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Overexpression of epithelial cell adhesion molecule as a predictor of poor outcome in patients with hepatocellular carcinoma

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Abstract. Cancer growth, metastasis and development are regulated by a number of genes, whose expression mediates important processes, including cellular plasticity, motility and internal interactions in the tumor microenvironment. The epithelial cell adhesion molecule (EpCAM) serves an important role in cell-cell migration and tumorigenicity, particularly metastasis. The aim of the present study was to measure EpCAM expression using immunohistochemistry and to investigate the association between clinicopathological features and prognosis in hepatocellular carcinoma (HCC). The results revealed that EpCAM expression may be a biomarker for poor prognosis in patients with HCC and may therefore be used to predict clinical outcome. The present study suggests that EpCAM expression in HCC can be considered as a routine biomarker for unfavorable prognosis and may provide a basis for the future development of anti-EpCAM-targeted therapy.

Introduction

In Taiwan, hepatocellular carcinoma (HCC) is the second-most common cause of cancer-associated mortality and its incidence is increasing (1). A number of risk factors for HCC have been reported previously, including hepatitis B, hepatitis C, alcoholic liver disease and non-alcoholic fatty liver disease (2-4). In 2016, the reported outcome of HCC was poor, with a high mortality rate (35.5/100,000) in Taiwan (5). Current targeted and systemic therapies for HCC face a number of challenges, including drug toxicity and resistance (6). As such, the development of effective prediction and surveillance tools are necessary to enable early diagnosis, the prediction of clinical outcomes and personalized adjuvant treatments (7). Recent studies on HCC have focused on the development of high-throughput microarrays for predicting HCC prognosis (8-10). Several genes and proteins have been reported to be associated with HCC prognosis, including forkhead box M1 (FOXM1) (10), cyclin-dependent kinase 1 (Cdk1) (9), TP53BP1 and CDKN1B (8).

Typically, tumor progression occurs in two stages: Growth and distant metastases. Circulating tumor cells spread from the primary sites into the peripheral blood supply and arrive at the metastasis site; this process involves many genes (11,12). Recurrent, high-grade and distant metastases indicate invasive circulating tumor cells, which are responsible for poor prognosis in HCC patients (13). A transmembrane glycoprotein-epithelial cell adhesion molecule (EpCAM; CD326) serves an important role in the distant metastasis of HCC (14). Ber-EP4 is a monoclonal antibody that effectively labels EpCAM in epithelial tissues and has been widely used to differentiate mesothelioma, adenocarcinoma and basal and squamous cell carcinomas (15,16). However, few studies have analyzed the association between EpCAM expression and HCC prognosis. The aim of the present study was to analyze tumor and normal tissue samples from Taiwanese patients with HCC who had not previously received chemotherapy or targeted therapy in order to determine whether EpCAM expression is an independent clinicopathological indicator of HCC.

Patients and methods

Patients. A prospective search was performed in Changhua Christian Hospital to identify patients with HCC diagnosed

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between July 2011 and November 2013 at the Division of General Surgery, Department of Surgery, Changhua Christian Hospital, Taiwan. The present study was approved by the Institutional Review Board of Changhua Christian Hospital (CCH IRB number: 120504). Informed consent was obtained from all included patients. The inclusion criteria were as follows: HCC diagnosis, no previous chemotherapy or targeted therapies. The exclusion criteria were as follows: Age <18 years, pregnancy, previous chemotherapy, targeted therapy, or surgical intervention. All included patients received curative surgical treatment and long-term follow-up. The demographic and clinical data, including pathological stage, operative procedure and surgical outcome, were recorded and patients were monitored until death, censorship, or loss to follow-up. The Child-Pugh score predicted the clinical outcome. Following surgical resection, primary tumor tissues and matched adjacent normal tissues were collected and analyzed using a tissue microarray. The follow-up duration was defined as the period between the date of surgical intervention to the date of last visit or death. Patients were categorized into the low expression and high expression groups depending on EpCAM expression. The median EpCAM expression in the tumor group was used as the cut off value.

Immunohistochemistry (IHC) and scoring. To detect EpCAM expression, tumor specimens were embedded in paraffin, cut into 4-μm-thick sections and mounted on poly-l-lysine-coated slides. Subsequently, 10 mM Tris-HCl (pH 7.4) and 150 mM sodium chloride were used to deparaffinize and rinse slides. Tissue was fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min at room temperature, blocked with 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min at room temperature and incubated with EpCAM (Ber-EP4) monoclonal antibodies (cat. no. 61-0132-2; 1:100; Genemed Biotechnologies, Inc., South San Francisco, CA, USA) in blocking buffer for 1 h at room temperature. The slides were washed thrice with PBS and antibodies were detected using the EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse kit (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and observed under a light microscope (BX50; Olympus Corp., Tokyo, Japan) at a magnification of x20 and x40. EpCAM expression was visualized in negative controls (adjacent normal tissues) by performing the same IHC steps, excluding the addition of the monoclonal antibodies. IHC results were evaluated by a professional pathologist and the scoring system considered two aspects: Staining intensity and percentage of positive cells. The staining intensity was scored using 4 grades as described previously (17,18): 0, no expression; 1, weak expression; 2, moderate expression; and 3, strong expression. The IHC score ranged from 0 to 300 and was calculated using the following formula: Staining intensity x percentage of positive labeled cells.

Statistical analysis. The association between EpCAM expression and the clinical and pathological parameters was analyzed using Chi-square and paired-sample t-tests. SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Survival curves were plotted using the Kaplan-Meier method and compared using log-rank test. Cox's proportional hazards regression model was used to analyze the association between the variables and survival data. P<0.05 was considered to indicate a statistically significant difference.

Table I. Clinicopathological characteristics of patients with hepatocellular carcinoma.

Characteristics	Patient data
Age (years)	62.8±10.8
Sex, n (%)	
Female	50 (27.0)
Male	135 (73.0)
Recurrence rate, n (%)	20 (10.8)
Survival days	857±342.5
Child-Pugh score	4.79±1.8
Differentiation, n (%)	
Well	12 (6.5)
Moderate	97 (52.4)
Poor	76 (41.1)
Clinical stage, n (%)	
Stage I	143 (76.4)
Stage II	21 (11.4)
Stage III	21 (11.4)
Tumor size (mm)	45.9±37.2
Hepatitis B, n (%)	101 (54.6)
Hepatitis C, n (%)	62 (33.5)

Results

Patient characteristics. Patient characteristics are listed in Table I. A total of 185 patients (aged 26-81 years; mean, 62.9±10.8 years; male: Female, 73:27) were included in the present study. The majority of patients had Child-Pugh score A (Child-Pugh points: 4.79±1.8), with a mean tumor size of 45.9 mm. The prevalence of hepatitis B and C was 54.6 and 33.5%, respectively. Following surgical excision, pathology revealed moderately differentiated tumors in most patients (n=97; 52.4%) and well-differentiated tumors in 12 patients (6.5%), with poorly differentiated tumors in 76 patients (41.1%). A total of 143 patients (76.4%) were determined to have HCC at clinical stage I. The mean follow-up time was 857 days. During this period, 20 (10.8%) patients experienced tumor recurrence.

EpCAM expression. IHC staining revealed that all 185 HCC and normal adjacent tissues were positive for EpCAM expression. EpCAM expression was upregulated in tumor tissues compared with matched adjacent normal liver tissues (Fig. 1A). In addition, EpCAM expression was significantly higher in HCC tissues compared with the paired adjacent normal liver tissues (P<0.001; Fig. 1B). The authors of the current study hypothesized that high EpCAM expression promotes poor clinical outcomes in the low-expression group. Among the clinicopathological parameters assessed, the differentiation grade was positively associated with high EpCAM expression (P<0.05; Table II). No significant association was observed between high EpCAM expression and hepatitis B or C.

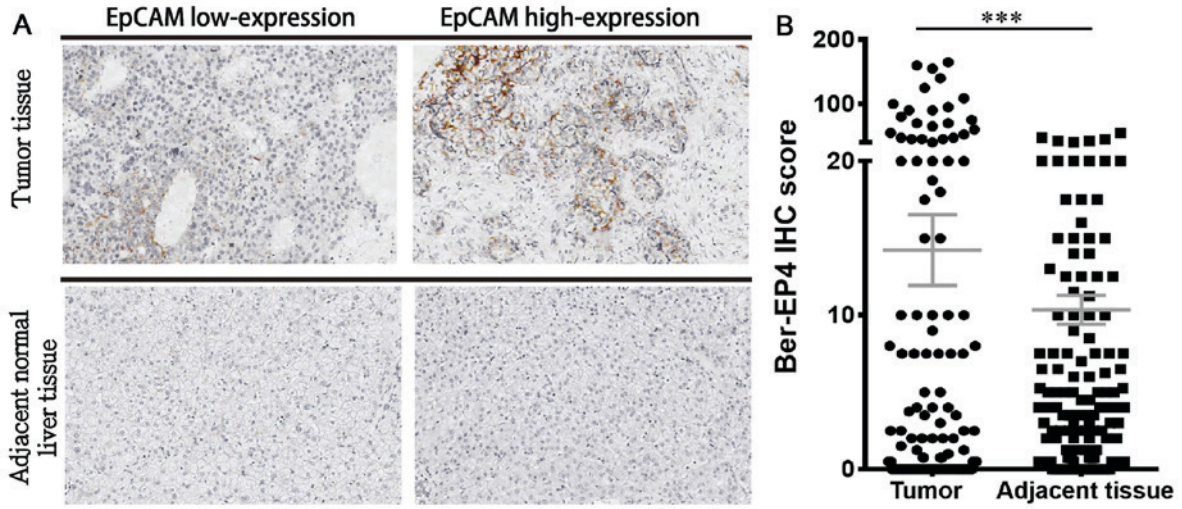

Figure 1. EpCAM expression in tumor and matched adjacent normal liver tissues from patients with hepatocellular carcinoma. (A) Representative IHC tissue samples from the EpCAM low-expression and high-expression groups. (B) Quantified EpCAM expression in tumor and adjacent normal liver tissues. Magnification, x100. ***P<0.001. EpCAM, epithelial cell adhesion molecule; IHC, immunohistochemistry.

Table II. Association between clinicopathological characteristics and epithelial cell adhesion molecule expression in patients with hepatocellular carcinoma.

	EpCAM		
Variables	Low expression	High expression	P-value
Age (years)	63.2±11.6	62.1±9.6	0.292
Sex, n			0.511
Female	27	23	
Male	65	70	
Recurrence, n			0.354
Negative	80	85	
Positive	12	8	
Differentiation, n			0.042
Well	6	6	
Moderate	40	57	
Poor	46	30	
Clinical stage, n			0.497
Stage I, II	84	80	
Stage III, IV	12	9	
Tumor size (mm^2)	46.2±39.6	45.6±34.9	0.384
Hepatitis B, n			1.000
Negative	42	42	
Positive	50	51	
Hepatitis C, n			1.000
Negative	61	62	
Positive	31	31	

Table III. Univariate analysis of overall survival for patients with hepatocellular carcinoma.

	Overall survival		
Variables	Median survival days (n)	Survival (%)	Log-rank
Sex			0.904
Female	895	86	
Male	1,034	85.2	
Recurrence			0.099
Negative	935	83.6	
Positive	634	100	
Differentiation			0.175
Well	860	100	
Moderate	918	86.6	
Poor	832	81.6	
Clinical stage			0.003
Stage I, II	928	87.8	
Stage III, IV	642	66.7	
Hepatitis B			0.967
Negative	919	85.7	
Positive	909	85.1	
Hepatitis C			0.567
Negative	915	84.6	
Positive	895	87.1	
Epithelial cell adhesion molecule			0.043
Low	976	90.3	
High	832	80.4	

Survival analysis. Overall survival analysis revealed a significant difference in two factors: Clinical stage and EpCAM expression (P<0.05; Table III). The median survival time was 928 days for patients with stage I and II tumors and 642 days for patients with stage III or IV tumors. The median survival

Table IV. Univariate and multivariate analyses of clinical characteristics in patients with hepatocellular carcinoma.

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Sex						
Female	-	-	-			
Male	1.054	0.446-2.493	0.904			
Recurrence						
Negative	-	-	-			
Positive	0.043	0.000-13.801	0.285			
Clinical stage						
Stage I, II	-	-	-	-	-	-
Stage III, IV	3.487	1.465-8.302	0.005	3.255	1.365-7.762	0.008
Differentiation						
Well or moderate	-	-	-			
Poor	1.734	0.815-3.690	0.153			
EpCAM						
Low	-	-	-	-	-	-
High	2.238	1.004-4.989	0.049	2.108	0.943-4.712	0.069

HR, hazard ratio; CI, confidence interval; EpCAM, epithelial cell adhesion molecule.

time was 832 days in the group with high EpCAM expression (survival rate, 80%) and 976 days in the group with low EpCAM expression (survival rate, 90%).

The association between high EpCAM expression and clinical outcomes in HCC patients was investigated (Table II). Overall survival analysis revealed that patients with a high clinical stage and high EpCAM expression had lower survival rates and reduced survival duration, compared with those at clinical stages III and IV, and low EpCAM expression. Cox regression analysis confirmed the prognostic significance of a high clinical stage and high EpCAM expression (Table IV). Kaplan-Meier analysis revealed that patients with high EpCAM expression had a shorter overall survival time compared with those with low EpCAM expression (Fig. 2A); similar results were noted for high clinical stage and differentiation grade in patients with HCC (Fig. 2). These results suggest that high EpCAM expression serves an important role in determining the clinical outcomes of patients with HCC.

Discussion

EpCAM is a transmembrane glycoprotein that regulates Ca^{2+}-independent cell-cell adhesion via several functions, including cell migration, proliferation and differentiation (19-21). In addition, EpCAM is involved in c-Myc- and cyclin A/E-mediated cell cycle progression and proliferation (22). High EpCAM expression has been reported to be regulated by Wnt/β-catenin signaling, which is responsible for the tumorigenic and invasive abilities of HCC (23). High EpCAM expression is reportedly associated with poor clinical outcomes in breast (24), ovarian (25) and esophageal squamous cell carcinoma (26). Furthermore, Schmelzer *et al* (27) has proposed EpCAM as a hepatic stemness marker. Together, these reports suggest that EpCAM may be a good marker for HCC prognosis.

In the present study, EpCAM expression in primary HCC was investigated, as well as its impact on clinical outcomes. The results revealed that high EpCAM expression was significantly associated with high differentiation grade. Bae *et al* (28) previously reported that high EpCAM expression was associated with high histologic grade, while EpCAM downregulation inhibited HCC proliferation. These findings support the results of the present study, which indicate that high EpCAM expression is associated with high differentiation grade and poor outcome. EpCAM immunoreactivity has been reported in 15.6-35% of HCCs (29-31) and is associated with young age, poor differentiation grade and high clinical stage (28,30,32,33). In the present study, EpCAM expression was significantly associated with differentiation grade; however, a high proportion of patients enrolled in the present study had stage I tumors (76.4%) compared with previous studies (28,34,35). Furthermore, no significant association was observed between EpCAM expression and clinical stage. However, Kaplan-Meier analysis demonstrated that EpCAM serves an important role in patients with tumors of high clinical stage. With regards to long-term patient follow-up, EpCAM expression was not significant in multivariate analysis.

The carrier rate of hepatitis B and C in Taiwan is high and these viruses induce rapid development of HCC (36). A number of oncogenes and DNA micromutations, including WNT, β-catenin, p53, Janus kinase, signal transducer and activator of transcription and mitogen-activated protein kinase-1, have been investigated in the progression and

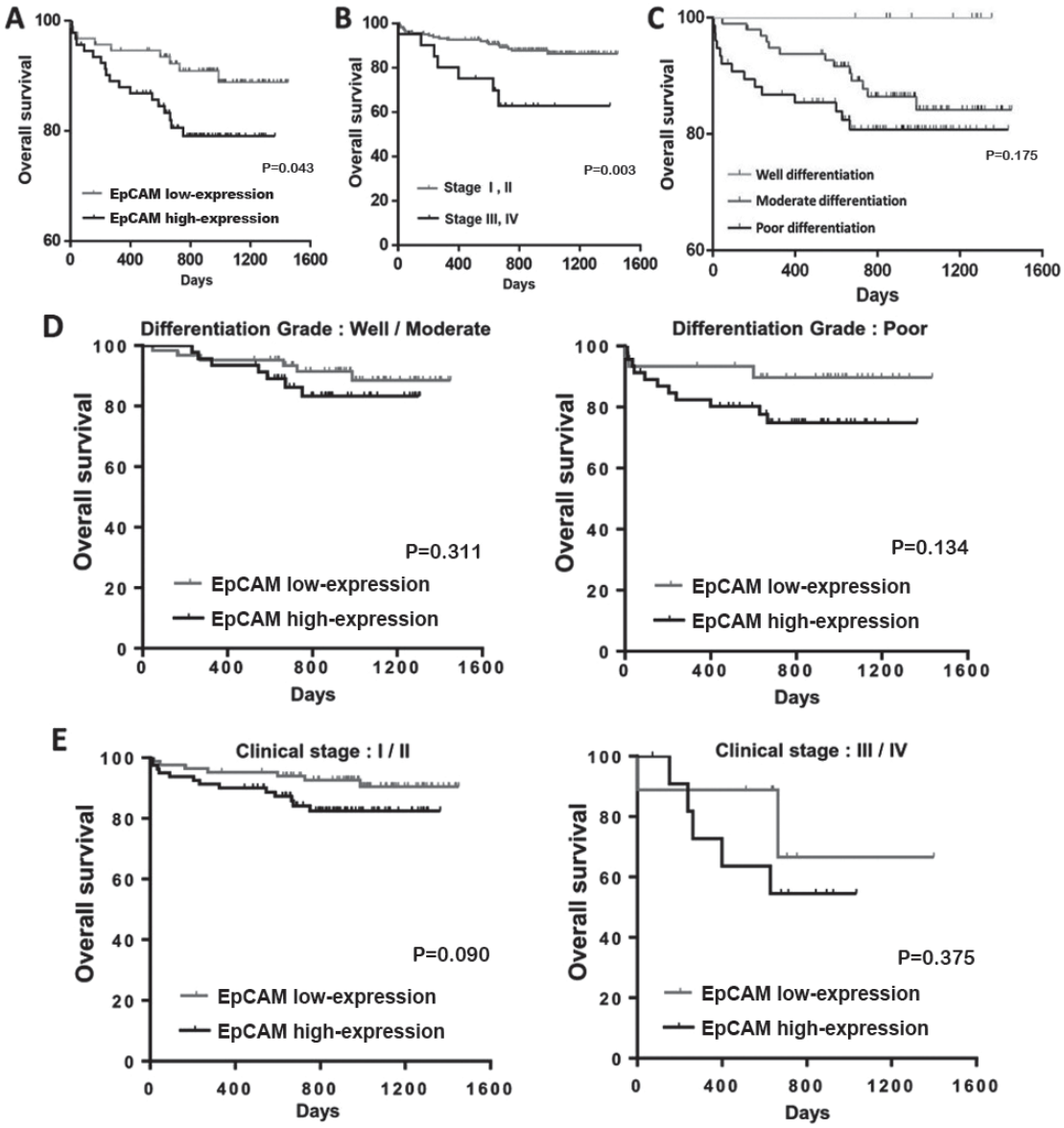

Figure 2. Kaplan-Meier analysis of the association between overall survival and (A) EpCAM expression, (B) clinical stage and (C) differentiation grade in patients with hepatocellular carcinoma. Kaplan-Meier analysis of the association between overall survival and (D) differentiation grade and (E) clinical stage in the EpCAM low-expression and high-expression groups. All comparisons were performed using the log-rank test. EpCAM, epithelial cell adhesion molecule.

development of hepatocarcinogenesis in patients with hepatitis B and C (37-39). However, few studies have investigated the novel biomarker EpCAM. Kimura *et al* (40) reported that high EpCAM expression is frequently observed in patients with hepatitis B virus. In addition, they demonstrated that EpCAM-expressing cells have high anti-cancer drug resistance. This trend was not observed in the present study. Furthermore, no significant difference was observed in overall survival analysis and Kaplan-Meier analysis. This may be due to a number of reasons; firstly, the patient sample was small and the effects of high-expression of EpCAM may not have been accurately detected. Secondly, a larger number of patients with stage I and II tumors were included in the present study compared with previous studies (28,34,41). Previous studies have reported that EpCAM expression is more significantly associated with clinical outcome in high-stage tumors (28,34,41). Thirdly, liver cirrhosis rates and Child-Pugh score were lower in in the present study compared with previous reports (28,34,41). Although no association was observed between high EpCAM expression and poor clinical outcome in HCC patients with hepatitis B and C virus infection in the present study, we believe that such association may exist. Further studies are required to confirm the role of high EpCAM expression in HCC. The authors of the present study also believe that high EpCAM expression may be associated with poor prognosis in HCC. Thus, high EpCAM expression may be a prognostic factor of poor outcome in patients with HCC.

In conclusion, the present study suggests a potential role of EpCAM as an important risk factor for poor survival in HCC and EpCAM expression can be measured using routine IHC. Further studies are required to investigate EpCAM as a biomarker for HCC. The preliminary data herein suggests that HCC patients with high EpCAM expression may benefit from

targeted therapy and immunotherapy. Thus, anti-EpCAM therapy is an appealing strategy for HCC and should be explored in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CJK and CJL were involved in the acquisition and analysis of data. PYC and MYW were involved in the design of the study and organized the manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of Changhua Christian Hospital (Changhua, Taiwan, R.O.C.). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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