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Effect of EpCAM, CD44, CD133 and CD166 expression on patient survival in tumours of the ampulla of Vater

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ABSTRACT

Background Carcinomas of the Vaterian system are rare and presumably arise from pre-existing adenomas. According to the cancer stem cell (CSC) hypothesis, only a small subset of tumor cells has the ability to initiate and develop tumor growth. In colorectal cancer, CD44, CD133, CD166 and EpCAM have been proposed to represent CSC marker proteins and their expression has been shown to correlate with patient survival.

Aims To evaluate a potential role of these CSC proteins in tumors of the ampulla of Vater, we investigated their expression in 175 carcinoma, 111 adenoma and 152 normal mucosa specimens arranged in a Tissue Microarray format.

Materials and methods Membranous immunoreactivity for each protein marker was scored semi-quantitatively by evaluating the number of positive tumor cells over the total number of tumor cells. Median protein expression levels were used as cut-off scores to define protein marker positivity. Clinical data including survival time were obtained by retrospective analysis of medical records, tumor registries or direct contact.

Results The expression of all evaluated marker proteins differed significantly between normal mucosa, adenoma and carcinoma samples. In all markers, we found a tendency towards more constant expression from normal to neoplastic tissue. EpCAM expression was significantly correlated with better patient survival. The increased expression of CD44s, CD166 and CD133 from normal mucosa samples to adenoma and carcinoma was linked to tumor progression. However, there was no statistically significant correlation with survival.

Conclusion Our findings indicate, that in ampullary carcinomas, loss of expression of EpCAM may be linked to a more aggressive tumor phenotype.

INTRODUCTION

The ampulla of Vater combines the terminal and common segment of the bile and pancreatic duct before they enter the duodenum.[1] Carcinomas originating from this complex anatomical unit are uncommon and have an incidence of approximately four to six cases per million population.[2][3] Carcinomas of the papilla of Vater, defined as junction of the biliary, and pancreatic ducts within the duodenum account for 6%—20% of all peripancreatic tumours[4] and represent 10%—50% of all cancers resected by pancreaticoduodenectomy.[5] They can be sited in the ampulloduodenal part of

the papilla of Vater, which is lined by intestinal mucosa. They also can develop in deeper parts of the ampulla, which are lined by pancreaticobiliary duct mucosa. Clinically, tumours of the ampulla of Vater are rapidly detected due to biliary outflow obstruction.[6][7] Early symptoms as well as differences in tumour biology are held responsible for their favourable clinical outcome (median survival 30—50 months, 5-year survival rate 21%—64%).[8][9] Histologically, intestinal, pancreaticobiliary, intestinal-mucinous, invasive papillary and poorly differentiated subtypes can be distinguished.[10] The subtypes differ in several clinical and histological aspects including cell type-specific markers, oncogene expression, modes of tumour spread as well as extent and interaction with the extracellular matrix.[11] Most authors agree that local spread of the tumour (T stage) is the only significant and independent prognostic factor for this cancer, whereas the predictive value of tumour grade and lymph node metastases is still debated.[12][13] More recent research data suggest that the prognosis of ampullary cancer may be related to the histological differentiation in intestinal or pancreatobiliary types.[14] In the last years, several molecular markers have been proposed as additional prognostic factors. However, most of these studies have yielded conflicting results and have not been still validated by other reports.[15–19] Several sources of discrepancy between different reports have been acknowledged mainly due to non-standardised assays often performed on underpowered patient samples that are too small to enable meaningful conclusions to be drawn. Therefore, there is undoubtedly a need for additional prognostic markers for such neoplasia. Recent findings support the concept that cells with the properties of stem cells are integral to the development and perpetuation of several forms of human cancer.[20][21] Cancer stem cells (CSCs) have low replicative ability, multipotency and resistance to apoptosis and are responsible for tumour development.[22] In the different types of digestive tumours, different sets of markers have emerged as the most useful for the identification of CSC. In particular, in intestinal as well as in pancreatic cancer, some markers including CD44, EpCAM, CD166 and CD133 have been indicated as possible CSCs markers. Furthermore, in colorectal cancer, we have shown that their expression inversely correlated with patient survival.[23] However, conflicting results have been

reported about the role of some putative CSC markers in gastrointestinal tract tumours. In particular, contradictory findings have been reported about the association of CD44, in particular of its v6 splicing variant, and tumour progression.[24–26] Furthermore, while CD133 molecule was initially identified as a reliable CSC marker in human colorectal cancers,[27 28] a subsequent study has shown that in both mouse and human colorectal cancers, CD133 expression is not restricted to rare cell subsets, but it is detectable in a large majority of tumour cells, irrespective of their tumourigenicity.[29] Because of the lacking studies dealing with CSC markers in ampullary tumours, the aim of this study was to elucidate the expression and the prognostic role of CD133, CD166, CD44s, EpCAM expression in ampullary tumours by using a tissue microarray (TMA) including 175 carcinoma, 111 adenoma and 152 normal mucosa specimens of the papilla of Vater.

MATERIAL AND METHODS
Ethics
The study has been approved by the institutional review board of the Department of Pathology, University of Basel, Switzerland. All the analyses were performed according to the ethical standards required by each local ethic committee.

Patients' characteristics and tissue samples
Patients' characteristics have been previously described by our study group.[30 31] Briefly, the files of the Institute of Pathology, University Hospital Basel (Switzerland), the Institute of Pathology, University of Regensburg (Germany), the Institute of Pathology Nuernberg and the Anatomic Pathology Unit, Department of Human Morphology, University of Insubria, Varese (Italy), were searched for adenomas or carcinomas of the ampulla of Vater over the period from 1985 to 2005. In total, 175 carcinoma, 111 adenoma and 152 normal mucosa samples were retrieved. Sufficient paraffin-embedded tissue for TMA construction was available in all cases. The male-to-female ratio was 3:2; mean age at diagnosis was 63 years (range 15–81 years). To our knowledge, no case was associated with Familial adenomatous polyposis (FAP).

TMA construction
TMAs were constructed from formalin-fixed and paraffin-embedded specimens using a custom-built instrument (Beecher Instruments, Silver Spring, Maryland, USA) as previously described.[30 31] Briefly, H&E-stained sections were obtained from each selected primary block (donor block) to define representative tissue regions. Core biopsies (0.6 mm cylinders) were taken from the selected tissue regions and then transferred to a paraffin recipient block. The resulting TMA was cut into 4 μm sections, which were used for immunohistochemistry. The number of punches per patient ranged from one to three for both normal tissue and carcinoma and from one to five for adenomas. If more than one punches was obtained, the additional punches were taken from different representative blocks.

Histology and immunophenotyping
All tumours were classified according to the guidelines of the Armed Forces Institute of Pathology using only H&E stains.[10] Mild dysplasia was designated as low-grade dysplasia, whereas moderate and severe dysplasia was considered as high-grade dysplasia. Carcinomas histologically indistinguishable from colorectal carcinomas were classified as intestinal types, whereas carcinomas showing a dense desmoplastic stroma surrounding small glands or solid nests of tumour cells were referred to as the pancreaticobiliary subtype. Invasive papillary carcinomas typically formed papillary and micropapillary structures in their invasive component and poorly differentiated adenocarcinomas lacked histologic features of glandular or other differentiation. Additionally, an intestinal-mucinous subtype, characterised by any mucinous differentiation, was defined.

For immunohistochemistry, sections were pre-treated with CC1 (Ventana Medical Systems, Tucson, Arizona, USA) and incubated with primary antibodies against CD133, CD44s, CD166 and EpCAM (table 1). Staining procedures were performed on a Benchmark immunohistochemistry staining system (Ventana Medical Systems) using iVIEW-DAB as chromogen.

Membranous immunoreactivity for each protein marker was scored semiquantitatively by evaluating the number of positive tumour cells over the total number of tumour cells. Scores were assigned using 5% intervals and ranged from 0% to 100%. All tissues were scored by an experienced pathologist (LT), blinded to clinicopathological information. To define interobserver agreement, all samples were examined independently by a second pathologist (DB).

Statistical analysis
Statistical correlations between categorical variables were tested using a χ^2 or Fisher exact test, where appropriate. Differences in patient survival were demonstrated using the Kaplan–Meier method and analysed using the log-rank test in univariate analysis. All tests were two sided. p Values <0.05 were considered statistically significant. Cut-off scores were selected by evaluating the receiver operating characteristic curves for each protein marker and the end-point survival. The point on the curve with the shortest distance to the coordinate (0, 1) was selected as the threshold value to classify cases as 'positive/overexpressing' or 'negative/loss'.[32] Analysis was performed using SAS V.9.1 (SAS Institute).

RESULTS
Patients' characteristics and tissue samples
Overall, 175 carcinoma, 111 adenoma and 152 normal mucosa samples were retrieved. In patients with carcinomas, we found 19 pT1 (11%), 59 pT2 (34%), 63 pT3 (36%) and 13 pT4 (7%) tumours as well as 17 G1 (10%), 82 G2 (47%) and 55 G3 (31%) cases (no data concerning T stage and grading in 21 samples). Seventy-three (42%) carcinoma patients were node positive and two (1%) had haematogenous metastases at initial diagnosis.

Full clinical data including survival time were available in 133 patients with ampullary carcinoma (76%). Patients were studied up to 164 months after operation. Median follow-up time was 36 months.

The histological classification of 175 ampullary carcinomas identified 85 intestinal types, 42 pancreaticobiliary types, 23 poorly differentiated adenocarcinomas, 16 intestinal-mucinous types and nine invasive papillary types.

Table 1 Primary antibodies against CD133, CD44, CD166 and EpCAM

Antibody	Dilution/detection	Pretreatment
CD44 (Dako, DF1485)	1:50/BOND	BOND ER2
CD133 (cell signalling, C24B9)	1:100/BOND	Steamer 120°C, pH8
CD166 (Novocastra, MOG/07)	1:200/BOND	BOND ER2
EpCAM (Novocastra, VU-1D9)	1:200/BOND	BOND ER2

Original article

Figure 1 Immunophenotyping in tissue samples from patients with ampullary carcinoma (400×). (A) CD44. (B and C) CD133. (D and E) CD166. (F) EpCAM.
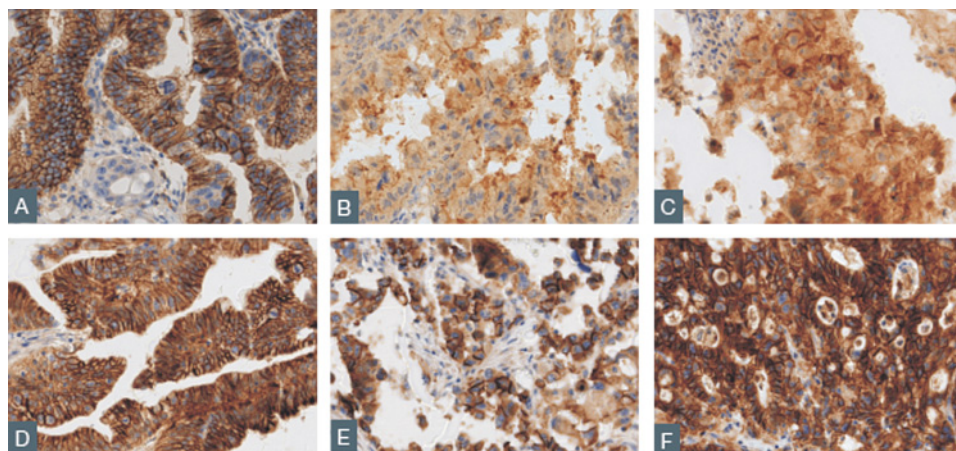
Histological grading was evaluable in 76 of 111 (68%) adenomas and disclosed low-grade dysplasia in 57 of 76 (75%) and high-grade dysplasia in 19 of 76 (25%) cases. All adenomas demonstrated tubular or tubulovillous architecture. Seventy-eight of 111 (70%) adenoma samples were derived from patients with coexisting carcinoma.

Immunophenotyping

Tissue samples of ampullary carcinoma patients expressing CD44, CD133, CD166 and EpCAM are shown in figure 1. Moreover, in table 2, the distribution of the different biomarkers across different diagnostic categories is shown.

The expression of all marker proteins differed significantly between carcinoma, adenoma and normal mucosa samples (table 3).

We have also evaluated the positivity in the two principal histologic types (intestinal type vs pancreatobiliary type). EpCAM was significantly more expressed in intestinal type (table 4).

We have tried also to evaluate if there is some difference between adenomas without coexisting carcinoma and adenomas without coexisting carcinomas. Only CD44 was significantly more expressed in adenomas with coexisting carcinomas (p=0.043).

No difference was found between low-grade and high-grade adenomas (data not shown).

Survival

Five-year survival (95% CI) was 45.2 (34 to 56) in EpCAM-positive versus 28.2 (11 to 48) in EpCAM-negative patients (p<0.05). EpCAM was not an independent prognostic factor after adjusting for pT and pN stages. Survival curves of both patient groups using the Kaplan−Meier method are demonstrated in figure 2.

DISCUSSION

Tumours of the papilla of Vater are a relatively rare neoplastic entity that came into focus in recent years. Significant overlap exists in phenotypic and molecular characteristics between ampullary and colorectal carcinomas. As in colorectal cancer, the development of ampullary carcinoma from adenomas as precancerous lesions has been well documented, and studies investigating molecular alterations associated with the proposed adenoma−carcinoma sequence have been also performed, including our group.[33] However, still missing is a comprehensive analysis of the expression of putative CSC markers in very large groups of patients, amenable to detailed statistical analysis. Moreover, the prognostic significance of the co-expression of multiple CSC markers within the same tumour has not been evaluated so far.

This is the first systematic study assessing the prognostic value of four CSC markers, namely EpCAM, CD44, CD133 and CD166, in a large series of patients with ampullary tumours.

Table 2 Distribution of biomarkers between different diagnostic categories

	Number of cases within each expression category							
	0%	1%−5%	6%−20%	21%−40%	41%−60%	61%−80%	81%−100%	Sum
Normal								
CD133	9	63	30	0	1	1	0	104
CD44	67	20	8	7	3	6	4	115
CD166	81	5	6	3	6	1	13	115
EpCAM	7	1	0	0	2	2	89	101
Adenoma								
CD133	10	61	14	0	0	0	0	85
CD44	25	13	17	5	3	14	12	89
CD166	58	9	5	1	4	3	6	86
EpCAM	0	0	0	1	2	1	89	93
Cancer								
CD133	16	83	45	6	1	0	0	151
CD44	38	15	27	19	9	18	18	144
CD166	84	19	14	9	5	7	10	148
EpCAM	2	0	1	1	3	3	136	146

Table 3 Expression of all evaluated marker proteins in normal mucosa, adenoma and carcinoma samples

	Normal	Adenoma	Carcinoma	p Value
CD44+ (>5%)	29/118 (24.6%)	52/90 (57.8%)	91/145 (62.8%)	<0.001
CD133+ (>5%)	30/104 (28.9%)	11/74 (12.9%)	44/151 (29.1%)	0.012
CD166+ (>0%)	34/120 (28.3%)	28/87 (32.2%)	64/151 (42.4%)	0.044
EpCAM +(100%)	89/104 (85.6%)	88/93 (94.6%)	115/145 (79.3%)	0.005

The overexpression of EpCAM was significantly correlated with better survival time. The increasing expression of CD44, CD166 and CD133 from normal mucosa samples to adenoma and carcinoma was linked to tumour progression. However, there was no statistically significant correlation with survival.

EpCAM is a glycosylated, 30—40 kDA type I membrane protein, which is expressed in a variety of human epithelial tissue cancers, as well as in progenitor and stem cells. It is composed of an extracellular domain with epidermal growth factor and thyroglobulin repeat-like domains, a single transmembrane domain and a short 26 amino acid intracellular domain called EpICD. In normal cells, EpCAM is predominantly located in intercellular spaces, where epithelial cells form very tight junctions. Therefore, on normal epithelia, it is sequestered and may be much less accessible to antibodies than in cancer tissue, where it is homogeneously distributed on the cell surface. Furthermore, EpCAM is part of the signature of cancer-propagating cells in numerous solid tumours as well as in normal progenitor and stem cells.[34]
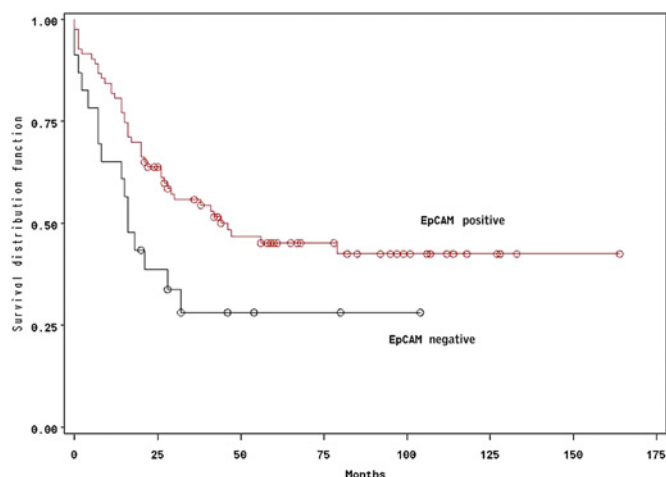
EpCAM was one of the first tumour-associated antigens identified in the late 1970s. Systematic analysis of EpCAM expression for intensity and frequency showed that EpCAM is expressed on essentially all human adenocarcinoma, on certain squamous cell carcinoma, on retinoblastoma and on hepatocellular carcinoma.[35]

Importantly, EpCAM is part of the signature of cancer-propagating cells in numerous solid tumours and of normal progenitor and stem cells.[34] The controversial biological role of EpCAM has recently been discussed by van der Gun et al.[36] It is of interest that EpCAM overexpression has been associated with both decreased and increased survival time. EpCAM exerts different effects on cell adhesion, either promoting or preventing metastasis.[36] The correlation of EpCAM expression and poor survival has been described in several tumour types, including invasive breast cancer,[37] urothelial carcinoma of the bladder,[38] gallbladder carcinoma[39 40] and squamous cell carcinoma of the oesophagus.[41] In different tumours, studies on EpCAM-directed immunotherapeutic therapies are currently in clinical development. Therapeutic trials of monoclonal antibodies directed against EpCAM have shown that they may induce antibody-based cellular cytotoxicity by adhering to cytokines such as interleukin 2 or complement-based cytotoxicity by activating T cytotoxic cells.[42 43]

Figure 2 Survival of patients with EpCAM-positive and -negative ampullary carcinoma using the Kaplan—Meier method.

Previous studies have yielded conflicting results regarding EpCAM expression and survival in ampullary carcinoma. By Scheunemann et al,[44] EpCAM expression has been associated with poorer survival in tumours of the papilla of Vater. This study, however, was mainly focused on the frequency and prognostic impact of minimal tumour cell spread in lymph nodes classified as 'tumour free' in routine histopathologic evaluation, and EpCAM expression in primary tumours was not analysed.

More recently, Fong et al[45] were unable to prove a prognostic value of EpCAM overexpression in pancreatic and ampullary carcinoma. In contrast, in our study, we showed that decreased EpCAM membranous expression significantly correlated with biological features of aggressive tumour behaviour. Our study therefore suggests that diminished EpCAM expression is related to tumour invasiveness and progression and it is linked to a more aggressive tumour phenotype. This could also be confirmed by the more frequent expression of EpCAM in intestinal-type than in pancreatobiliary-type tumours. Intestinal-type tumours are in fact associated with a better prognosis.[14]

Several reasons for the discrepancies between our results and previous studies can be hypothesised including differences in sample size (power for detecting prognostic differences), methodology (TMA vs whole tissue sections), different clones of antibody and, most importantly, the choice of cut-off scores for the definition of positive staining or staining intensity.

EpCAM is intensely used as a therapeutic target for antibody-based approaches. Future development of EpCAM-directed therapeutics may profit from newly identified functions of EpCAM as mitogenic signal transducer in various ways. An important insight is that EpCAM is apparently needed to maintain distinct cancer cell attributes[46] and, potentially, the CSC phenotype as well. This function can reduce the risk of immune escape by loss of EpCAM target expression from cancer cells. EpCAM-directed therapies may be selective for those cancer cells with the strongest negative impact on prognosis and for cancer-propagating subsets of malignant cells.

Discrepant results have also been reported regarding the effect of *CD44* gene or protein and its splice variants on survival in tumour patients and it is an important receptor that binds hyaluronan (HA). CD44 has previously been considered to be a marker of tumour invasiveness and metastasis. Only recently, it has been described as putative colorectal CSC marker.

Table 4 Expression of evaluated markers in the two principal histologic subtypes of ampullary carcinoma

	Intestinal	Pancreatobiliary	p Value
CD44+ (>5%)	82/99 (82.8%)	33/41 (80.5%)	0.805
CD133+ (>5%)	27/88 (30.7%)	12/40 (30.0%)	0.938
CD166+ (>0%)	73/97 (76.8%)	32/44 (72.7%)	0.672
EpCAM+ (100%)	68/75 (90.7%)	25/33 (75.8%)	**0.039**

The bold value means that EpCAM overexpression is statistically significant.

Original article

Take-home messages

▶ CD44, CD133 and CD166 but not EpCAM immunohistochemical expression increased from normal mucosa to adenoma to carcinoma.

▶ EpCAM was more expressed in intestinal-type ampullary carcinoma.

▶ Loss of EpCAM may be linked to a more aggressive tumour phenotype.

▶ Loss of expression of EpCAM, but not of CD44, CD133 or CD166, is linked to poor survival.

However, CD44 does not seem to belong to the group of genes, such as OCT4 and NANOG, that are central for maintaining stem cell characteristics. Nonetheless, two connections between CD44 and genes that regulate stem cell characteristics have been described. First, CD44 is a target of the WNT pathway. Loss of adenomatous polyposis coli (APC) function leads to the constitutive activation of β catenin, a constituent of the WNT signalling pathway. CD44s and CD44v6 expression is restricted to the intestinal crypts in non-transformed tissue, but both CD44 isoforms are strongly overexpressed in dysplastic crypts and adenomas in humans and mice with mutant APC.[47] Second, HA–CD44 binding promotes protein kinase C (PKC) activation and this increases NANOG phosphorylation and translocation to the nucleus. Here, it associates with Drosha and an RNA helicase, p68, leading to the transcription of the oncogenic microRNA (miRNA) miR-21 and a reduction in the expression of the tumour suppressor programmed cell death 4. These events initiate the upregulation of the inhibitor of apoptosis (IAP) proteins and multidrug resistant protein 1 (MDR1). CD44, in turn, associates with and stabilises MDR1 expression.[48] This could be one mechanism through which CD44 contributes to stem cell resistance to chemotherapy, as MDR1 exports several drugs from cells.

In several gastrointestinal tumours, including colorectal cancer,[23] stromal[49] as well as neuroendocrine tumours,[50] loss of CD44 expression has been associated with disease progression and reduced survival. In our study, CD44 expression was more frequently associated with adenomas with coexisting carcinomas.

Our study is also the first to evaluate the prognostic impact of CD166 in ampullary carcinoma. We found an increasing expression of CD166 from normal tissue to carcinoma, suggesting that the increased expression of this marker might be linked to tumour progression. Our data are supported by a recent study in colorectal cancer patients, where a similar increasing expression of CD166 from normal to neoplastic tissue has been described by Weichert et al.[51]

We speculate that our findings of decreased rather than increased expression of membranous EpCAM expression and its association with features of tumour progression are mainly a consequence of its cell adhesion function.

In colorectal cancer, Kojima et al[52] and Horst et al[53] reported a significant correlation of increased CD133 expression and poor clinical outcome. In contrast, in a study on non-small-cell lung cancer, CD133 expression was not a prognostic factor for survival.[54] Consistent with the latter study, we found no significant impact of CD133 on survival in our series of ampullary cancer patients.

In summary, we have provided evidence that in ampullary carcinoma, loss of expression of EpCAM, but not of CD44, CD133 or CD166, is linked to poor survival.

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