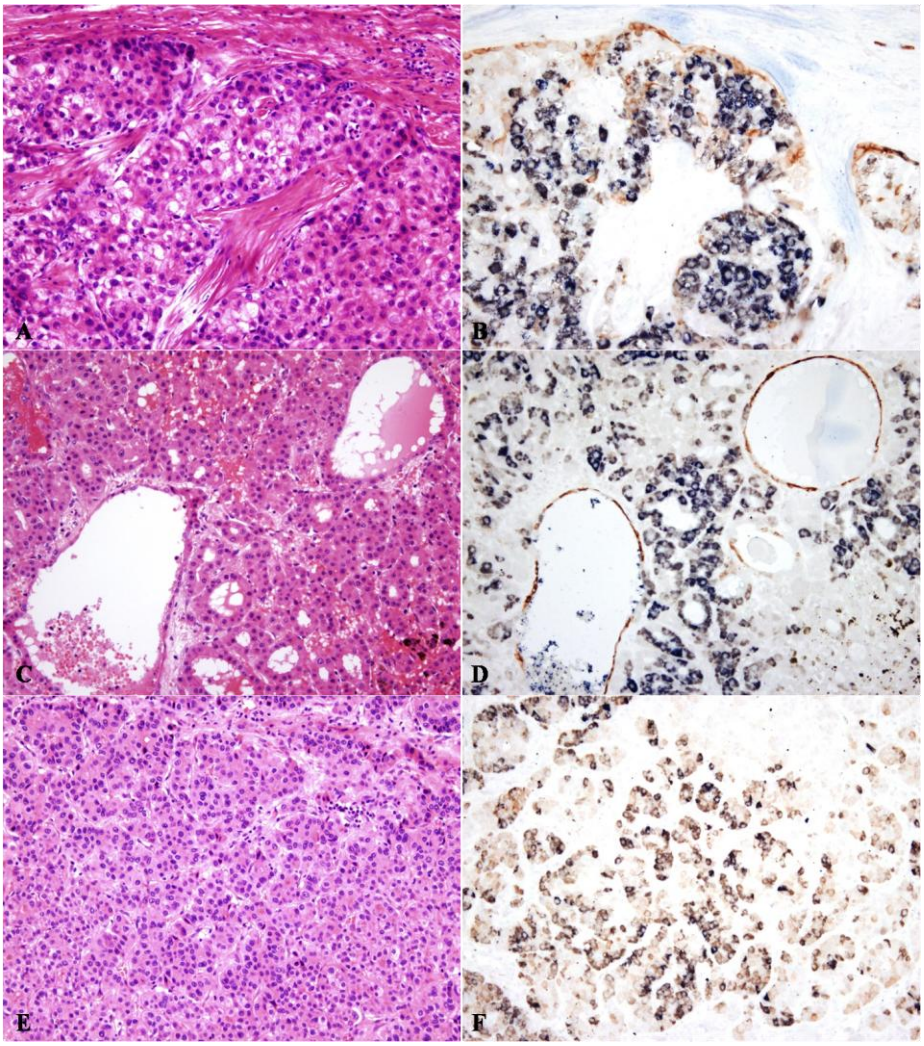


Figure 11. Expression patterns of liver stem/progenitor cell markers and hepatocytic differentiation marker. (A) The scirrhous HCCs show mainly stroma-facing (peripheral) pattern (HE, x200). (B) In stroma-facing (peripheral) pattern K7-positive tumor cells (brown) are arranged at the periphery of the tumor cell nests facing the scirrhous stroma and Hep Par 1-positive tumor cells (blue) are located inside the tumor cell nests. (C) The pseudoglandular pattern is distinct in classical HCCs (HE, x100). (D) In pseudoglandular pattern K7-positive tumor cells (brown) arranged along the pseudoglandular structure of tumor cells. (E) The random pattern does not

show specific histologic character (HE, x100). (F) In random pattern K7-positive tumor cells (brown) are admixed with Hep Par 1-positive tumor cells (blue).

Table 5. Expression rates of liver stem/progenitor cell markers (K7, K19, EpCAM and CD56) and hepatocytic differentiation marker (Hep Par 1) pattern

	Scirrhous HCC (n=19)	Classical HCC (n=24)	P-value
Stroma-facing (peripheral)	9 (47.4%)	2 (8.3%)	0.002*
Pseudoglandular	0 (0%)	6 (25%)	
Random	4 (21.1%)	2 (8.3%)	

*P<0.05

5. TGF-β signal

A. The mRNA and protein expression levels of TGF-β signaling pathway molecules

The mRNA expression levels of TGF-β signaling pathway molecules including TGF-β ($p<0.0001$), TGFβRI ($p<0.0001$), TGFβRII ($p=0.006$) and Smad4 ($p<0.0001$) were significantly high in scirrhous HCCs than in classical HCCs (Fig. 12).

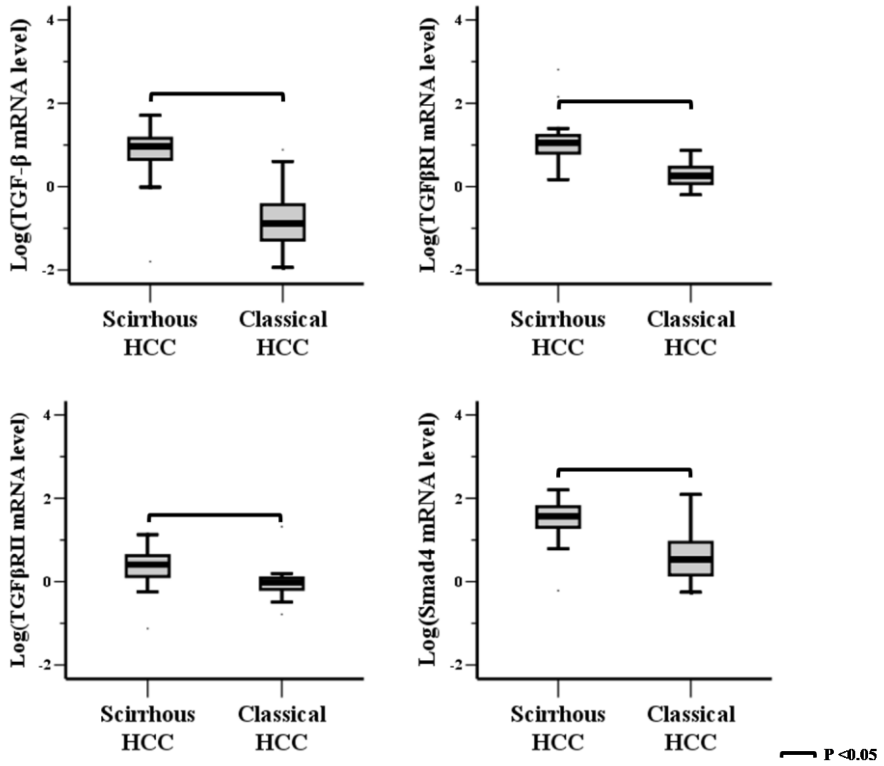


Figure 12. mRNA expression levels of TGF-β signaling pathway molecules. The mRNA of TGF-β signaling pathway molecules (TGF-β, TGFβRI, TGFβRII and Smad4) are significantly up-regulated in scirrhous HCCs than in classical HCCs.

The protein expression rate of Smad4 was significantly higher in scirrhous HCCs than in classical HCCs. The difference between scirrhous HCCs and classical HCCs was statistically significant (p=0.017) (Table 6 and Fig. 13).

Table 6. Protein expression rates of Smad4

		Scirrhous HCC (n=19)	Classical HCC (n=24)	P-value
Smad4	Negative	2 (10.5%)	7 (29.2%)	0.017*
	<10%	5 (26.3%)	13 (54.2%)	
	10~50%	8 (42.1%)	3 (12.5%)	
	>50%	4 (21.1%)	1 (4.2%)	

*P<0.05

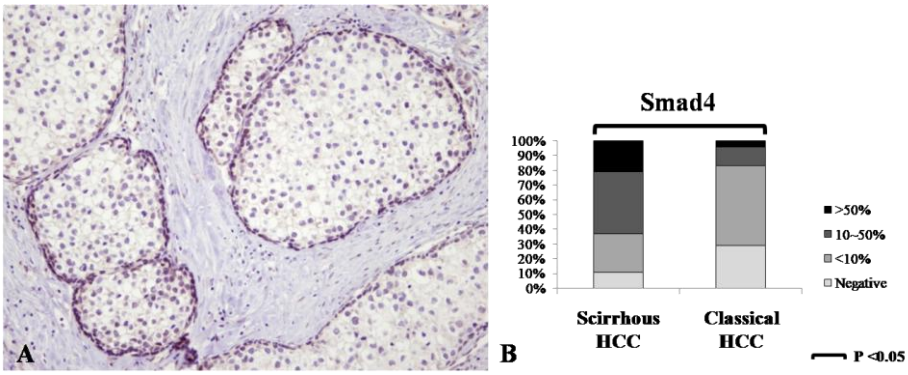
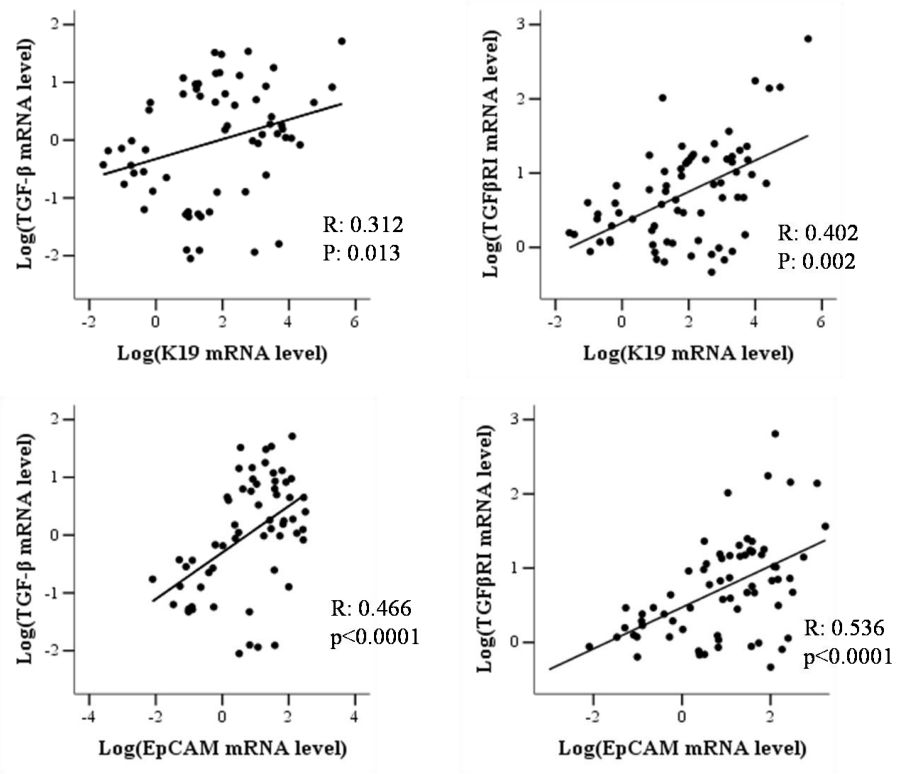


Figure 13. Protein expression of Smad4. (A) The Smad4 is expressed in nucleus and cytoplasm of tumor cells. (B) The expression rates of Smad4 are higher in scirrhous HCCs than in classical HCCs.

The mRNA expression levels of TGF-β signaling pathway molecules were highly correlated with liver stem/progenitor cell markers (Fig. 14). The mRNA expression levels of TGF-β were correlated with K19 (P=0.013, R=0.312), EpCAM (P<0.0001, R=0.466) and cMET (p<0.0001, R=0.569). The mRNA expression levels of TGFβRI were correlated with K19 (P=0.002, R=0.402), EpCAM (P<0.0001, R=0.536), Oct3/4 (P=0.002, R=0.426) and cMET (P<0.0001, R=0.637). The mRNA expression levels of TGFβRII were correlated with cMET (P<0.0001, R=0.509). The mRNA levels of Smad4 were correlated with EpCAM (P=0.003, R=0.378) and cMET (P<0.0001, R=0.637). The fibrous stroma area within tumor was correlated with TGF-β (P<0.0001, R=0.475) and TGFβRI (P=0.015, R=0.338).



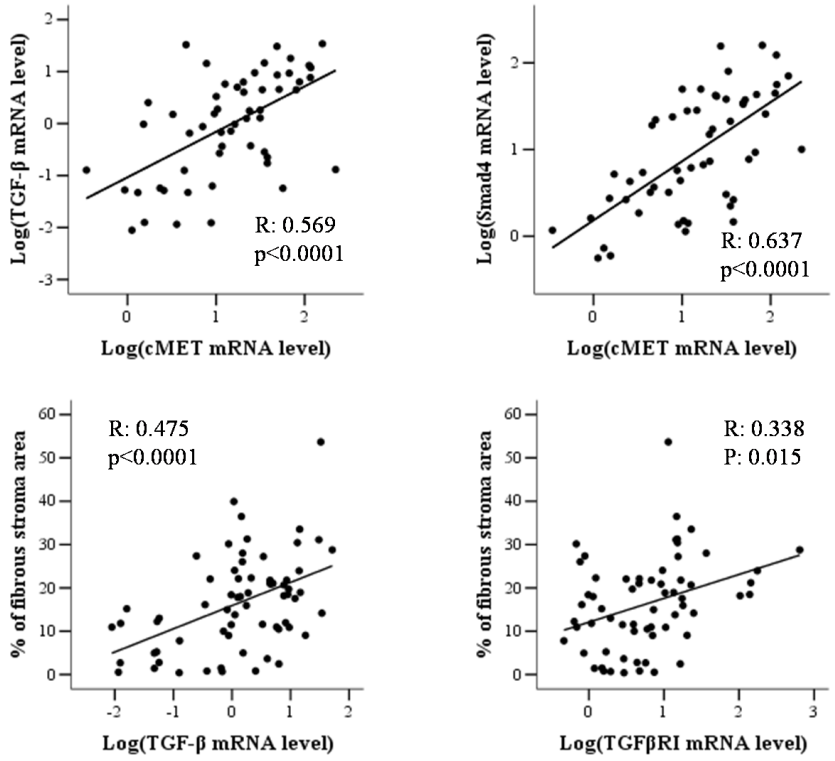


Figure 14. Scatter plots of TGF-β signaling pathway molecules with liver stem/progenitor cells markers and fibrous stroma area within tumor. The TGF-β signaling pathway molecules were highly correlated with liver stem/progenitor cell markers and fibrous stroma area within tumor.

6. EMT-related genes

A. The mRNA expression levels of EMT-related genes

The mRNA expression levels of EMT-related genes including Snail ($p<0.0001$) and Twist ($p=0.015$) were significantly high in scirrhous HCCs than in classical HCCs (Fig. 15).

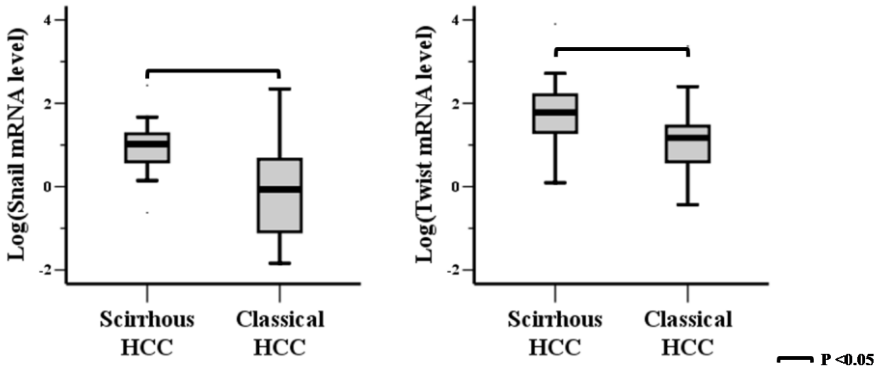


Figure 15. mRNA expression levels of EMT-related genes. The mRNA of EMT related genes (Snail and Twist) are significantly up-regulated in scirrhous HCCs than in classical HCCs.

B. The protein expression levels of EMT-related genes

The protein expression rates of Snail were significantly higher in scirrhous HCCs than in classical HCCs. The difference between scirrhous HCCs and classical HCCs was statistically significant (p=0.006) (Table 7 and Fig. 16). The protein expression loss of E-cadherin was not statistically significant between scirrhous HCC and classical HCC (Table 7 and Fig. 17).

Table 7. Protein expression rates of EMT-related genes (Snail and E-cadherin)

		Scirrhous HCC (n=19)	Classical HCC (n=24)	P-value
Snail	Negative	6 (31.6%)	18 (75%)	0.006*
	<10%	10 (52.6%)	6 (25%)	
	10~50%	3 (15.8%)	0 (0%)	
	>50%	0 (0%)	0 (0%)	
E-cadherin	<75% (reduced)	15 (78.9%)	22 (91.7%)	0.226
	>75% (preserved)	4 (21.1%)	2 (8.3%)	

*P<0.05

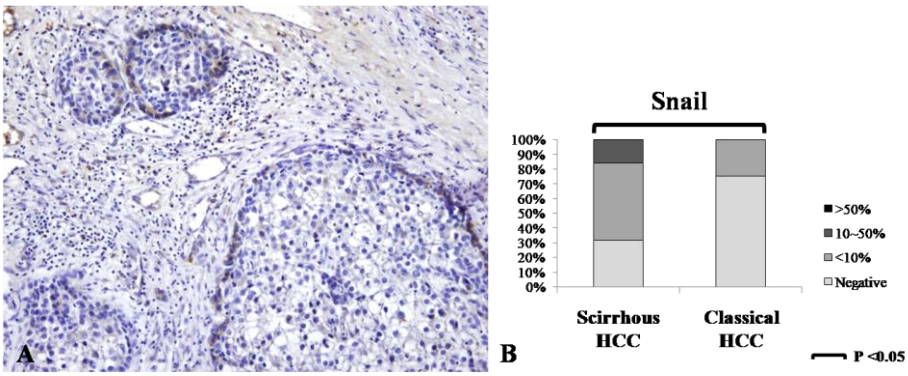
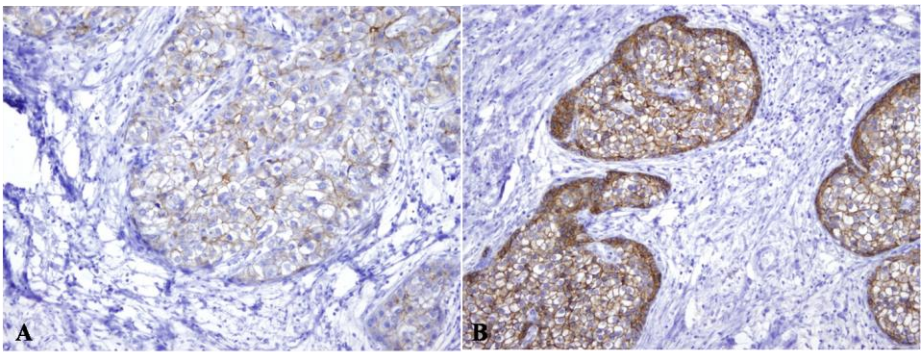
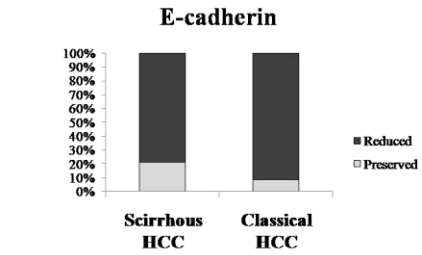


Figure 16. Protein expression of Snail. (A) The Snail is expressed in nucleus and cytoplasm of tumor cells. (B) The expression rates of Snail are higher in scirrhous HCCs than in classical HCCs.





C

Figure 17. Protein expression loss of E-cadherin. (A) In reduced pattern the E-cadherin is expressed in less than 75% of tumor cells. (B) In preserved pattern the E-cadherin is expressed in more than 75% of tumor cells. (C) The difference of expression loss rate of E-cadherin is not statistically significant.

The mRNA expression levels of EMT-related genes (Snail and Twist) were highly correlated with liver stem/progenitor cell markers (Fig. 18). The mRNA expression levels of Snail were correlated with EpCAM ($P<0.0001$, $R=0.475$) and cMET ($P<0.0001$, $R=0.614$). The mRNA expression levels of Twist were correlated with K19 ($P<0.0001$, $R=0.895$), EpCAM ($P<0.0001$, $R=0.652$), CD133 ($P<0.0001$, $R=0.749$) and Oct3/4 ($P<0.0001$, $R=0.915$). The mRNA expression levels of EMT-related genes (Snail and Twist) were highly correlated with TGF-β signaling pathway molecules (Fig. 19). The mRNA expression levels of Snail were correlated with TGF-β ($P<0.0001$, $R=0.670$), TGFβRI ($P<0.0001$, $R=0.618$), TGFβRII ($P<0.0001$, $R=0.473$) and Smad4

(P<0.0001, R=0.684). The mRNA expression levels of Twist were correlated with TGF-β (P=0.042, R=0.268) and TGFβRI (P=0.001, R=0.428). The fibrous stroma area within tumor was correlated with Snail (P=0.008, R=0.384).

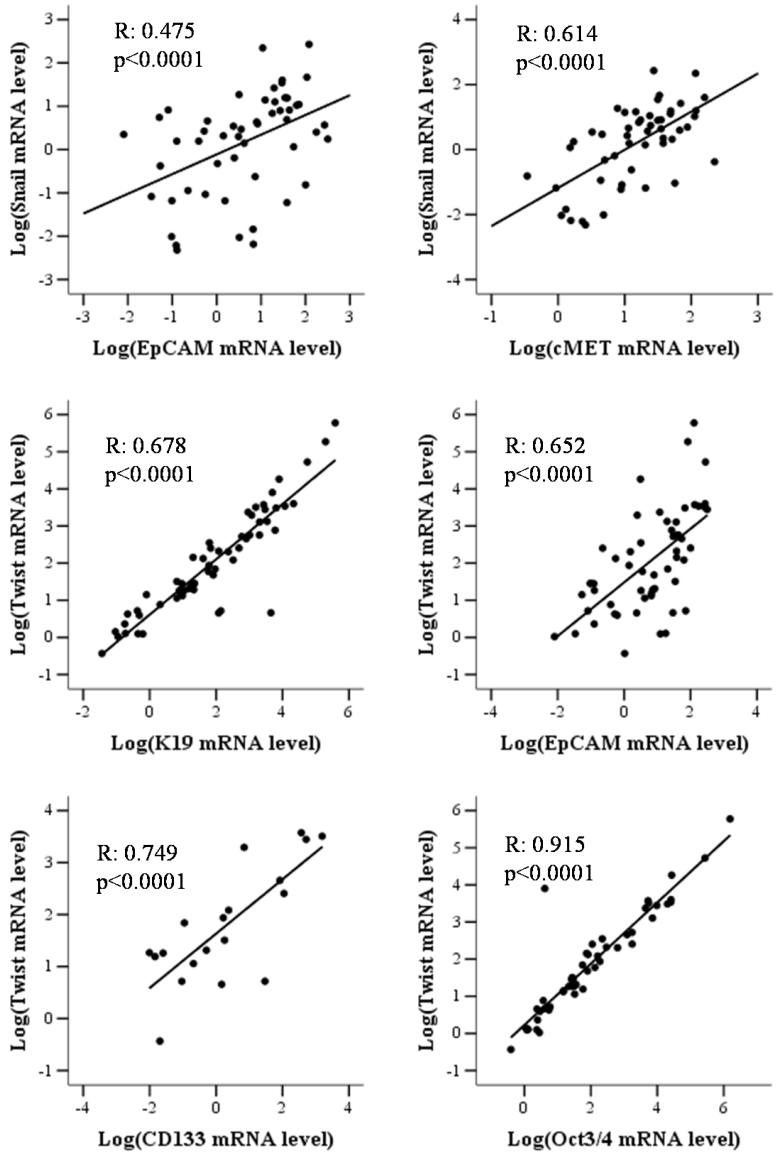


Figure 18. Scatter plots of EMT-related genes (Snail and Twist) and liver stem/progenitor cells markers. The EMT-related genes (Snail and Twist) were highly correlated with liver stem/progenitor cell markers (EpCAM, K19, CD133, Oct3/4 and cMET).

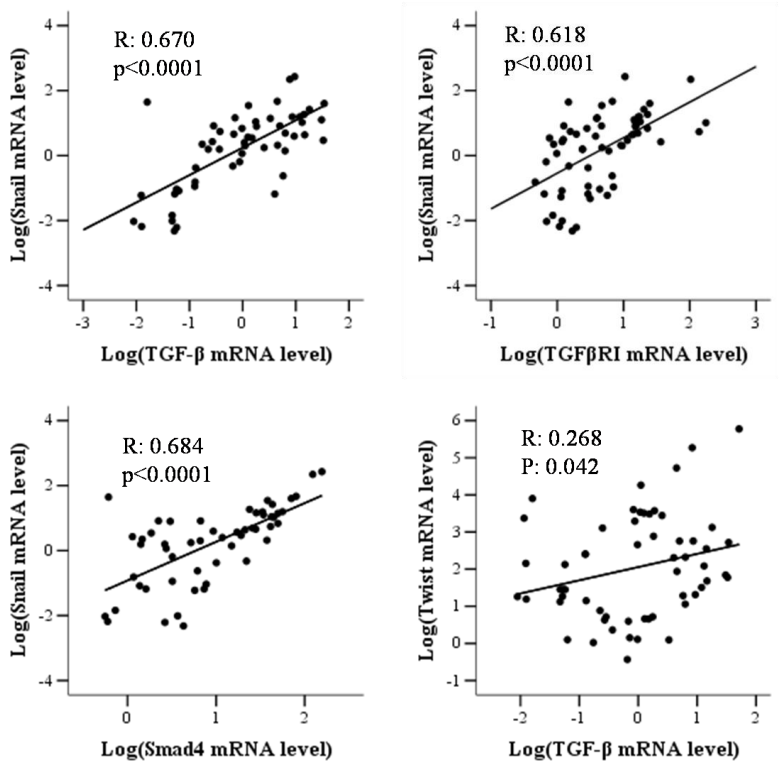


Figure 19. Scatter plots of EMT-related genes (Snail and Twist) and TGF-β signaling pathway molecules. The EMT-related genes (Snail and Twist) were highly correlated with TGF-β signaling pathway molecules.

C. The topographical analysis of liver stem/progenitor cell markers and Snail expression using double immunohistochemical staining

The snail protein expression showed a characteristic pattern, resembling the stroma-facing (peripheral) pattern of stem/progenitor cell markers. The snail-positive tumor cells were located along the periphery of the tumor cell nests and formed cord or chain-like structure. The Snail-positive tumor cells co-expressed liver stem/progenitor cell markers (K19 and EpCAM). This pattern was predominantly seen in scirrhous HCCs (P=0.020) (Table 8 and Fig. 20).

Table 8. Co-expression rate of Snail and liver stem/progenitor cell markers

	Scirrhous HCC (n=19)	Classical HCC (n=24)	P-value
Co-expression with stem/progenitor cell marker	9 (47.4%)	4 (16.7%)	0.020*
No co-expression	4 (21.1%)	2 (8.3%)	

*P<0.05

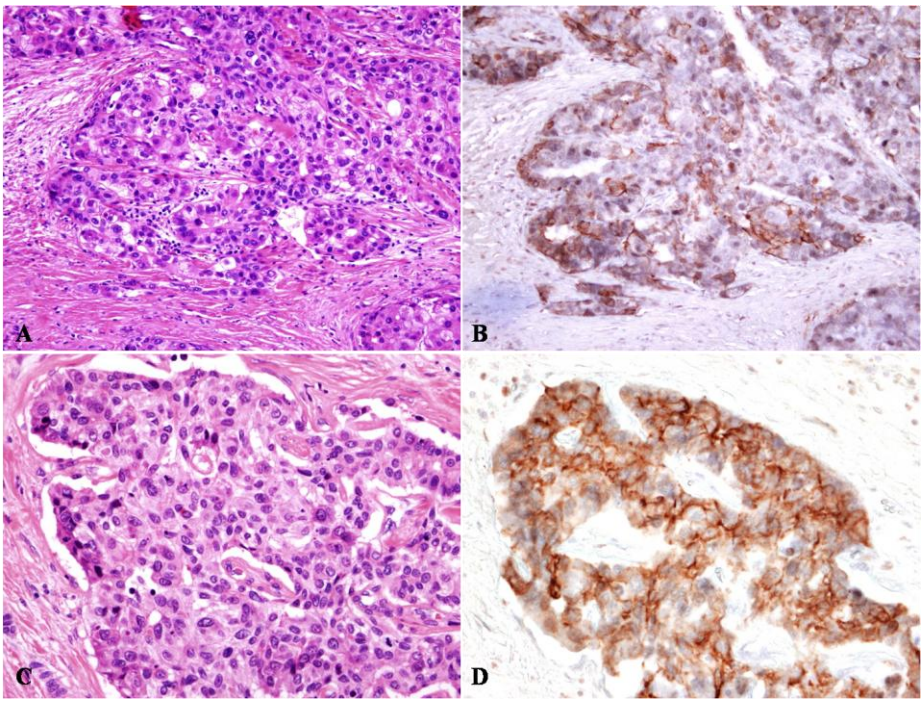


Figure 20. Co-expression of Snail and liver stem/progenitor cell markers (EpCAM and K19). (A) The scirrhous HCCs show mainly co-expression of Snail and liver stem/progenitor cell markers (HE, x200). (B) The EpCAM-positive tumor cells (brown) are located at the periphery of tumor cell nests and the Snail is co-expressed (blue). (C) The periphery of the tumor cell nests are arranged with small tumor cells with dark scant cytoplasm (HE, x400). (D) The K19-positive tumor cells (brown) are co-expressed with Snail (blue).

IV. Discussion

According to WHO classification the scirrhous HCC is a histological pattern which shows abundant fibrous stroma between tumor cell trabeculae[1], but the definition of scirrhous HCCs was vague and the lower cut value of scirrhous area within tumor showed difference from 25% to 50%[3, 56]. Some investigators evaluated the degree of fibrosis compared to the maximal tumor dimension[3, 56] and in another study the scirrhous area was evaluated with morphometry with Masson trichrome staining[5, 6]. In this study the scirrhous area was evaluated grossly at first and selected the cases which had more than 60% of scirrhous area within tumor and then the fibrotic area compared to tumor cell area was calculated with morphometry using Masson Trichrome staining. For the definition of scirrhous HCC, it is necessary to include following criteria, 1) no preoperative treatment such as transcatheter arterial embolization or percuatneous ethanol injection therapy, which reportedly results in secondary sclerotic changes, 2) to exclude fibrolamellar subtype, and 3) the scirrhous area, more than 60% compare to greatest dimension of tumor.

The previously reported data about the prognosis of scirrhous HCC are variable. Kurogi et al. reported that the overall survival rate of scirrhous HCC was significantly higher, but the recurrence-free survival rate was not significantly different compared to classical HCCs[3]. Matuura et al. reported that there was no significant difference between the survival curves[6]. On the other hand, Kim et al. reported that scirrhous HCCs showed absence of tumor capsule and more frequent portal vein invasion and it tended to be higher tumor recurrence rate and lower survival rate[56]. And Okamura et al. reported that MMP-7 was more frequently expressed in scirrhous HCC than in classical HCCs[5]. MMP-7 has a proteolytic effect of extracellular matrix and it has been known to be a poor prognostic indicator[57]. Fujii et al. demonstrated prognostically heterogeneous groups within scirrhous HCCs and one of them

showed poor prognosis and the other two showed favorable prognosis[4].

The possible reasons for the variable prognostic data are considered as follows. The definition of scirrhous HCC showed difference from study to study. Most of the previous studies were limited to clinical data without pathological prognostic indicator such as vascular invasion. Or the scirrhous HCC might be genuinely heterogeneous group to have biologically different behavior. One possible mechanism of variable prognosis is the diverse TGF-β effect on tumors. TGF-β can suppress tumor growth and also can promote tumor progression and metastasis[36]. In this study scirrhous HCCs showed high expression of EMT-related genes, Snail and Twist, which are late signature of TGF-β signal. Fujii et al. reported the close relation of the scirrhous HCC and TGF-β expression but in their study the EMT was not evident[4]. The distinct mechanism of switching the TGF-β signal from tumor suppressor to promoter is not clearly demonstrated yet. Liver cancer-derived hepatitis C virus core protein and different thresholds of Smad3 activation are some of the switching factor of TGF-β signal from tumor suppression to EMT[58].

In this study scirrhous HCC showed high expression of liver stem/progenitor cell markers including K7, K19, EpCAM, CD56, CD133, Oct3/4 and cMET, and low expression of hepatocytic differentiation marker Hep Par 1. These results are consistent with the most recent previous study[4]. But Kurogi et al. reported that all of the scirrhous HCCs were negative for K19[3] and Matsuura et al. reported that the immunoreactivity of K7 in scirrhous HCCs was significantly higher than classical HCCs but K19 showed no difference[6].

HCCs with liver stem/progenitor cell marker expression have been subclassified as a poor prognostic group in many studies and in these tumors various signals are up-regulated including TGF-β signal[12]. Candidate for cancer stem cells were reported to produce TGF-β[59, 60].

Yamashita et al. demonstrated that EpCAM-positive tumor cells were

located at the invasion border zones and were disseminated at the invasive front[21, 22] and Fujii et al. reported that liver stem/progenitor cell markers and TGF-β were observed at the periphery of the tumor cell nest facing the fibrous stroma[4]. In this study that the liver stem/progenitor cell markers and hepatocytic differentiation marker, Hep Par 1 expression showed three unique patterns, stroma-facing (peripheral), pseudoglandular and random pattern. The stroma-facing (peripheral) pattern reminded bile ductular structures embedded in fibrous connective tissue, differentiating to mature hepatocytes in diseased livers like cirrhosis. The pseudoglandular pattern was specific for classical HCCs and the lumen resembled dilated canaliculi in which bile is collected. Maeda et al. reported the K7 expression is related to pseudoglandular architecture[61]. In advance of these findings it is demonstrated that the tumor cells facing the fibrous stroma were frequently expressed Snail, the key molecule of EMT with liver stem/progenitor cell markers at the same time. Recently Mani et al. reported that EMT is closely associated with stem cell properties in mammary epithelial cells and carcinoma[62].

Recently the concept of mixed hepatobiliary carcinoma has been strongly suggested with increasing evidence. This implies that within one primary hepatic carcinoma diverse morphological variation of HCCs, CCs and tumor cells having intermediate features can be observed. Even among the experts of liver pathology the reproducibility of pathologic diagnosis of primary hepatic carcinoma is poor especially when the tumor has fibrous stroma[63]. The most typical form is known as combined HCC and CC which shows foci of typical HCC and foci of typical CC[64]. Intermediate carcinoma is composed of nests of small oval cells with high nuclear:cytoplasmic ratio and hyperchromatic nuclei[65]. The cholangiolocellular carcinoma shows small tumor cells growing in small cords and nests embedded in a fibrous stroma resembling ductular reaction[66]. It has been reported to be strongly related to liver stem/progenitor cells[66] and whether the bipotent hepatobiliary stem cells are the cell of origin

or the tumors undergo dedifferentiation is still debating[10]. In this study the HCC producing abundant fibrous stroma which showed tumor cell nests and trabeculae embedded in fibrous stroma having mostly mature hepatocytic tumor cells inside the nests and peripheral stroma-facing arranged small tumor cells with distinct immuno-reactivity for liver stem/progenitor cell markers (K7, K19, EpCAM and CD56) were described. According to the previous description of WHO classification this tumor should be called scirrhous HCC but the finding of this study implies more things beyond the mere scirrhous HCC. The tumor cells have definite HCC feature at the inside of the tumor cell nests. But the abundant fibrous stroma, small tumor cells expressing liver stem/progenitor cell markers, less frequent tumor capsule formation, more frequent vascular invasion and more aggressive biologic behavior are not typical features of ordinary HCCs. These finding are more CC-like feature in the histological, phenotypical and biological aspects (Fig. 21). If the primary hepatic carcinomas are arranged in a row as a spectrum from HCC with most hepatocytic differentiation to CC with most cholangiocytic differentiation, scirrhous HCCs in this study might be put in the center of the row between HCC and CC.

V. Conclusion

The scirrhous HCC showed more CC-like features than classical HCC in the following aspects: abundant fibrous stroma, less frequent capsule formation, more frequent vascular invasion (portal vein and microvessel) and intrahepatic metastasis, and worse disease free survival.

In scirrhous HCCs, the liver stem/progenitor cell markers including K7, K19, EpCAM, CD56, CD133, Oct3/4 and cMET were highly expressed, and the hepatocytic differentiation marker, Hep Par 1 was low-expressed.

The scirrhous HCC showed activation of TGF-β signal than classical HCC, which is the possible mechanism of abundant fibrous/scirrhous stroma within

tumor.

The scirrhous HCC showed elevated mRNA expression of Snail and Twist, the EMT-related genes, than classical HCC. The tumor cells expressing liver stem/progenitor cell markers were coexpressed with Snail, the key molecule of EMT.

The aggressive biologic behavior of scirrhous HCC showing less frequent capsule formation, more frequent vascular invasion and intrahepatic metastasis and worse disease free survival rate might be related to the activation of TGF-β signal and EMT related genes.

The HCC producing abundant fibrous stroma, so-called scirrhous HCC might be categorized as a distinct subgroup of HCC.

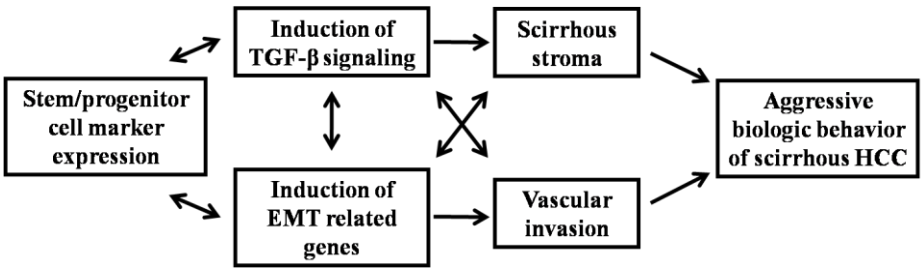


Figure 21. Schematic concept of this study. In scirrhous HCCs the liver stem/progenitor cells markers expression is associated with TGF-β signal and EMT-related genes. The histologic feature of abundant scirrhous stroma is related to the high expression of TGF-β signaling pathway molecules. The frequent vascular invasion of scirrhous HCCs in this study might be associated with up-regulation of EMT related genes. The scirrhous HCCs with stem/progenitor cell feature showed distinct histologic features and biologically aggressive behavior, possibly resulting from the high expression of TGF-β signaling and EMT.

V. References

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ABSTRACT (IN KOREAN)

경화성 간세포암종에서 간줄기세포 표지자, 전환성장인자 베타 신호 체계, 상피간엽전환 관련 유전자 발현

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최근 암줄기세포가 암종의 불량한 예후 및 치료 저항성에 관여하며, 상피간엽전환 (epithelial mesenchymal transition)이 암줄기세포의 발생을 촉진함이 보고되었다. 또한 상피간엽전환은 종양세포의 혈관 침입 및 전이에 중요하며, 전환성장인자 베타 (transforming growth factor-β, TGF-β)가 상피간엽전환에 중요한 유전자로 알려져 있다. 전형적인 간세포암종은 섬유성 간질조직의 형성이 거의 없는 것이 특징이고, 종양세포 사이에 섬유성 간질의 형성이 풍부한 조직학적 소견을 보이는 경우 경화성 간세포암종이라 하며, 그 임상병리학적 의의는 아직 확실히 알려져 있지 않다. 본 연구에서는 경화성 간세포암종의 병리학적 특성을 밝히고자, 수술전 치료없이 절제된 경화성 간세포암종 19예를 대상으로 임상병리학적 소견, 간줄기세포 표지자 [EpCAM, CD133, keratin 19 (K19), K7, CD56, alpha-fetoprotein (AFP)], 간세포분화 표지자 (Hep Par 1), TGF-β 신호 물질(TGF-β, TGF-β Receptor I, TGF-β Receptor II, Smad4)과 상피간엽전환 관련 유전자 (Snail, Twist)의 발현을 실시간 역전사 중합효소연쇄반응 및 면역조직화학염색으로 검색하여 전형적인 간세포암종 (24예)과 비교 검색하였다.

경화성 간세포암종은 전형적인 간세포암종에 비하여 섬유성 간질조직 형성 ($p<0.0001$), 종양세포의 미세혈관 침범 ($p=0.004$) 및 문맥혈관침범 ($p=0.047$), 간내 전이 ($p=0.044$)가 의의있게 높았으며, 종양피막의 형성 ($p<0.0001$)은 더 낮았다. 간줄기세포 표지자 (EpCAM, K19, K7, CD56, AFP)에 대한

면역염색 결과 경화성 간세포암종의 26.3~94.7% 및 전형적인 간세암종의 4.2~50%에서 양성발현을 보여, 경화성 간세포암종에서 간줄기세포 표지자의 발현이 의의 있게 높았다 ($p<0.05$). 또한 경화성 간세포암종에서는 EpCAM, K19, CD133, Oct3/4, cMET의 mRNA발현이 전형적인 간세포암종에 비하여 의의있게 높았다 ($p<0.05$). 반면 간세포 분화의 표지자인 Hep Par 1는 경화성 간세포암종에서 68.4%, 전형적인 간세포암종에서 100% 발현되어, 경화성 간세포암종에서 더 낮게 발현되었다 ($p<0.05$). TGF-β 신호 물질 (TGF-β, TGF-β Receptor I, TGF-β Receptor II, Smad4) 및 상피간엽전환 관련 유전자 (Snail, Twist)의 mRNA발현은 모두 경화성 간세포암종에서 전형적인 간세포암종 보다 의의 있는 증가를 보였다 ($p<0.05$). 이중면역조직화학염색으로 간줄기세포 표지자와 Snail의 발현양상을 검색하였다. 간줄기세포 표지자는 섬유성 간질과 맞닿은 종양세포 군집의 변연부에서 발현하는 경향을 보였으며, 대부분의 경우 (69%)에서 상피간엽전환의 핵심 인자인 Snail과 함께 발현되었다. 환자의 추적관찰 결과 경화성 간암종은 전형적인 간세포암종에 비하여 의의있게 높은 재발율을 보였다 ($P=0.030$).

이상의 소견으로 경화성 간세포암종은 간줄기세포 표지자의 발현, 상피간엽전환 및 TGF-β 신호의 발현증가로 침습적인 병리생물학적 특성 및 불량한 예후를 보이는 것으로 생각되며, 이러한 특성을 근거로 경화성 간세포암종은 단순히 간세포암종의 조직학적 소견의 하나가 아니라, 간세포암종의 특징적인 아형으로 분류하는 것이 좋을 것으로 생각한다.

핵심되는 말: 경화성 간세포암종, 간줄기세포 표지자, 전환성장인자 베타 신호 체계, 상피간엽전환