

# Overexpression of Epithelial Cell Adhesion Molecule Antigen in Gallbladder Carcinoma Is an Independent Marker for Poor Survival

Meri Varga,<sup>1</sup> Peter Obrist,<sup>2</sup>  
Stephan Schneeberger,<sup>3</sup> Gilbert Mühlmann,<sup>3</sup>  
Christina Felgel-Farnholz,<sup>3</sup> Dominic Fong,<sup>1</sup>  
Mathias Zitt,<sup>3</sup> Thomas Brunhuber,<sup>2</sup>  
Georg Schäfer,<sup>2</sup> Guenther Gastl,<sup>1</sup> and  
Gilbert Spizzo<sup>1</sup>

<sup>1</sup>Division of Hematology & Oncology, <sup>2</sup>Department of Pathology, and <sup>3</sup>Department of Surgery, University of Innsbruck, Innsbruck, Austria

## ABSTRACT

**Purpose:** Gallbladder carcinoma is an aggressive type of cancer that is difficult to cure by conventional procedures. There thus is a need to identify novel molecular markers for the assessment of prognosis and as potential therapeutic targets. This retrospective study was designed to investigate the prognostic significance of epithelial cell adhesion molecule (Ep-CAM) overexpression in human gallbladder carcinoma.

**Experimental Design:** Ep-CAM expression was examined immunohistochemically on paraffin-embedded tissue specimens from 99 patients who underwent surgical treatment for gallbladder carcinoma in the period between August 1988 and May 1999.

**Results:** Ep-CAM overexpression was found in 63 (63.6%) of the tumor samples. Kaplan-Meier curves showed that Ep-CAM overexpression was significantly related to decreased overall survival ( $P < 0.01$ ). Overall survival gradually worsened with increasing Ep-CAM scores. Notably, in the subgroup of pT1 tumors ( $n = 17$ ), patients without Ep-CAM overexpression had a 5-year overall survival rate of 100% compared with 38% ( $P = 0.01$ ) for patients with Ep-CAM-overexpressing tumors. By univariate analysis, no correlation was found with conventional clinicopathological parameters. Multivariate analysis, including Ep-CAM expression, pT stage, tumor grade, and resection margin involvement, showed that Ep-CAM overexpression was an

independent prognostic marker in gallbladder carcinoma ( $P = 0.03$ ; relative risk, 1.8).

**Conclusions:** These results demonstrate for the first time that Ep-CAM overexpression is an independent prognostic marker in gallbladder carcinoma and that its prognostic impact should be validated prospectively. Furthermore, the Ep-CAM antigen represents an attractive target for specific therapies with monoclonal antibodies or specific vaccines in patients with Ep-CAM-overexpressing gallbladder carcinoma.

## INTRODUCTION

Gallbladder carcinoma (GBC) is an aggressive and mostly lethal malignancy. It represents the fifth most common malignant neoplasm of the digestive tract and is often diagnosed incidentally after laparotomy or laparoscopy performed for benign gallbladder disease. The precise etiology is not known, but the presence of gallstones has been reported to be associated with an increased risk for GBC (1). Decisions regarding the use of surgical or palliative treatment in patients with GBC mostly rely on prognostic factors such as the T stage (2), defined by the depth of invasion in the gallbladder wall. Other factors predicting poor prognosis are a high grade and lymph node involvement. To date, no reliable molecular prognostic factors have been established for GBC. Data are limited regarding consistent molecular changes associated with gallbladder carcinogenesis and disease progression. In recent years, several molecular markers with prognostic impact on GBC have been described, such as Ki-67 (3), cyclin D1 (4), MUC1 (5), p53 (6, 7), p16 (7), p21 (8), p27Kip1 (9), RCAS1 (10), K-ras (11), and c-erb-B2 (12).

Epithelial cell adhesion molecule (Ep-CAM; also called GA733-2, 17-1A, and KSA) is a 40-kDa transmembrane glycoprotein expressed on most human epithelial cells (13, 14) that functions as a homotypic intercellular adhesion molecule (15). Overexpression of Ep-CAM has been described in many types of carcinoma (16). We have recently reported that Ep-CAM overexpression correlates with prognosis in breast cancer (17). Others have found that progressive transformation in cervical intraepithelial neoplasia is accompanied by increasing Ep-CAM antigen expression (18). Likewise, in squamous cell cancers of the lung, Ep-CAM expression increases with worsening grade and TNM stage (19). Thus, most studies support the view that Ep-CAM is linked to the progression of various epithelial cancers.

Recently, Ep-CAM has attracted major interest as a molecular target for therapeutic interventions. A chimeric mouse monoclonal antibody named edrecolomab used in patients with Dukes C colorectal carcinoma reduced the recurrence and overall mortality rates (20). Further work has led to a fully humanized form of an IgG1 monoclonal antibody recognizing Ep-

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**Requests for reprints:** Gilbert Spizzo, Division of Hematology and Oncology, Anichstrasse 35, A-6020 Innsbruck, Austria. Phone: (43) 512 504 80642; Fax: (43) 512 504 5615; E-mail: Gilbert.Spizzo@uibk.ac.at.

Table 1 Correlation of epithelial cell adhesion molecule with conventional clinicopathological parameters in 99 patients with gallbladder carcinoma

Characteristics	Total patients (n)	Ep-CAM <sup>a</sup> overexpression				P <sup>b</sup>
		No		Yes		
		n	%	n	%	
Age at diagnosis						
<70 years	39	14	36	25	64	0.938
≥70 years	60	22	37	38	63	
Histological type						
Adenocarcinoma	91	32	35	59	65	0.528
Squamous cell carcinoma	5	2	40	3	60	
Neuroendocrine carcinoma	3	2	67	1	33	
Histological grade						
I	18	8	44	10	56	0.677
II	38	14	37	24	63	
III	43	14	33	29	67	
pT						
1	17	8	47	9	53	0.712
2	36	11	31	25	69	
3	35	13	37	22	63	
4	11	4	36	7	64	
Sex						
M	27	7	26	20	74	0.186
F	72	29	40	43	60	
Resection margin						
Negative	36	16	44	20	56	0.117
Positive	53	15	28	38	72	
Unknown <sup>c</sup>	10					

<sup>a</sup> Ep-CAM, epithelial cell adhesion molecule.

<sup>b</sup>  $\chi^2$  test.

<sup>c</sup> Excluded from P calculations.

CAM, named MT201, that was shown to mediate higher antibody-dependent cellular cytotoxicity than edrecolomab *in vitro* (21). Other promising Ep-CAM antibodies conjugated to interleukin-2 (22) or immunotoxins (23) are being evaluated in clinical trials.

GBC is one of the most aggressive type of cancers and is difficult to cure by conventional procedures. There thus is an urgent need to identify novel molecular markers for the assessment of prognosis and as potential therapeutic targets. In this retrospective study, we evaluated the prognostic impact of Ep-CAM expression for GBC.

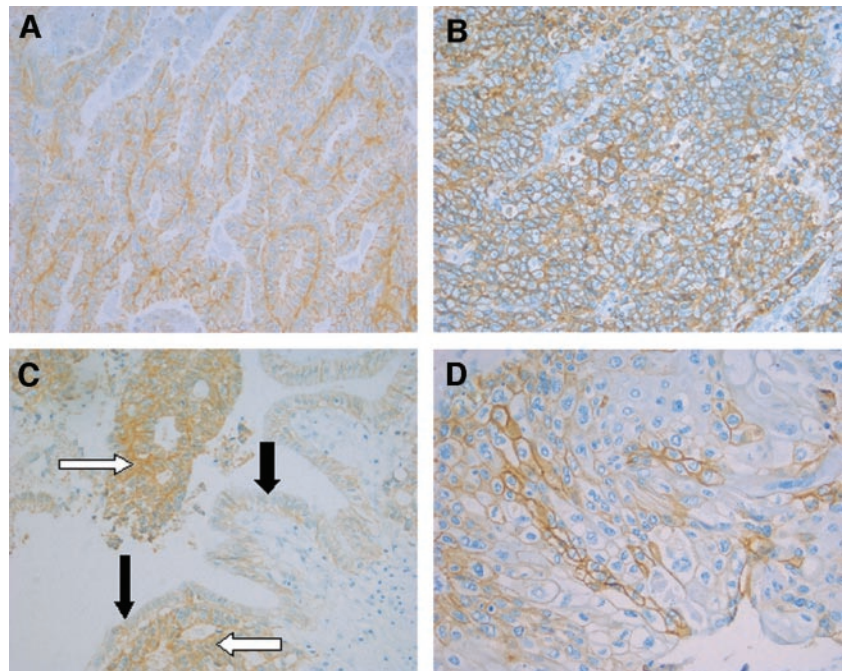
## PATIENTS AND METHODS

**Patients.** Specimens from 99 patients with GBC who consecutively underwent surgery between August 1988 and May 1999 in different Tyrolean hospitals were included in the present study. All specimens had been collected, diagnosed, and stored by the Central Pathology Laboratory at the Department of Pathology, Innsbruck University Hospital. Samples were taken from 72 females and 27 males (median age, 71 years; range, 37–89 years). GBC was diagnosed on the basis of histological findings and was staged according to the TNM system (nodal status and depth of wall infiltration) according to the American Joint Committee on Cancer (24). The slides of all tumors were reviewed by one pathologist (P. O.) to define the histological grade according to the criteria described by Albores-Saavedra *et al.* (25). In 19 patients lymph nodes were dissected and the nodal status was evaluated. The clinicopathological features of the patients are summarized in Table 1. Forty-one patients underwent simple cholecystectomy for chronic cholecystitis

and/or cholelithiasis, and the diagnosis of GBC was incidental. The other 58 patients underwent surgery after finding of clinical or radiological features for GBC. Follow-up time ranged from 1 to 144 months (median, 19 months). Before May 2002, 80 of the 99 patients had died. Seventy-six patients died from progressive disease, whereas 4 patients died from other disease (3 patients died from cardiac and 1 patient from pulmonary disease).

**Immunohistochemistry.** Ep-CAM expression was determined by immunohistochemistry on paraffin-embedded tissue specimens, using the murine monoclonal antibody ESA (NovoCastra, Medac GmbH, Hamburg, Germany) as described previously (17). Briefly, 5- $\mu$ m sections were cut from paraffin-embedded tissue blocks, mounted on adhesive-coated glass slides, deparaffinized, and rehydrated. Endogenous peroxidase was blocked with methanol containing 30% hydrogen peroxide over 20 min. Pretreatment consisted of a 20-min incubation period in Pronase solution. After washing in Tris buffer, slides were incubated for 30 min at room temperature with the primary antibody (ESA; 1:50 dilution), after which a peroxidase-conjugated goat antimouse antibody ready for use (EnVision; DAKO, Vienna, Austria) was added for immunostaining for 60 min. Slides were then placed in the chromogen, which consisted of diaminobenzidine solution containing 30% hydrogen peroxide. Finally, slides were counterstained with Mayer's Hemalum solution. Ep-CAM overexpression was evaluated by two independent assessors (M. V. and P. O.) by use of light microscopy. Breast cancer samples with different Ep-CAM expression (no, low, moderate, or high expression) and breast cancer cell lines (Ep-CAM positive, MCF-7; Ep-CAM negative, Hs-578T) were used as negative and positive controls. Reading of tissue slides

**Fig. 1** *A*, highly differentiated gallbladder adenocarcinoma stained for epithelial cell adhesion molecule (Ep-CAM) by immunohistochemistry. Expression was moderate (intensity score of 2) in 40% of tumor cells (proportion score of 2). Shown is a tumor sample without Ep-CAM overexpression (total score of 4). *B*, poorly differentiated gallbladder carcinoma with strong Ep-CAM expression (intensity score of 3) in 90% of tumor cells (proportion score of 4). Shown in a tumor sample with Ep-CAM overexpression (total score of 12). *C*, gallbladder adenocarcinoma with Ep-CAM overexpression (white arrows) and adjacent normal gallbladder epithelium (black arrows) with weak Ep-CAM expression. *D*, squamous cell carcinoma presenting with strong Ep-CAM expression (intensity score of 3) in 20% of tumor cells (proportion score of 2). Shown is a tumor sample with Ep-CAM overexpression (total score of 6).



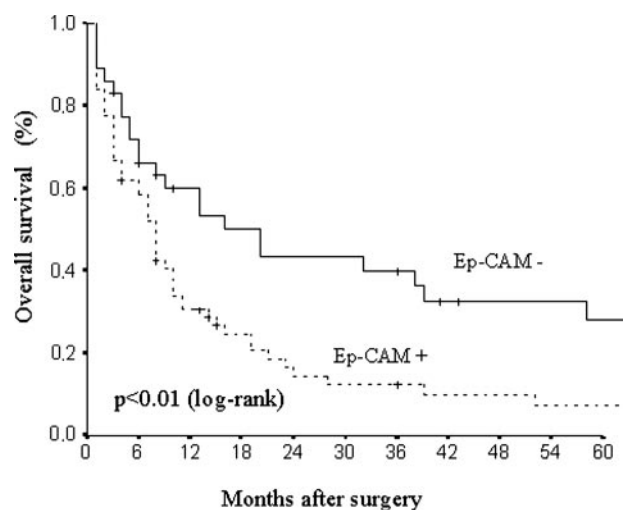
was blinded, and both assessors were unaware of clinical outcomes. In case of discrepant evaluation, cases were reevaluated on a double-headed microscope to achieve a consensus. Antigen expression was defined as the presence of specific staining on the surface membranes of tumor cells. Ep-CAM overexpression was evaluated by calculating a total immunostaining score as the product of a proportion score and an intensity score. The proportion score described the estimated fraction of positive stained tumor cells (0, none; 1, <10%; 2, 10–50%; 3, 50–80%; 4, >80%). The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranged from 0 to 12. As described previously, Ep-CAM “overexpression” was defined as a total score >4 (17). For detailed expression analysis, Ep-CAM expression was divided in four subgroups (group 1, score = 0; group 2, score = 1–4; group 3, score = 6 and 8; group 4, score = 9 and 12).

**Statistical Analysis.** The associations between Ep-CAM expression and clinicopathological variables were assessed by the  $\chi^2$  test. Survival curves were plotted according to the Kaplan-Meier method and compared by the log-rank test. The significance of various parameters for survival was analyzed by the Cox proportional hazards model in the multivariate analysis.  $P < 0.05$  was considered statistically significant. Lymph node involvement was not considered for statistical analysis because of the few patients with reported nodal status ( $n = 19$ ).

## RESULTS

Using our previously defined criteria (17), we found Ep-CAM overexpression (Fig. 1) in 63 (63.6%) of the 99 patients. By subgroup analysis 9 tumor samples (9.1%) lacked Ep-CAM expression (score 0), 27 samples (27.3%) showed weak expression (scores 1–4), 49 samples (49.5%) stained moderately (scores 6 and 8), and 14 samples (14.1%) exhibited strong

Ep-CAM expression (score 9 and 12). By univariate analysis comparing Ep-CAM expression to age, sex, grade, pT, resection margin status, and histological subtype, no correlation was found between Ep-CAM expression and conventional clinicopathological features (Table 1). Kaplan-Meier analysis and the log-rank test were used to calculate the impact of classical clinicopathological features and Ep-CAM expression on survival. As expected, T stage ( $P < 0.01$ ), grade ( $P < 0.01$ ), and positive resection margins ( $P < 0.01$ ) were of prognostic value,



**Fig. 2** Prognostic significance of epithelial cell adhesion molecule (Ep-CAM) overexpression in 99 patients with gallbladder carcinoma regarding overall survival. *Ep-CAM+*, patients with Ep-CAM-overexpressing tumors ( $n = 63$ ); *Ep-CAM-*, patients with tumors not overexpressing Ep-CAM ( $n = 36$ ).



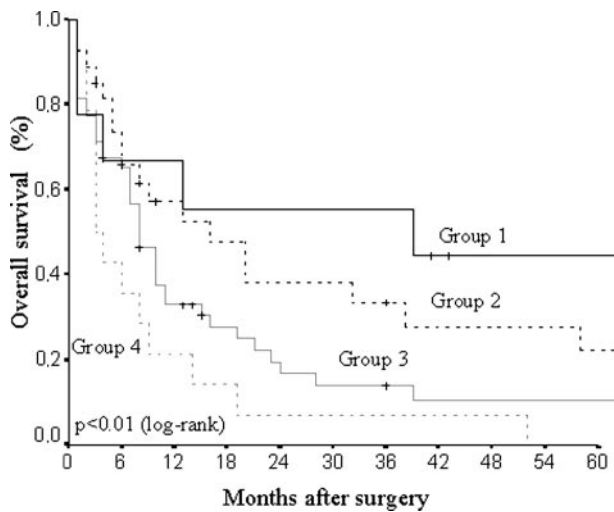


Fig. 3 Prognostic significance of epithelial cell adhesion molecule (Ep-CAM) overexpression in 99 patients with gallbladder carcinoma regarding overall survival. Group 1, patients with gallbladder carcinoma presenting with an Ep-CAM score of 0 (black solid line;  $n = 9$ ). Group 2, patients with gallbladder carcinoma presenting with an Ep-CAM score of 1–4 (black dashed line;  $n = 27$ ). Group 3, patients with carcinomas presenting with Ep-CAM scores of 6 and 8 (gray solid line;  $n = 49$ ). Group 4, patients with gallbladder carcinoma presenting with Ep-CAM scores of 9 and 12 (gray dashed line;  $n = 14$ ).

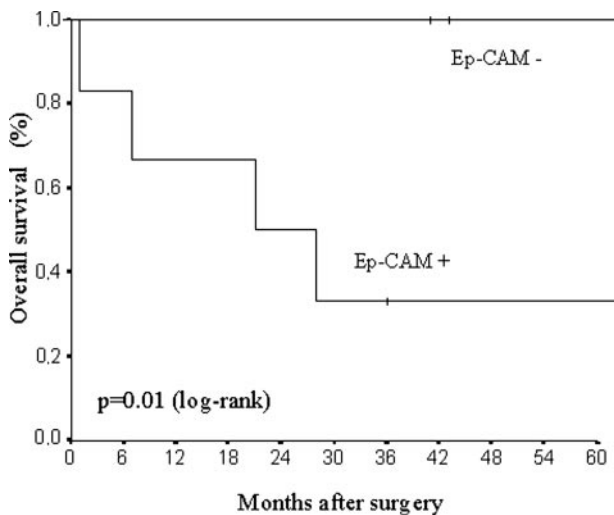


Fig. 4 Prognostic significance of epithelial cell adhesion molecule (Ep-CAM) overexpression in 17 patients with pT1 gallbladder carcinoma regarding overall survival. Ep-CAM+, patients with Ep-CAM-overexpressing tumor ( $n = 9$ ); Ep-CAM-, patients with tumors not overexpressing Ep-CAM ( $n = 8$ ).

whereas histological subtype and patient age and sex were prognostically not relevant. Of note, Ep-CAM overexpression was associated with a poor prognosis (Fig. 2;  $P < 0.01$ ). The median survival times of patients presenting tumors with and without Ep-CAM overexpression were 8 and 16 months, respectively. Accordingly, the estimated 5-year overall survival rates were 7% and 33%. By subgroup analysis, the prognosis regard-

ing overall survival gradually worsened with increasing Ep-CAM scores (Fig. 3;  $P < 0.01$ ). The median survival decreased from 39 months in group 1 (score 0) to 16 months in group 2 (scores 1–4), to 8 months in group 3 (scores 6 and 8) and was 3 months in group 4 (scores 9 and 12).

The prognostic impact of Ep-CAM overexpression differed among various pathological T stages. In patients with pT1 tumors ( $n = 17$ ), Ep-CAM overexpression ( $n = 9$ ) was highly significant for prognosis (Fig. 4;  $P = 0.01$ ). Estimated 5-year overall survival rates were 38% and 100% in patients with and without Ep-CAM overexpression, respectively. The median survival time of patients with Ep-CAM-overexpressing tumors was 52 months, but had not reached for patients without Ep-CAM overexpression by the time of this report. In contrast, Ep-CAM overexpression was not predictive for prognosis in the subgroup of patients with pT2, pT3, or pT4 tumors.

In the subgroup of patients with histological types other than adenocarcinoma (five patients with squamous cell carcinoma and three patients with neuroendocrine carcinoma), no prognostic impact of Ep-CAM overexpression was observed. In the subgroup of patients with adenocarcinoma, the median survival times of patients with tumors with and without Ep-CAM overexpression were 8 and 20 months, respectively. Multivariate analysis including pT stage, grade, resection margin involvement, and Ep-CAM overexpression (Table 2) identified Ep-CAM overexpression as an independent prognostic marker ( $P = 0.03$ ; relative risk, 1.8; 95% confidence interval, 1.0–3.2). Additionally, pT stage and the involvement of resection margins but not tumor grade were independent prognostic markers.

## DISCUSSION

This is the first report demonstrating an independent prognostic value of Ep-CAM overexpression for GBC. Similar results were obtained by our group (17) and by Tandon *et al.* (26) in human breast cancer. It remains to be seen whether in these carcinomas Ep-CAM expression represents merely a surrogate marker for prognosis or whether it plays a pathogenic role for carcinogenesis and tumor progression. Although Ep-CAM functions as a homotypic adhesion molecule in normal epithelial morphogenesis, it also appears to contribute to the malignant phenotype of cancer and to affect prognosis. To date, however, final proof for a direct carcinogenic or tumor promoting role of Ep-CAM expression is lacking.

Table 2 Multivariate analysis (Cox regression) of different prognostic parameters in patients with gallbladder carcinoma

	OS <sup>a</sup>		
	P	RR	95% CI
pT	0.006		
1 vs. 2	0.003	6.9	2.0–24.5
1 vs. 3	0.001	11.0	2.8–42.8
1 vs. 4	0.001	12.3	2.8–54.3
Resection margin	0.02	2.2	1.1–4.4
Ep-CAM overexpression	0.03	1.8	1.0–3.2
Grade	NS		

<sup>a</sup> OS, overall survival; RR, relative risk; CI, confidence interval; Ep-CAM, epithelial cell adhesion molecule; NS, not significant.

Circumstantial evidence for a tumor-promoting role comes from data indicating a correlation of Ep-CAM expression with proliferation in cervical intraepithelial neoplasia (18) and squamous cell cancer of the lung (19). Even in normal liver, tissue regeneration is associated with increased Ep-CAM expression on hepatocytes (27). In transformed epithelial cell lines, enhanced cell proliferation coincided with increased Ep-CAM expression (28). Interestingly, in GBC molecular markers associated with enhanced cell proliferation, such as strong expression of Ki67, cyclin D1, p16, and p21 and low expression of p27KIP1, were all indicative of a poor prognosis. Thus, further studies need to address the relationship between the expression of these proliferation-associated markers and Ep-CAM expression as well as their contributions for predicting prognosis.

In breast cancer, we noted that Ep-CAM, Her-2/neu (29), and cyclooxygenase-2 (30) have an independent and additive prognostic impact. Her-2/neu was found to be predictive for prognosis in GBC as well (12). Studies are in progress at our institution to develop a prognostic model for GBC with a panel of immunohistochemically detectable molecular markers comprising Ep-CAM, Her-2/neu, and cyclooxygenase-2 and proliferation-associated molecules such as Ki67.

The only potentially curative therapy for GBC is surgical resection. Unfortunately, most patients with this type of cancer present with unresectable disease. Decisions regarding the extent of surgical management of patients with GBC strongly depends on the pT status. Patients with potentially resectable pT2, pT3, or pT4 stage disease who underwent radical cholecystectomy showed a significantly better survival than patients treated with simple cholecystectomy (for a review, see Ref. 31). However, the decision to treat T1 disease with simple cholecystectomy or with radical cholecystectomy is not clearly defined. In this study, Ep-CAM overexpression was highly significant in patients with pT1 disease (Fig. 4). Thus it would be tempting to suggest radical cholecystectomy for all operable Ep-CAM-overexpressing GBC and dependent on the nodal status administer additional adjuvant radiation and systemic chemotherapy within prospectively randomized clinical trials.

If our preliminary results can be validated in larger series, Ep-CAM status may indeed become a means to guide treatment decisions regarding surgical management of GBC as well as the additional use of adjuvant local radiation and systemic chemotherapy. Furthermore, the Ep-CAM antigen represents a potential GBC target for specific therapies with monoclonal antibodies or vaccines in the adjuvant and palliative setting.

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