Transcriptional Repressor Snail and Progression of Human Hepatocellular Carcinoma[1]

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ABSTRACT

Purpose: **Snail protein is a suppressive transcriptional factor of E-cadherin that mediates cell-to-cell adhesion, tumor progression, and metastases. We explored the expression and function of Snail and its family member Slug in human hepatocellular carcinoma (HCC) to identify its role in tumor progression.**

Experimental Design and Results: **Transfection of Snail cDNA in Li-7, endogenous E-cadherin-positive human HCC cells, selectively induced the loss of E-cadherin protein expression. We then investigated the expression of Snail and Slug mRNA in 43 human tissue samples of HCC. Using *in situ* hybridization, Snail mRNA was determined to dominantly express in HCC cells, but not in bile duct cells, blood vessels or infiltrating leukocytes. The mRNA of Snail and Slug were quantified using real-time reverse transcriptase-PCR, and correlations with E-cadherin expression and clinicopathological factors were investigated. Snail mRNA was overexpressed in 7 cases (16%) of HCC compared with adjacent noncancerous liver tissue. E-Cadherin protein expression determined in the same 43 cases by immunohistochemistry was significantly down-regulated in those cases with Snail mRNA overexpression ($P = 0.04$). The tumor and nontumor ratio of Snail mRNA independently correlated with tumor invasiveness ($P = 0.04$). However, Slug mRNA correlated with neither E-cadherin expression nor tumor invasiveness.**

Conclusions: **The data indicate that Snail both down-regulates E-cadherin expression and promotes the invasion in human HCC.**

INTRODUCTION

HCC[3] is the third most common malignant tumor in Japan and shows relatively poor prognosis and rapid progression (1, 2). Intrahepatic recurrence, the main cause of the poor prognosis, depends on the potential for invasiveness of the primary HCC (3, 4). A general consensus is that portal vein or capsular invasion is one definition of tumor invasiveness in HCC (4, 5), but the mechanisms through which HCC acquires such invasive potentials are not well understood (6).

E-Cadherin-mediated cell-to-cell adhesion plays a critical role in the maintenance of cell polarity and environment (7, 8). E-Cadherin was reported to be down-regulated and closely related to tumor invasion and metastasis in HCCs and other cancers (7). Genetic and epigenetic alteration of E-cadherin was also reported (7). Somatic mutation, loss of heterozygosity of the *E-cadherin* gene, and CpG methylation around the promoter region of the *E-cadherin* gene were noted in human gastric cancer, breast cancer, and HCC (9–13). However, E-cadherin promoter hypermethylation is not always associated with loss of expression (13), and evidence has been presented that E-cadherin expression could be repressed by mechanisms other than promoter hypermethylation (14). The heterogeneity and reversibility of E-cadherin protein expression are both controversial areas (7).

Recently, the Snail transcription factor was reported to directly repress E-cadherin expression in many epithelial cancers associated with epithelial-mesenchymal transitions (15, 16). The zinc finger transcription factor Snail was first identified in *Drosophila* and is essential for mesoderm formation (17). *Drosophila* embryos homozygous for Snail mutations fail to develop a mesoderm layer and die early in embryogenesis (18), and there is an association with impaired down-regulation of E-cadherin (19). In *Drosophila* and mouse gastrulation, Snail is expressed in ectodermal epithelial cells during transition into mesenchymal cells (18, 20). Reverse correlation of Snail and E-cadherin expression has been noted in cultured malignant cells of melanomas, squamous cell carcinomas, and HCCs (21–23). Recently, Hajra *et al.* (24) reported that Slug, also a zing-finger protein and a Snail family member, is a likely repressor of E-cadherin in breast cancer cell lines. However, we can find

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; EGFP, enhanced green fluorescent protein; Ab, antibody; TRITC, tetramethylrhodamine isothiocyanate; DIG, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRES, internal ribosome entry site; HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 1 Clinicopathological features of 43 HCCs

	Well[a] (n = 6)	Mod (n = 23)	Poor (n = 14)	Overall (n = 43)
Mean age (yr) (mean ± SD)	64.5 ± 5.8	65.8 ± 10.5	59.8 ± 9.2	63.8 ± 9.8
Men/women	2/4	18/5	11/3	31/12
Tumor size (cm)	2.6 ± 1.3	4.0 ± 2.0	6.5 ± 3.7	4.6 ± 2.9
Virus marker				
HBV (+)	0	1	4	5
HCV (+)	6	15	9	30
HBV (+) and HCV (+)	0	2	1	3
Noncancerous liver tissue				
Normal or mild fibrosis	0	2	1	3
Fibrosis	1	11	8	20
Precirrhosis	0	3	1	4
Cirrhosis	5	7	4	16
Capsule formation	4	21	13	38
Capsular invasion	1	20	13	34
Portal vein invasion	1	13	9	23
Hepatic venous invasion	0	1	5	6
Bile duct invasion	0	2	3	5
Intrahepatic metastasis	0	10	8	18

[a] Well, well-differentiated HCC; mod, moderately differentiated HCC; poor, poorly differentiated HCC.

no documentation regarding the expression of Snail or Slug in human HCC tissue.

In this study, we investigated whether Snail represses E-cadherin expression in human HCC cells. The levels of expression and the localization of Snail and Slug mRNA were detected in a series of human HCC samples, and correlations between Snail/Slug expression and clinicopathological factors were analyzed. Our evidence suggests that Snail, rather than Slug, may contribute to both E-cadherin expression and to the progression of HCCs.

MATERIALS AND METHODS

Subcloning of Human Snail and Slug cDNA and Construction of Expression Plasmids. The full coding region of human *Snail* (GenBank accession no. NM005985) was amplified by PCR using primers (5′-ACTATGCCGCGCTCTTTCCT-3′ and 5′-AGTCCTGTGGGGCTGATGTG-3′) from cDNA of human HCC. The PCR product was cloned into the pCRII vector (Invitrogen, Carlsbad, CA) that contains the priming sites of SP6 and T7 transcriptional factors. The full coding region of human *Slug* (GenBank accession. no. BC014890) was amplified by PCR using primers (5′-GCTGTAGGAACCGCCGTGTC-3′ and 5′-ATTTGTCATTTGGCTTCGGAGTG-3′) from cDNA of human HCC, and the product was cloned into the pT7Blue vector (Novagen, Madison, WI). Isolated DNA sequences were determined using a cycle sequencing procedure, as described previously (8). Snail cDNA was then subcloned into the bicistronic expression vector pIRES2-EGFP (Clontech, Palo Alto, CA), which allows for translation of both the genes of interest and the EGFP.

Cell Culture and Transient Transfection of Snail. Li-7 cells, a cultured human HCC cell line, were supplied from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). These cells were cultured at 37°C in 5% CO_2 in RPMI 1640 (Life Technologies, Inc., Rockville, MD), containing 10% FBS (Life Technologies, Inc.). Li-7 cells (1×10^6) were grown in 3.5-cm dishes and transiently transfected with 2 μg of the pIRES2-Snail-EGFP plasmid, as well as the empty pIRES2-EGFP (mock) plasmid using Lipofectamine (Life Technologies, Inc.), according to the manufacturer's instructions. At 72 h after transient transfection, Snail-transfected cells, which expressed both Snail and EGFP, were confirmed by epiluminescence fluorescence microscopy (Axioscop2, Zeiss, Germany) and then processed for fluorescent immunohistochemistry.

Fluorescent Immunohistochemical Staining of E-Cadherin. Cells were fixed using 4% paraformaldehyde for 30 min at 4°C, then incubated with primary Ab against E-cadherin (Transduction Laboratories, Lexington, KY) diluted 1:500 in 0.05% Saponin/PBS (Sigma Chemical, St. Louis, MO) for 90 min at 25°C. Cells were then incubated with TRITC-conjugated secondary Ab to mouse IgG (ICN Pharmaceuticals, Aurora, OH) for 30 min at 25°C. The samples were visualized by epiluminescence fluorescence microscopy with appropriate filter combinations. Snail or mock-transfected cells were visualized with EGFP (green), and E-cadherin expression was investigated using a TRITC-labeled (red) secondary Ab. These two fluorescences were synthesized using an *in situ* imaging system (Meta Systems, Altlussheim, Germany).

Patients. This present retrospective study was based on data obtained using surgically resected tissues from 43 consecutive Japanese patients who underwent hepatectomy for primary HCCs. Written informed consent was obtained from each patient before tissue acquisition. All data were collected in the Department of Anatomical Pathology, Graduate School of Medicine of Kyushu University (Fukuoka, Japan) from July 1995 to July 1999. Clinicopathological features of these 43 patients are summarized in Table 1. All tumors were defined as HCC, and pathological features of the tumors were determined histologically based on classifications of the Liver Cancer Study Group of Japan (25). Other pathological features such as portal vein invasion, capsular invasion, and bile duct invasion were also defined histologically. Histological grades of the tumors consisting of more than two features were defined by the most

prominent feature, and those components were selected for immunohistochemical studies.

Riboprobe Preparation and *in Situ* Hybridization of Snail. The pCRII-Snail vector was linearized with restriction enzymes that cut the multiple cloning site in two orientations, and this served as a template for *in vitro* transcription of antisense or sense (negative control) riboprobes, using T7 and Sp6 RNA polymerase (Boehringer-Mannheim, Mannheim, Germany), respectively. The cRNA transcripts were labeled using a DIG RNA Labeling Kit (Boehringer-Mannheim) according to the manufacturer's instructions. The *in vitro* transcription method was monitored by control plasmids pSPT18 and pSPT19 (Boehringer-Mannheim).

Fresh frozen samples of HCC were embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA), cut in 5-μm sections, and mounted on silanated slides. Sections were rehydrated, digested with proteinase K (1 μg/ml), and then fixed in 4% paraformaldehyde/PBS. Acetylation was done in 0.1 M triethanolamine in 0.25% acetic anhydride for 15 min. Slides were hybridized at 42°C for 16 h in 100 μl of hybridization buffer containing 50% formamide, 10% dextran sulfate, 1 μg/μl sonicated salmon sperm DNA, 1 μg/μl yeast total RNA, 1 μg/μl BSA, 2× SSC [300 mM NaCl, 30 mM sodium citrate (pH 7.2)] and 100 ng/ml DIG-labeled RNA probe. The posthybridization washes were as follows: twice at 42°C for 1 h in 50% formamide and 2× SSC; 5 min in NTE buffer [0.5 M NaCl in 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA] at 37°C; 30 min in NTE buffer containing 20 μg/ml RNase A (Sigma Chemical) at 37°C; 15 min in NTE buffer; and 20 min three times in 0.1× SSC at 42°C. Sections were incubated with a 1:5000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (sheep polyclonal, Fab fragments; Boehringer-Mannheim) for 30 min at room temperature. After several washes in PBS, sections were incubated in 150 μl of detection buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer-Mannheim) for 36 h at room temperature. Finally, the color reaction was stopped by washing the sections for 5 min in 10 mM Tris (pH 8) and 1 mM EDTA, then the sections were counterstained with 1% methylene green. *In situ* hybridization was done twice using both antisense and sense probes to confirm the results.

Real-Time Quantitative RT-PCR of Snail and Slug. Total RNA was extracted and purified from 43 paired samples of fresh frozen cancerous tissues and noncancerous liver tissues using Trizol Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. For reverse transcriptase reaction, we used 5 μg of the RNA, random hexamers, and Superscript II reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instructions. The oligonucleotide primers and TaqMan probes designed for Snail and Slug were as follows: Snail (5′-ACCACTATGCCGCGCTCTT-3′ and 5′-GGTCGTAGGGCTGCTGGAA-3′); Slug (5′-TGTTGCAGTGAGGGCAAGAA-3′ and 5′-GACCCTGGTTGCTTCAAGGA3′); and TaqMan probe (Snail, 5′-6FAM-TCGTCAGGAAGCCCTCCGACCC-TAMRA-3′ and Slug, 5′-6FAM-AGGCTTCTCCCCCGTGTGAGTTCTAATG-TAMRA-3′). Each primer was placed in a different exon to avoid amplification of contaminating genomic DNA. Primers and probe for GAPDH (TaqMan GAPDH control reagent kit) were purchased from Perkin-Elmer Applied Biosystems (Foster City, CA).

Real-time quantitative PCR was done using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems), as described previously (26). Briefly, each PCR mixture contained 1 μl of cDNA, TaqMan Universal PCR master mix (Perkin-Elmer Applied Biosystems), primer pair, and TaqMan probe in a final volume of 50 μl. The PCR conditions were an initial denaturation step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles consisting of 15 s at 95°C, and a 1 min at 60°C. Serial 1:10 dilutions of plasmid DNA (pCRII-Snail and pT7Blue-Slug) were analyzed for each target cDNA, and these served as standard curves from which we determined the rate of change of threshold cycle values. The amount of target gene expression was calculated from the standard curve, and quantitative normalization of Snail and Slug cDNA in each sample was done using GAPDH as an internal control. Real-time PCR assays were done in triplicate, and the mean values were used for calculations of mRNA expression. Finally, the Snail and Slug mRNA expression ratios for tumorous (T) and nontumorous (N) tissues were calculated as follows: R = [Snail or Slug (T) / GAPDH (T)] / [Snail or Slug (N) / GAPDH (N)] × 102. Cases were designated as either overexpression ($R > 100$) or nonoverexpression ($R \leq 100$) cases.

Immunohistochemical Staining of E-Cadherin. Formalin-fixed, paraffin-embedded tissue sections from 43 HCC cases that corresponded to the RNA extracted cases were processed for immunohistochemical staining, as described previously (27). A primary monoclonal Ab against E-cadherin (diluted 1:1000; Transduction Laboratories) was used. Positive immunoreactivity of normal bile duct epithelium was confirmed as a positive control for each specimen (28).

Immunohistochemical staining was examined under a light microscope by two pathologists (Keis. S. and K. T.). The cell membranous staining of E-cadherin was evaluated semiquantitatively, and tumors were divided into two groups: (*a*) preserved pattern: >75% of tumor cells showed equivalent membranous staining to adjacent normal bile duct epithelium and (*b*) reduced pattern: <75% of tumor cells showed membranous staining, as described elsewhere (27).

Statistical Analysis. Comparisons between Snail/Slug expression levels (R; >100 or ≤100) and E-cadherin expression patterns were evaluated using χ^2 test, and comparisons between the Snail/Slug expression ratios and clinicopathological parameters were evaluated using Student's t or Welch test. P of <0.05 was considered to have statistical significance.

RESULTS

Ectopic Expression of Snail to Down-Regulate E-Cadherin Expression in Human HCC Cells. The effect of Snail expression was first investigated using human HCC cells. We used a bicistronic expression vector pIRES2-EGFP (Clontech) that carries the IRES of the encephalomyocarditis virus. The expression cassette contains a single promoter, which, in combination with the IRES, allows for translation of the genes of interest and the EGFP from the same mRNA. The schematic maps of pIRES2-Snail-EGFP (Snail) and pIRES-EGFP (mock) are shown in Fig. 1*a*. Under fluorescence microscopy, Snail

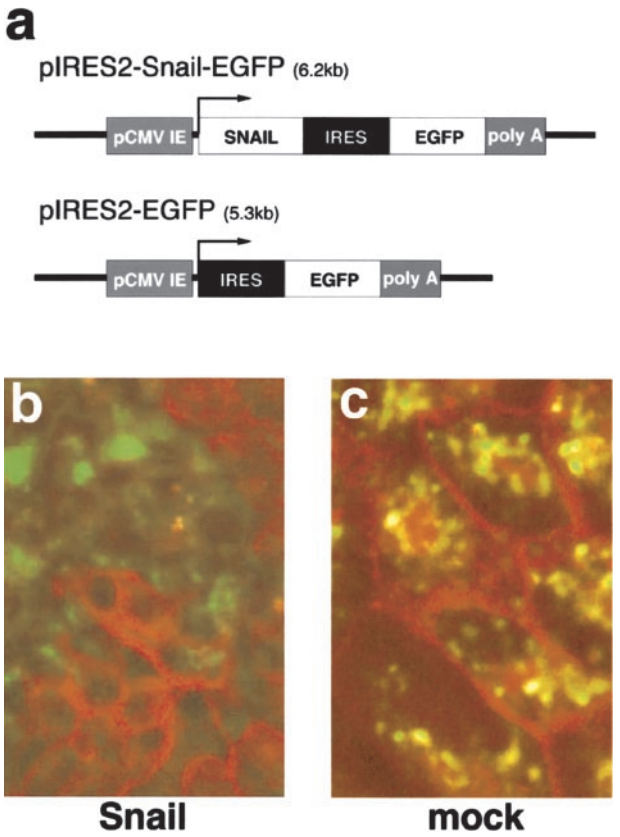

Fig. 1 Construction and expression of Snail and EGFP proteins. *a*, schematic drawings of pIRES2-Snail-EGFP and pIRES2-EGFP expression vectors. The pIRES2-Snail-EGFP expression plasmid was obtained by insertion of the entire Snail coding sequence into the pIRES2-EGFP vector, upstream of the IRES and the EGFP coding region. *b* and *c*, fluorescent immunohistochemistry of E-cadherin in Snail transfected (*b*) and mock-transfected (*c*) Li-7 cells. E-Cadherin (*red*) was negative for Snail transfected cells (*b*), whereas E-cadherin was positive for mock-transfected cells (*c*). Original magnification, ×200.

expression cells were distinguished using a green filter set because pIRES2-Snail-EGFP-transfected cells expressed EGFP (green). On the other hand, E-cadherin/TRITC (red) expression was detected by immunofluorescence using a rhodamine filter set. Because the human HCC cell line Li-7 carries endogenously functional E-cadherin protein (29) and expresses quite low amounts of Snail mRNA (data not shown), we used Li-7 cells to determine the effects of the transient expression of Snail. In Fig. 1*b*, the green fluorescent color indicates Li-7 cells transfected with pIRES2-Snail-EGFP, whereas the red fluorescent color indicates the protein expression of E-cadherin. Snail transfectants showed a remarkably reduced expression of E-cadherin protein, whereas positive E-cadherin expression was observed in nontransfected Li-7 cells. On the other hand, E-cadherin expression was homogeneously preserved in mock-transfected cells (Fig. 1*c*). These observations provided direct evidence that Snail repressed E-cadherin expression in human HCC cells.

Snail mRNA Localization in Human Tissue Samples of HCC. The expression and localization of Snail mRNA were determined in human tissue samples of HCC using *in situ* hybridization. Frozen samples of HCC tissues were hybridized with the DIG-labeled Snail RNA probe and incubated with alkaline phosphatase-conjugated anti-DIG antibody followed by incubation with detection buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. As shown in Fig. 2, positive signal expression was seen exclusively in HCC carcinoma cells and not in bile duct cells, blood vessels, or even infiltrating leukocytes, using the antisense probe (Fig. 2*a*). No signals with a sense probe indicated the specificity of detection (Fig. 2*b*). Additional examination revealed hepatocytes to be the dominant cells expressing Snail mRNA in human adult liver tissues.

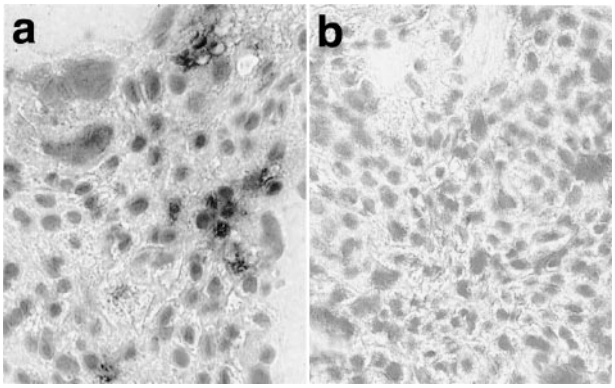

Fig. 2 Representative RNA *in situ* hybridization on a frozen section of HCC with reduced E-cadherin pattern using DIG-labeled cRNA probes coding for human Snail. *a*, an antisense cRNA probe was used (magnification, ×125). *b*, a sense cRNA probe was used (magnification, ×270). Intense staining of HCC cells was observed only when using the antisense probe.

Snail and Slug mRNA Expression in Human Tissue Samples of HCC. We quantified the copy numbers of Snail and Slug mRNA in 43 pairs of HCC tissue and noncancerous liver tissues using a TaqMan probe on ABI Prism 7700 Sequence Detection System, as described previously (26). The copy number of Snail, Slug, and GAPDH mRNA ranged from 133.16 to 16985, 227.36 to 91871, and 12684.07 to 6439090, respectively. Snail and Slug expression were standardized using the expression of the GAPDH housekeeping gene as the internal control. The cancerous (T)/noncancerous (N) ratio of mRNA (R) was then calculated to determine Snail and Slug mRNA levels in each case.

Snail mRNA levels in cancerous tissue ranged from 0.892 to 67.9 (mean ± SE: 14.6 ± 2.6) and that of noncancerous tissue from 3.17 to 158 (mean ± SE: 45.6 ± 5.5). The ratio (R) of Snail ranged from 0.02 to 664 (mean ± SE: 69.2 ± 20.2). Seven (16%) of 43 samples examined were defined as cases overexpressing Snail mRNA; all these samples were from men and had a mean tumor size of 3.0 ± 0.9 cm. There were 1 well, 5 moderately, and 1 poorly differentiated HCCs. The clinicopathological features of the 7 Snail overexpression cases were as follows: 5 showed capsular formation with capsular invasion; 5 portal vein invasion; 1 hepatic venous invasion; and 2 bile duct invasion. The HBV- and HCV-associated viral causes were HBV(+) in one case and HCV(+) in 5 cases. The adjacent

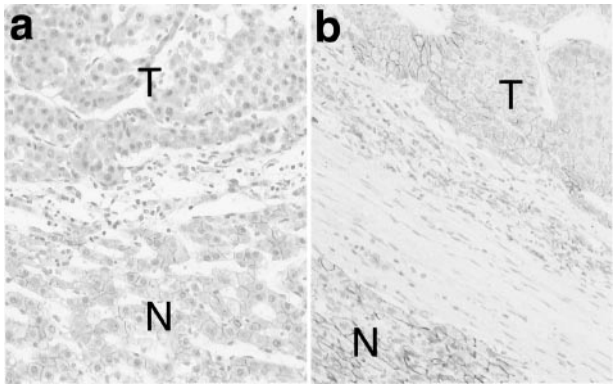

Fig. 3 Representative example of the E-cadherin expression patterns, determined by immunohistochemistry. *a*, carcinoma cells showed diffuse strong membranous expression (preserved pattern) in the Snail nonoverexpression case. *b*, carcinoma cells showed weak membranous expression (reduced pattern) in the Snail overexpression case. T, tumorous area; N, nontumorous area.

noncancerous liver tissues of these 7 were hepatic fibrosis in 4, precirrhosis in 1, and portal cirrhosis in 2.

Slug mRNA levels were from 1.767 to 368 (mean ± SE: 53.6 ± 11.4) in cancerous tissue and from 7.29 to 179 (mean ± SE: 60.5 ± 7.20) in noncancerous tissue. The ratios (R) of Slug ranged from 2.34 to 1374 (mean ± SE: 162 ± 44.1). Fifteen (35%) of 43 examined samples were defined as cases overexpressing Slug mRNA. There were 3 well, 7 moderately, and 5 poorly differentiated HCCs. The clinicopathological features of the 15 Slug mRNA overexpression cases were as follows: 12 showed capsular formation with capsular invasion; 6 with portal vein invasion; 2 with hepatic venous invasion; and bile duct invasion was nil. There was no statistical correlation between mRNA level of Snail and that of Slug.

E-Cadherin Protein Expression in Human HCC Tissue Samples with or without Snail/Slug mRNA Overexpression. Expression of E-cadherin protein was also analyzed immunohistochemically. Although membranous expression of E-cadherin was preserved in all of the adjacent noncancerous hepatocytes, 16 of 43 HCCs (37%) had a reduced expression pattern (Fig. 3). These findings did not significantly correlate with clinicopathological features such as histological differentiation, capsular invasion, portal vein invasion, and bile duct invasion. The relationship between Snail/Slug mRNA expression and E-cadherin protein expression patterns was then determined in the HCC samples. Snail mRNA overexpression significantly correlated with E-cadherin reduced expression (Table 2). Five (71%) of 7 cases overexpressing Snail showed a reduced E-cadherin expression pattern, whereas only 11 of 36 cases of Snail nonoverexpression (31%) had a reduced pattern, with a statistically significant difference ($P = 0.04$). However, there was no significant correlation between Slug overexpression and E-cadherin expression (Table 2).

Snail and Slug mRNA Expression and Clinicopathological Features. The relationship between Snail mRNA expression ratios and clinicopathological features is summarized in Table 3. The mean Snail mRNA ratio was significantly higher in cases of capsular invasion (73.2 *versus* 18.3, $P = 0.04$; Table 3, Fig. 4*a*). Cases of portal vein and bile duct invasion also had high Snail mRNA ratios compared with the cases without invasion, although there was no statistical significance because of the broad distribution of the ratio [98.2 *versus* 35.8 ($P = 0.10$), 188 *versus* 53.6 ($P = 0.14$), respectively; Table 3, Fig. 4*b*]. Among the 7 Snail overexpression cases, 5 cases (71%) showed portal vein invasion and 2 (29%) showed bile duct invasion, whereas there were only 18 (50%) with portal vein invasion and 3 (8%) with bile duct invasion in 36 cases of Snail nonoverexpression. In addition, 4 showed remarkably high Snail mRNA levels ($R > 200$), and these were all advanced HCCs with portal vein invasion (Fig. 4*b*). The relationship between the Slug mRNA expression ratio and clinicopathological features is summarized in Table 3. There was no statistical significance of Slug expression on clinicopathological parameters. Because both Slug overexpression and E-cadherin reduction did not correlate with clinicopathological features, the Snail transcriptional re-

Table 2 Comparison of Snail and Slug expression between preserved and reduced patterns of E-cadherin

	Preserved pattern ($n = 27$)	Reduced pattern ($n = 16$)	P
Snail mRNA			
Overexpression ($n = 7$)	2 (29)	5 (71)	
Nonoverexpression ($n = 36$)	25 (69)	11 (31)	0.04
Slug mRNA			
Overexpression ($n = 15$)	11 (73)	4 (27)	
Nonoverexpression ($n = 28$)	16 (57)	12 (43)	0.30

Table 3 Comparison of clinicopathological variables dependent on Snail and Slug mRNA ratios

	Snail mRNA (mean ± SE)	P	Slug mRNA (mean ± SE)	P
Differentiation				
Mod and poor[a]	74.8 ± 23.2		170 ± 51.1	
Well	35.0 ± 20.5	0.21	110 ± 19.2	0.64
Tumor size				
≥3.0 (cm)	80.0 ± 29.8		184 ± 65.0	
<3.0	50.0 ± 16.6	0.36	122 ± 36.3	0.51
Capsule formation				
(+)	67.4 ± 22.3		176 ± 49.5	
(−)	82.7 ± 44.1	0.81	61.6 ± 15.9	0.41
Capsular invasion				
(+)	73.2 ± 24.8		186 ± 55.1	
(−)	18.3 ± 10.1	0.04	72.1 ± 14.6	0.30
Portal vein invasion				
(+)	98.2 ± 36.3		121 ± 48.8	
(−)	35.8 ± 9.06	0.10	209 ± 76.4	0.33
Hepatic venous invasion				
(+)	104 ± 89.6		75.5 ± 33.4	
(−)	63.5 ± 19.2	0.49	176 ± 50.7	0.43
Bile duct invasion				
(+)	188 ± 127		25.1 ± 6.94	
(−)	53.6 ± 15.5	0.14	180 ± 49.2	0.26
Intrahepatic metastasis				
(+)	74.0 ± 37.4		227 ± 98.9	
(−)	65.7 ± 22.8	0.84	116 ± 25.4	0.22

[a] Mod, moderately differentiated HCC; poor, poorly differentiated HCC; well, well-differentiated HCC.

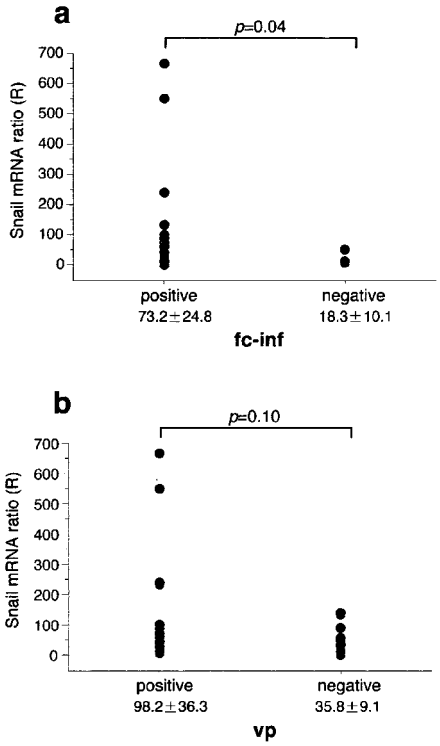

Fig. 4 Comparison of distribution of Snail mRNA ratio (R) between HCCs with and without capsular invasion (fc-inf; *a*), and portal vein invasion (vp; *b*). *a*, the mean R of fc-inf0positive cases was significantly higher than that of negative cases ($P = 0.04$). *b*, the mean R of vp-positive cases tended to be higher than that of negative cases ($P = 0.10$). All cases of $R > 200$ were both fc-inf- and vp-positive ones (*a* and *b*).

pressor apparently mediates the aggressive progression of human HCC.

DISCUSSION

Recent direct evidence shows that Snail transcription factor and its family protein Slug repress E-cadherin expression in human cancer cell lines (21–24). Down-regulation of E-cadherin causes loss of cell-to-cell adhesion. Impaired adhesion characterizes the potential of invasion and metastases, crucial steps for progression of HCC (3). Thus, the down-regulation of E-cadherin promotes invasion and metastases of HCC and *vice versa* (9). To confirm the function of Snail in HCC, we used E-cadherin-positive Li-7 cells. Jiao *et al.* (23) reported that E-cadherin and Snail inversely express in HCC cell lines with various phenotypes. Our data revealed direct evidence that transient Snail expression can suppress E-cadherin protein expression in HCC cells.

We investigated the localization of Snail mRNA using *in situ* hybridization and confirmed that Snail mRNA is expressed in HCC cells. We then quantitatively analyzed the mRNA expression levels of Snail in both cancerous and noncancerous tissues of HCCs. Snail was reported to be expressed in mesenchymal cells (30), therefore, our reverse transcriptase-PCR analysis contained Snail mRNA levels in both mesenchymal and HCC cells. We used the cancerous/noncancerous ratio of Snail mRNA to evaluate Snail expression levels in each case. Seven (16%) were determined to be Snail overexpression cases, and this overexpression significantly correlated with reduced E-cadherin expression. Our data show that Snail, rather than Slug, functions as a suppresser of E-cadherin in human HCC tissue, as well as in cultured HCC cells. Recently, Hajra *et al.* (24) reported that Slug contributed to the down-regulation of E-cadherin expression in breast cancer cell lines. Although both proteins are produced in all vertebrate species, their functions are different among various species and different cells (31, 32). These data suggest that E-cadherin production of carcinoma cells should be regulated by the different transcriptional repressors among the different cells or tissues.

We found significant E-cadherin reduction in Snail overexpression cases, however, there were 11 (69%) with reduced E-cadherin expression but without Snail overexpression. Kanai *et al.* (33) reported that 48% show DNA hypermethylation of the E-cadherin promoter region and 42% show loss of heterozygosity at the locus adjacent to the E-cadherin gene in HCC. Genetic mutation of the E-cadherin gene was detected in breast, gastric, and gynecological cancers, which showed a uniform loss of E-cadherin expression (34–36). To date, a genetic mutation of the E-cadherin gene has not been reported in cases of HCC in which loss of E-cadherin expression is considered to be heterogeneous and reversible (7, 12). Therefore, E-cadherin expression in HCC may be regulated not just by the Snail transcriptional factor but also by other genetic and/or epigenetic alterations such as DNA mutation and/or methylation. Additional studies are required to reveal the entire regulatory mechanism of E-cadherin expression in HCC tumors.

In this study, Snail mRNA overexpression correlated with capsular and portal vein invasion of surgically resected human HCC. Interestingly, among 4 cases with remarkably high Snail mRNA levels ($R > 200$), all were accompanied by portal vein invasion and 3 by capsular invasion. In addition, 2 of 7 cases with Snail overexpression ($R > 100$) showed an E-cadherin preserved pattern, but portal vein invasion and capsular invasion were evident. Our data show that Snail, rather than Slug, negatively regulates E-cadherin expression, but it may also regulate the expression of other genes involved in the invasive potential of HCC. E-Cadherin has been reported to involve in tumor invasiveness, including HCC (28, 37, 38), but the relationships between E-cadherin and clinicopathological factors were not consistent among these studies. In this study, E-cadherin was not found to be related to any clinicopathological factors. Differences of etiology and methods of evaluation might cause this discrepancy (28, 37, 38). Additionally, the reversibility of E-cadherin expression should be considered. Snail and other family proteins bind to specific target genes and function as transcriptional repressors, but it is considered that the repression of E-cadherin alone is not sufficient to explain the role of Snail in cell migration and cancer development (30). The possible involvement of rhoA, rhoB, and other molecules, as well as E-cadherin, in the Snail pathway that controls cell motility has been considered for *Drosophila*, *Caenorhabditis elegans*, and vertebrate (30, 32, 39, 40). Additional investigations are needed to fully understand the functions and target genes of Snail protein in HCCs.

In summary, the Snail transcriptional factor is expressed and functions in human HCC *in vitro* and *in vivo*. Our results indicate that Snail expression plays an important role in both the regulation of E-cadherin expression and in the acquisition of invasive potential in human HCC. Snail is possibly a potential target for an antitumor therapy blocking the functions of invasion and metastasis in human HCCs. It is expected that further investigation of the Snail function that mediates HCC progression will be rewarding.

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