Expression and role of epithelial cell adhesion molecule in dysplastic nodule and hepatocellular carcinoma

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Received June 13, 2012; Accepted August 8, 2012

DOI: 10.3892/ijo.2012.1631

Abstract. Epithelial cell adhesion molecule (EpCAM) has been proposed as a marker for cancer stem cells in human hepatocellular carcinoma (HCC). However, the function and clinical significance of EpCAM in HCC is largely unknown. We examined EpCAM expression and localization in 28 dysplastic nodules (DNs) and their corresponding cirrhotic nodules, 79 HCC tissue sections and 132 HCC tissue microarray cores by immunohistochemistry and determined the relationship to clinicopathologic findings. We also examined the role of EpCAM in HCC using synthetic small interfering RNA to silence EpCAM gene expression in Huh-7 cells. EpCAM expression was very rare in DNs but dominantly appeared in a distinctly nodular type of small HCC. Expression of EpCAM was observed in 39% (31/79) of HCC tissue sections and in 34.1% (45/132) of tissue microarray sections. EpCAM expression in HCC was significantly associated with high tumor grade and serum α-fetoprotein level. Silencing EpCAM gene expression significantly decreased the proliferative activity and invasiveness of HCC cells. EpCAM expression was an independent prognostic factor for survival in patients with T1 HCC. The data indicate that EpCAM expression occurs at distinct nodular stage of HCC and could play an important role in HCC progression.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor and the third leading cause of cancer-related mortality worldwide (1). Considerable progress has been made over the past few decades for diagnosing and treating HCC. However, HCC is still associated with a high rate of mortality, and its recurrence is often problematic and even lethal (2). Accumulating evidence suggests that tumor maintenance and growth are sustained by a minority population of cancer stem cells (CSCs) or tumor-propagating cells (TPCs) (3-6). CSCs are posited to be responsible for tumor initiation and for the generation of distant metastasis and relapse after therapy (7). Despite the current progress in understanding the contribution of CSCs to tumorigenesis, it remains elusive whether CSCs are derived from tissue-derived stem cells, bone marrow stem cells or differentiated mature cells that have undergone a de-differentiation or a trans-differentiation process (3-7).

The development of HCC usually follows a multistep sequence and the carcinogenic sequence of chronic hepatitis, cirrhosis, dysplastic nodule (DN), and HCC has been well established. Nodular lesions that differ from the surrounding liver parenchyma and that are characterized by cytological or structural atypia are termed DNs. DNs are classified as low-grade (LGDN) or high-grade (HGDN) depending on the degree of atypia (8). If CSCs in HCC arise from hepatic progenitor cells (HPCs), the progenitors would be expected to be already present in DN, a well-known precancerous lesion of HCC. Clarifying the histogenesis of CSCs is very important, because it may provide a rationale for novel therapeutic approaches to HCC.

Epithelial cell adhesion molecule (EpCAM) is a transmembrane intercellular cell-adhesion molecule that is expressed in many human epithelial cells (9). EpCAM has been identified as a marker of human hepatic stem/progenitor cells in the liver that is absent in mature hepatocytes (10-13). EpCAM is frequently expressed in most epithelial cell tumors, including HCC (9). For this reason, EpCAM has attracted major attention as a potential therapeutic target for cancer patients. Indeed, the use of the EpCAM-specific monoclonal antibody has been successful in treatment of malignant tumors associated with EpCAM positive carcinomas patients (14,15). Recent studies have suggested that the role of EpCAM is not limited to cell adhesion, but it is also involved in cellular signaling, cell differentiation, proliferation, and migration (12,14,16). Treatment of EpCAM-positive human breast cancer cells with EpCAM-specific small interfering RNA (siRNA) reduces cell proliferation, migration and invasion (17). Increased expression of EpCAM is associated with tumor angiogenesis and poor prognosis of HCC (13,18,19). However, the function and clinical significance of EpCAM in HCC is largely unknown.

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Key words: epithelial cell adhesion molecule, hepatocellular carcinoma, dysplastic nodule

In the present study, we examined the location and expression of EpCAM in surgical specimens of DNs and HCCs, the relationship between EpCAM expression and clinicopathologic factors in HCCs, and whether EpCAM silencing by siRNA affects cell growth, migration, and invasiveness in HCC cells. We also investigated whether EpCAM expression affects tumor angiogenesis in HCC.

Materials and methods

Patients. To investigate the location and expression pattern of EpCAM, we used 28 DNs (13 LGDNs and 15 HGDNs) and their corresponding cirrhotic nodules, and 79 HCC specimens collected from September 2004 to August 2008 at the Chonbuk National University Hospital. This study was approved by the ethics committees of Chonbuk National University. Written informed consent was exempted by the board due to the retrospective nature of the study. Representative 4-μm blocks were prepared from 10% formalin-fixed, paraffin-embedded tissue sections for immunohistochemical staining. In each case, clinicopathological features including patient age at diagnosis, gender, etiology, serological data including serum albumin level, α-fetoprotein (AFP), presence of ascites, tumor size, Edmonson-Steiner grade, microvessel invasion, presence of intrahepatic metastasis and follow-up data were obtained from hospital records. Tumors were staged according to the 2010 American Joint Committee on Cancer tumor-node-metastasis classification (20). The follow-up period was determined from the date of initial surgery until the date of the last follow-up or death. A previous existing tissue microarray (TMA) comprising 132 HCC cases was used to compare the concordance rates of EpCAM expression in HCC between whole tissue and TMA (21).

HCC cell lines. Human HCC cell lines HLE, HLF and Huh-7 were purchased from the Health Science Research Resources Bank (Osaka, Japan). HepG2 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). In addition, we used the sarcomatoid HCC cell line, designated SH-J1, which was established in our laboratory (22). All HCC cell lines were cultured according to the recommendations of the cell banks.

Immunohistochemistry. Immunohistochemical staining was performed by polymer intense detection system using the Bond-Max Automatic stainer (Leica, Bannockburn, IL, USA) in accordance with the manufacturer's instructions. Briefly, after antigen retrieval (microwave at high power for 10 min in 0.01 M citrate buffer, pH 6), the samples were incubated with anti-EpCAM antibody (Abcam, Cambridge, UK) for 30 min. Peroxidase activity was detected with the enzyme substrate 3-amino-9-ethyl carbazole. For negative controls, sections were treated the same way, except they were incubated with citrate buffered saline instead of the primary antibody. The samples subjected to immunostaining were rated according to a score calculated by adding the cancer area of the stain to the intensity of the stain. The area of staining was scored as 0 (<10%), 1 (11-30%), 2 (31-60%) and 3 (≥61%). The intensity of cell staining was grouped into four categories: 0, no immunostaining; 1, weak; 2, moderate and 3, strong. The maximum combined score was 6 and the minimum score was 0. If the combined score was ≥3, the tumor was considered positive, otherwise the tumor was considered negative. The cut-off score for determining positive expression for EpCAM was determined by receiver-operating characteristic (ROC) curve analysis. To study the relationship between EpCAM expression and tumor angiogenesis in HCC, we also examined the expression of CD34 (Dako, Carpinteria, CA, USA, for sinusoidal capillarization) and α-smooth muscle actin (Dako, for unpaired arteries) in 79 HCC specimens. The sinusoidal capillarization and number of unpaired arteries in HCC was measured as described previously (23).

Western blot analysis. Western blot analysis of EpCAM in HCC cell lines was performed as described previously (24). Briefly, cell lysates were subjected to denaturating sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electroblotting and immunoblotting for anti-EpCAM (Abcam). Blots were developed using secondary antibody, and immune complexes were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK). They were then analyzed using a LAS-3000 luminescent image analyzer (Fuji Film, Tokyo, Japan).

Small interfering RNA transfection. Small interfering RNA (siRNA) sequences were used to silence EpCAM expression. EpCAM siRNA and negative control were purchased from Bioneer Corporation (Daejeon, Korea). Sequences for EpCAM specific siRNAs and negative control siRNA were as follows: EpCAM: sense 5'-GUGAGAACCUACUGGAUCA(dTdT)-3', antisense 5'-UGAUCCAGUAGGUUCUCAC(dTdT)-3', and negative control: sense 5'-CCUACGCCACCAAUUUCGU (dTdT)-3', antisense antisense 5'-ACGAAAUUGGUGGCGUA GG(dTdT)-3'. Transfection of siRNA was performed with Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Cell growth and proliferation assay. Cell growth was determined by the colorimetric tetrazolium derived XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay (Roche Applied Science, Mannheim, Germany). DNA synthesis of cells was assessed by the bromodeoxyuridine (BrdU) incorporation assay (Roche Applied Science). For the cell growth and proliferation assay, 48 h after transfection of siRNA the cells of each group were reseeded in 96-well plates at a density of 0.3-1x10^4 cells per well. After 24-48 h, XTT and incorporated BrdU were measured colorimetrically using a microtiter plate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm.

In vitro migration and invasion assays. The migration assay was performed using a 24-transwell migration chamber (Corning Life Sciences, Acton, MA, USA) and the cell invasion assay was performed using a 24-transwell BioCoat Matrigel invasion chamber (BD Biosciences, San Jose, CA, USA) with an 8 μm-pore size polyvinyl-pyrrolidone-free polycarbonate membrane following the manufacturer's protocol. The cells that migrated or invaded to the lower surface of the filter were counted under a light microscope at x200 magnification in 10 randomly selected fields per well.

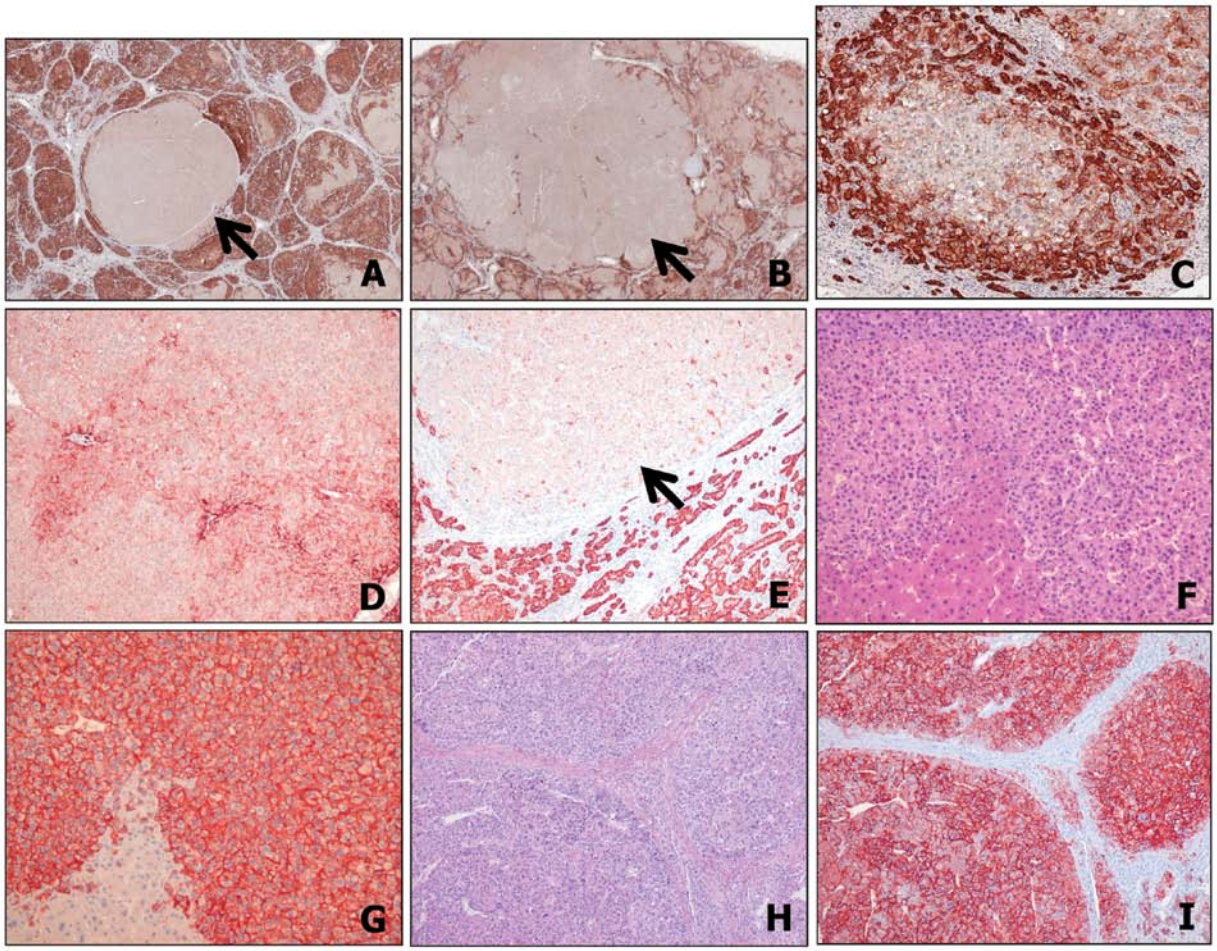

Figure 1. (A-E) EpCAM expression in dysplastic nodules and their corresponding regenerating nodules in liver cirrhosis. (A) Low grade dysplastic nodule (LGDN). (B and E) High grade dysplastic nodule (HGDN). Note the absence of EpCAM expression in neoplastic cells of dysplastic nodule (arrows), whereas reactive ductular cells and regenerating hepatocytes in periseptal and periportal area revealed strong EpCAM immunoreactivity in cirrhotic livers. (C) Regenerating nodule. The gradual loss of EpCAM expression in the center of regenerating nodule can be seen on differentiation from ductular cells into mature hepatocytes. (D) EpCAM expression in low grade dysplastic nodules. A geographic pattern of EpCAM with accentuated staining in cells around portal tracts in LGDN can be seen. (F-I) EpCAM expression in hepatocellular carcinoma (HCC). (F and G) Diffuse and intense membrane staining in vaguely nodular type HCC. (H and I) Diffuse and intense membrane staining in advanced HCC.

Statistical analyses. Comparisons between EpCAM expression and clinicopathological factors were assessed by the χ^2 test. Survival analyses were performed using the Kaplan-Meier method, and differences in survival between different clinical groups were determined by the log-rank test. A Cox proportional hazards regression analysis was performed to estimate the impact of clinicopathological factors on patient survival. P-values <0.05 were considered statistically significant. SPSS version 15.0 statistical software program (SPSS, Chicago, IL, USA) was used for the statistical analyses.

Results

Clinical features. The 175 patients with HCC were 25-79 years of age and consisted of 147 males and 28 females. A total of 126 patients were positive for hepatitis B virus surface antigen, 19 were alcohol related, 11 were positive for anti-hepatitis C virus antibody and 19 patients were of unknown etiology (Table I). The 175 HCCs were composed of 35 small HCCs (≤2 cm) and 140 advanced HCCs (>2 cm). Among the 35 small HCCs, six were vaguely nodular, 27 were distinctly nodular and two were infiltrative types.

Immunohistochemical results. Hepatocellular EpCAM expression in regenerating nodules showed strong cytoplasmic and/or membranous staining in all cirrhotic livers adjacent to DNs with the immunoreactivity depending on the degree of hepatocellular differentiation. Reactive ductular cells surrounding inflamed portal tract and periseptal areas showed stronger positivity for EpCAM (Fig. 1A and B). However, the expression of EpCAM was lost in the center of regenerating nodules, indicating the differentiation towards mature hepatocytes (Fig. 1C). Of 28 DNs, only one LGDN showed EpCAM expression. EpCAM expression in LGDN showed a geographic staining with weak intensity and accentuation in cells around the portal tracts (Fig. 1D and E). In 79 HCCs whole tissue sections, 31 were EpCAM-positive (39%). The pattern of EpCAM expression in HCC was more homogeneous and diffused than that of DN or regenerating nodules (Fig. 1F-I). Of the 175 HCC sections, EpCAM expression was detected in 72 (41%) HCCs.

Table I. Association between pathological features and EpCAM-positive patients with hepatocellular carcinoma (HCC).

	Overall HCC (n=175)			T1 HCC (n=65)		
Characteristics	Total	EpCam$^+$	P-value	Total	EpCam$^+$	P-value
Sex						
Male	147	56	0.060	51	20	0.230
Female	28	16		14	8	
Age (years)						
<55	66	29	0.559	18	5	0.123
≥55	109	43		47	23	
Etiology						
HBV	126	52	0.111			
HCV	11	1				
Alcohol	19	9				
Others	19	10				
Etiology						
Viral	137	53	0.210	50	19	0.131
Non-viral	38	19		15	9	
Liver cirrhosis						
Absence	86	33	0.464	24	12	0.388
Presence	89	39		41	16	
Ascites						
Absence	159	66	0.756	59	26	0.613
Presence	16	6		6	2	
Albumin, ng/dl						
<3.5	22	9	0.981	13	7	0.381
≥3.5	153	63		52	21	
Microvessel invasion						
Absence	72	31	0.667			
Presence	103	41				
Preoperative AFP, ng/ml						
≥100	115	40	0.018	51	21	0.555
>100	60	32		14	7	
Intrahepatic metastasis						
Absence	118	49	0.882	60	27	0.278
Presence	57	23		5	1	
Histologic grade						
1 and 2	110	34	<0.001	48	15	0.001
3 and 4	65	38		17	13	
pT stage						
1	65	28	0.894			
2	76	31				
3 and 4	34	13				

Among 35 small HCCs, EpCAM expression was detected in 19 (54%) HCCs. Nineteen EpCAM-positive small HCCs were composed of one vaguely nodular, 17 distinct nodular and one infiltrative type. In the validation study between whole tissue section and TMA samples, the concordance rate for EpCAM staining in HCC was 92% (33 of 36). Two EpCAM-positive HCCs in whole tissue sections changed to negative cases in TMA samples, whereas one EpCAM-negative case changed to positive case in TMA samples.

Correlation between immunohistochemical results and clinicopathological features. To elucidate the significance of EpCAM in HCCs, a correlation between EpCAM and the major clinicopathological features was evaluated (Table I).

Table II. Association with EpCAM expression and tumor angiogenesis in hepatocellular carcinoma.

	Overall HCC (n=79)			T1 HCC (n=65)		
Characteristics	Total	EpCam$^+$	P-value	Total	EpCam$^+$	P-value
SMA						
1+, 2+	31	14	0.953	14	6	0.769
3+, 4+	48	22		23	11	
CD334						
1+, 2+	9	5	0.523	5	2	0.774
3+, 4+	70	31		32	15	

SMA, α-smooth muscle actin for unpaired arteries staining; CD34 for sinusoidal capillarization.

Table III. Cox proportional hazard analyses of factors associated with hepatocellular carcinoma (HCC) in 175 patients.

	Overall HCC (n=175)				T1 HCC (n=65)		
Characteristics	HR	95%CI	P-value		HR	95%CI	P-value
Univariate Cox regression analysis				Univariate Cox regression analysis			
Intrahepatic metastasis	2.474	1.490-4.106	<0.001	EpCAM	3.284	1.003-10.759	0.049
Albumin	0.426	0.220-0.825	0.011				
Microvessel invasion	1.969	1.122-3.453	0.018				
pT stage			0.010				
	2.673	1.401-5.100	0.003				
	2.371	1.125-4.994	0.023				
Multivariate Cox regression analysis				Multivariate Cox regression analysis[a]			
pT stage			0.012	EpCAM	4.008	1.215-13.219	0.023
	2.756	1.359-5.589	0.005				
	1.627	0.695-3.808	0.262				
Intrahepatic metastasis	2.255	1.271-4.002	0.005				
Albumin	0.267	0.013-0.538	<0.001				

[a]Variables considered in the analysis were age, sex, intrahepatic metastasis, intravascular invasion, serum AFP and albumin level, histologic grade and EpCAM expression.

The clinicopathological analysis revealed that EpCAM-positive HCC was significantly associated with high histological grade (P<0.001) and serum AFP level (P=0.018). Other factors, including age, gender, etiology, background liver disease, albumin level, presence of intrahepatic metastasis, microvessel invasion and the presence of ascites were not correlated with EpCAM expression. No significant differences were observed between EpCAM expression and the sinusoidal capillarization or number of unpaired arteries in HCC (Table II).

Outcome. Follow-up intervals ranged from 1-142 months. Sixty-one patients died during the follow-up period. In univariate analysis, intrahepatic metastasis, serum albumin levels, microvessel invasion and T stage were significantly associated with poor patient survival (P<0.001, P=0.011, P=0.018 and P=0.010, respectively). Multivariate analysis revealed that T stage, intrahepatic metastasis and serum albumin levels were independent prognostic indicators (P=0.012, P=0.005 and P<0.001, respectively) (Table III). Among T1 HCC patients, mean survival of patients with EpCAM-positive HCC was 74.4 months, and EpCAM-negative HCC was 101.7 months. EpCAM expression was significantly associated with patient survival in T1 HCC patients in univariate and multivariate Cox survival analyses (P=0.049 and P=0.023, respectively) (Table III, Fig. 2).

Expression of EpCAM in HCC cell lines. The expression level of the EpCAM protein was higher in the Huh-7 and HepG2 cell lines (Fig. 3A). However, the expression of EpCAM was not evident in the SH-J1, HLE and HLF cell lines. Transfection with EpCAM siRNA resulted in a marked decrease of EpCAM protein expression at 48 h post-transfection in Huh-7 cells (Fig. 3B).

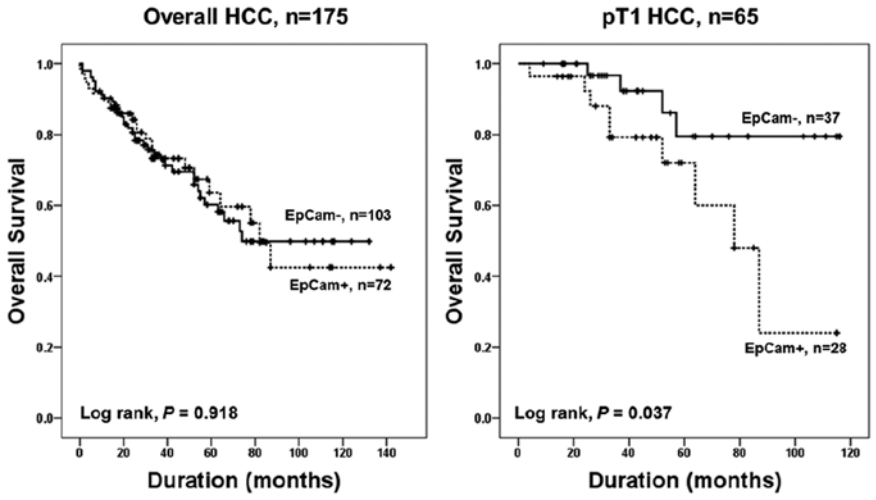

Figure 2. Kaplan-Meier analysis of overall survival in patients with EpCAM-positive hepatocellular carcinoma (HCC).

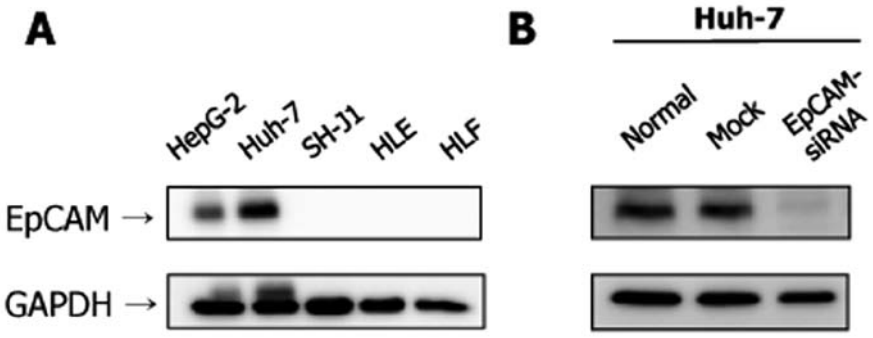

Figure 3. (A) Western blot analysis of EpCAM in hepatocellular carcinoma cell lines. (B) Huh-7 cells transfected EpCAM siRNA presented a dramatic decreased expression of EpCAM.

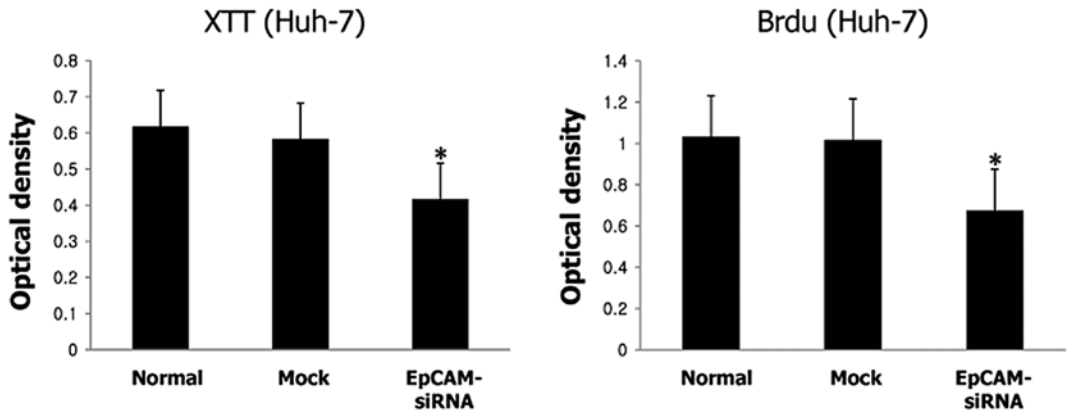

Figure 4. XTT and Brdu proliferation assay in hepatocellular carcinoma cell lines. The XTT assay demonstrated a significant decrease in cell growth compared to those of the control (p<0.001). In the BrdU assay, EpCAM downregulated Huh-7 cells showed significantly decreased cell proliferation compared to those of the control. The experiment was independently repeated three times.

Effects of EpCAM silencing on cell growth, proliferation, migration, and invasion. Silencing EpCAM gene expression in Huh-7 cells by EpCAM siRNA resulted in significant inhibition of cell growth compared to those of the control (P<0.001) (Fig. 4A). Silencing EpCAM gene expression also significantly decreased cell proliferation compared to those of the control (P<0.001) (Fig. 4B). Silencing EpCAM gene expression dramatically inhibited the migration and invasion ability of Huh-7 cells (Fig. 5).

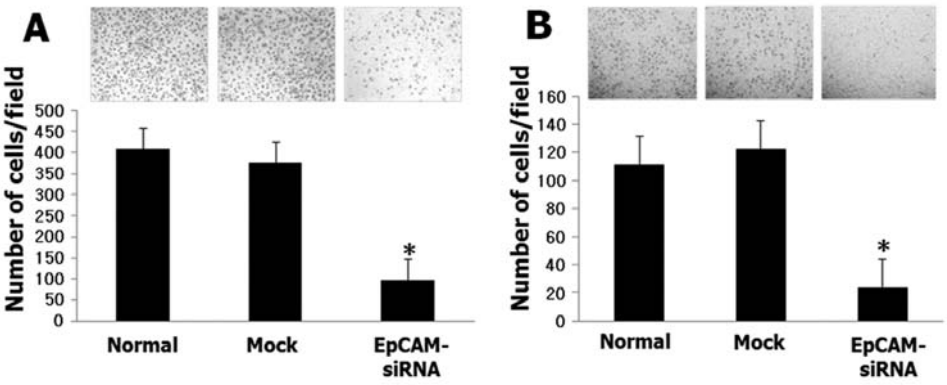

Figure 5. *In vitro* cell migration and invasion assay. (A) Cell migration of EpCAM silencing Huh-7 cells was decreased by 4.6-fold over the control ($p<0.001$). (B) The EpCAM silencing Huh-7 cell invasion was decreased by 4.5-fold over the control ($p<0.001$). The experiment was independently repeated three times.

Discussion

This study demonstrated that: i) EpCAM expression is very rare in DNs but predominates in a distinctly nodular type of small HCC; ii) EpCAM expression in HCC correlates with tumor cell de-differentiation and serum AFP levels; iii) EpCAM silencing induces significant inhibition in the growth and proliferation of HCC cells; and iv) EpCAM silencing decreases cell migration and invasion of HCC cells. We also found that EpCAM expression is an independent prognostic indicator of T1 HCC. However, we could not discern a significant association between EpCAM expression in HCC cells and tumor angiogenesis. These data strongly suggest that EpCAM expression occurs in the small nodular stage of HCC in hepatocarcinogenesis and indicate important roles of EpCAM in HCC progression.

We found that a strong expression of EpCAM in proliferating ductular cells and regenerating hepatocellular cells of regenerating nodules. On maturation of these regenerating hepatocytes, EpCAM expression was lost. There was a transient loss of hepatocellular EpCAM expression in DNs in regenerating nodules. EpCAM expression reappeared in distinctly nodular HCCs. Small HCCs (≤2 cm in diameter) can be subcategorized further into vaguely nodular and distinctly nodular HCCs based on macroscopic features. Vaguely nodular HCC is an early HCC, and distinctly nodular HCC is a small progressed HCC (8). Contrary to the notion that EpCAM-positive HCC may originate from HPCs, our finding of the dominant reappearance of EpCAM in distinct nodular HCC indicates that HCCs could obtain the EpCAM phenotype during a small progressed stage of HCC. Our findings are consistent with the results of Breuhahn *et al*, who reported the rarity of EpCAM expression in DN, the earliest known premalignant lesion in human HCC (11). The CSC model is essentially synonymous with the hierarchy model of carcinogenesis (25). However, the expression of stemness-related markers exists as a functional phenotype in the de-differentiation model and is evident by any member of the malignant population in the presence of the appropriate endogenous and exogenous factors (5). In HCC, EpCAM expression is regulated by Wnt/β-catenin signaling and tumorigenicity, invasiveness, and differentiation capabilities of EpCAM-positive HCC are regulated by Wnt/β-catenin signaling (12). Thus, EpCAM appears to be a common gene expressed in HCC with activated Wnt/β-catenin signaling (12). Taken together, these findings suggest that EpCAM expression is an acquired phenotype of cancer cells during HCC progression, although CSCs might be another contributor of EpCAM-positive HCC.

Presently, a proportion of HCCs expressed EpCAM and EpCAM expression correlated with the grade of malignancy. Moreover, EpCAM silencing resulted in a significant decrease in the rate of cell proliferation of HCC cells. These findings agree with previous studies demonstrating that the number of EpCAM-positive cells and expression levels of EpCAM correlate with de-differentiation and are associated with the proliferative activity of tumor cells (17,26,28). EpCAM is overexpressed in breast carcinoma and silencing of EpCAM gene expression with siRNA decreases proliferation of breast cancer cells (17). EpCAM blockage via siRNA also inhibits spheroid formation and tumorigenicity of Huh-1 cells (12). Taken together, these observations suggest that EpCAM is required for tumor cell de-differentiation and increased proliferative activity in HCC. This notion is supported by the fact that EpCAM has a direct impact on cell cycle and the ability to rapidly upregulate the proto-oncogene c-myc as well as cyclin A and E in human epithelial kidney 293 cells (27). Additionally, it has been shown that proteolytic cleavage of EpCAM releases an intracellular domain, which forms a complex with components of Wnt pathway and regulates gene transcription, resulting in cell proliferation and tumor formation (16).

We also found that EpCAM expression in HCC was significantly associated with high serum AFP level. A close relationship between EpCAM expression and high AFP levels has been demonstrated (12,13). Gene expression profiles have revealed that $EpCAM^+/AFP^+$ HCCs have progenitor features with poor prognosis, whereas $EpCAM^-/AFP^-$ HCCs have adult hepatocyte features with good prognosis (13). The latter study confirmed that $EpCAM^+$ HCC cells are highly invasive and tumorigenic, and activate Wnt/β-catenin signaling. The prognosis of patients with EpCAM-positive HCC is thought to be worse than those with pure HCC (13,18,19). In this study, EpCAM expression was not associated with the overall survival rate in all patients with HCC; however, we found that EpCAM expression was an independent prognostic indicator of T1 HCC. Because EpCAM expression was presently associated with high tumor grade and high AFP levels-factors that are well known

unfavorable prognostic factors in HCC-the finding that EpCAM appears to be a poor prognostic factor for HCC is reasonable. The collective findings suggest that EpCAM expression in HCC plays an important role in facilitating tumor cell proliferation, leading to high grade HCC with high AFP level.

This study demonstrates that EpCAM silencing by siRNA dramatically decreases cell migration and invasion of HCC cells. This is in agreement with previous studies showing that expression of EpCAM is related to the degree of invasion and/or metastasis in breast (29), lung (30) and pancreas cancers (31). Silencing of EpCAM gene expression decreased cell migration and invasion in a breast cancer cell line (17). Similarly, EpCAM blockage by siRNA decreases the population of EpCAM-positive cells and significantly inhibited cellular invasion of Huh-1 cells (12). Based on the above observations, it is clear that EpCAM is an important player in invasion and metastasis of tumor cells. However, the mechanisms of the promoting role of EpCAM in tumor invasion and metastasis are still not fully understood. EpCAM is a transmembrane glycoprotein that has been proposed to mediate homophilic adhesive interactions, thereby preventing cell scattering (9,10,26). From this, one might expect that EpCAM prevents cancer metastasis. However, in several tumor types, high expression of EpCAM has been inversely correlated with metastasis and poor clinical outcome (32-34). EpCAM is able to abrogate E-cadherin-mediated cell-to-cell adhesion by disrupting the link between α-catenin and cytoskeleton, resulting in promoting cell motility, proliferation and metastasis (32,35). EpCAM also interacts directly with CD44v4-v7, a tumor metastasis-promoting cell adhesion molecule, and with claudin-7, a tight cell junction protein (36,37). These complexes can influence cell-to-cell adhesion and cell matrix adhesion, and they appear to be involved in processes that promote metastasis. Another potential mechanism involves the possible links between EpCAM expression and activation of Wnt signaling. This contention is supported by the observation that EpCAM downregulation leads to a significant decrease in cytoplasmic β-catenin through an increase in its association with the E-cadherin adhesion complex (17). Consequently, the decreased available β-catenin for Wnt signaling leads to the shut-down of the activation of its target genes involved in tumor progression.

EpCAM has been targeted in clinical trials using monoclonal antibodies in various cancers (14,15,38) and we believe that EpCAM represents a novel target for gene therapy in HCC. In support of this hypothesis, we found that EpCAM was overexpressed in a proportion of HCCs. Furthermore, silencing EpCAM gene expression significantly decreased the proliferative capacity and invasive potential of HCC cells. siRNA can be used successfully for gene silencing *in vivo* (39,40). Since EpCAM antibody and/or siRNA can be easily synthesized, this study may provide a rationale for therapeutic approaches to HCC.

Our study did not find a statistically significant correlation between EpCAM expression and the number of unpaired arteries, or the degree of sinusoidal capillarization in HCC. On the other hand, some other studies have reported that high expression of EpCAM correlates with tumor angiogenesis in HCC (18,19). Further analysis of the EpCAM expression and tumor angiogenesis in clinical HCC tumor samples might provide useful information regarding prognosis and treatment.

In conclusion, our data indicate that EpCAM expression is very rare in DNs, but can reappear predominantly in distinctly nodular small HCC. Although we cannot conclude whether the EpCAM-positive HCCs originated from pre-existing CSC or from de-differentiation of HCC cells, our study suggests that the EpCAM phenotype might be an acquired feature of cancer cells during HCC progression. EpCAM expression is associated with tumor cell de-differentiation and serum AFP level, and is a negative prognostic factor in HCC. Moreover, silencing EpCAM gene expression significantly inhibits tumor cell proliferation and decreases the migration and invasiveness of HCC cells. These findings strongly suggest that EpCAM plays an important role in development and progression of HCC.

Acknowledgements

This study was supported by the National Research Foundation of Korea Grant funded by the Korean Government (no. 2011-0028223).

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