Expression of EpCAM^{MF} and EpCAM^{MT} variants in human carcinomas

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ABSTRACT

Aims Regulated intramembrane proteolysis has been shown to be an important mechanism for oncogenic activation of epithelial cell adhesion molecule (EpCAM) through nuclear translocation of the intracellular domain EpICD. Recent studies have identified new membrane-bound EpCAM variants. To evaluate the prevalence of two membranous EpCAM variants in human tumours, we performed a large-scale expression analysis using specific antibodies against the extracellular domain EpEX (MOC-31 clone) and the intracellular domain EpICD (9-2 clone) of the EpCAM antigen by immunohistochemistry.

Material and methods Two multi-tissue microarrays (TMA) series containing 1564 tissue samples each of 53 different histological tumour types were stained and compared. One TMA was stained for EpEX and one for EpICD. Membranous full-length EpCAM (EpCAM^{MF}) expression in tissues was defined by the expression of EpEX and EpICD, while the truncated variant of EpCAM (EpCAM^{MT}) was characterised by a significant loss of membranous EpICD expression compared with EpEX expression.

Results We defined tumours with high EpCAM^{MT} expression (ie, cancers of the endometrium and bladder), tumours with intermediate (ie, gastric, pancreatic, colorectal and oesophageal cancer) and tumours with low rates of expression of the EpCAM^{MT} variant (ie, lung, ovarian, gallbladder, breast and prostate cancer).

Conclusions Our results indicate that loss of membranous EpICD expression is a common event in human epithelial carcinomas, arguing for the expression of different degrees of EpCAM^{MF} and EpCAM^{MT} variants across the most important tumour entities. Future studies evaluating the prognostic and predictive role of these variants in human malignancies, especially in patients treated with EpCAM-specific antibodies, are clearly warranted.

INTRODUCTION

Epithelial cell adhesion molecule (EpCAM) is a type I transmembrane glycoprotein which is expressed on the basolateral membrane in most normal epithelial tissues and is overexpressed in many human carcinomas.[1] The corresponding EpCAM *gene* encodes a 40 kDa protein consisting of a 289 amino acid long extracellular domain called EpEX and a short intracellular domain (EpICD) of 26 amino acids.[2] Several biological functions of the carcinoma-associated antigen EpCAM have been described including cell adhesion as well as mitogenic signalling.[2 3] Recently, EpCAM has also been identified as a marker for cancer-initiating stem cells, making it an interesting target for cancer therapy.[4 5]

In 2009, the trifunctional anti-EpCAM-specific antibody catumaxomab has been approved for the treatment of malignant ascites in cancer patients with EpCAM positive tumours.[6] However, despite advances in the understanding of the biology of EpCAM, results from clinical trials using other EpCAM-specific targeting agents have been disappointing.[7] Recent studies have demonstrated that activation of EpCAM follows regulated intramembrane proteolysis.[3] Cleavage within the transmembrane domain of EpCAM results in shedding of the extracellular domain EpEX and accumulation of the intracellular domain EpICD to the nucleus. Hence, the cleaved intracellular domain EpICD has been proposed as a novel marker for treatment response in EpCAM positive tumours[8] and there is now growing evidence that subcellular compartmental accumulation of EpICD may be involved in development of epithelial carcinomas.[9] Very recently, different variants of the EpCAM protein have been described in cell lines with different biological functions.[10] A better characterisation of the expression of different EpCAM variants that may predict response to EpCAM-targeted therapies is crucial to optimise the selection of patients for these treatments. Routinely, EpCAM expression is determined by immunohistochemistry through evaluating its membranous staining intensity. However, diagnostic as well therapeutic antibodies that target EpCAM are usually directed against its ectodomain EpEX and these antibodies alone are not sufficient to discriminate between different EpCAM variants. By combining EpEX antibody with EpICD antibody staining on two separate tissue slides of the same tumour specimen, two EpCAM variants can be differentiated: the membrane-bound full-length protein (EpCAM$^{\text{Membranous full-length}}$; EpCAM^{MF}) and its truncated variant (EpCAM$^{\text{Membranous truncated}}$; EpCAM^{MT}) which have lost the intracellular domain but still have a remnant transmembranous and integral extracellular domain.

Defining EpCAM variants by this double-staining procedure, the aim of this study was to elucidate the expression of EpCAM^{MF} and EpCAM^{MT} in human cancers. By the use of two series of multi-tissue microarrays (TMA), we were able to investigate simultaneously the presence of the extracellular (EpEX) as well as the intracellular domain (EpICD) of EpCAM applying specific monoclonal antibodies and comparing the staining results under optimised conditions. Our results show for the first time that loss of membranous EpICD expression is a frequent event in human cancers across a large panel of tumour samples reflecting the different degree of expression of the membranous variants EpCAM^{MT} and EpCAM^{MF}.

MATERIAL AND METHODS

Ethics statement

This study was approved by the ethic committee of Basel, Switzerland, and oral and written consent was obtained from all patients. Retrieval of tissue and clinical data was performed according to the regulations of the local institutional review board and data safety laws.

Material and microarray construction

Formalin-fixed and paraffin-embedded tissue probes were sampled from the archives of the Institute for Pathology of the University Hospital Basel (Switzerland). A total of 1564 samples were arranged into a TMA format as described elsewhere.[11] Briefly, tissue cylinders with a diameter of 0.6 mm were punched from representative tumour areas of each 'donor' tissue block and brought into four different recipient paraffin blocks. Multiple 4 μm sections of the resulting TMA blocks were cut and mounted to an adhesive-coated slide system. In addition, whole tissue sections of tumours from the colon (n=5), stomach (n=5), endometrium (n=10), pancreas (n=5) and ovary (n=5) were analysed.

Calibration of EpICD and EpEX immunohistochemical staining

The development and specificity of the 9-2 antibody clone for the EpICD domain (distributed by HVD Life Science GmbH, Vienna, Austria; for more information: http://www.oncotyrol.at/business/produkte/anti-epicd-epcam-c-term/) were described previously.[12] The staining intensity of this antibody was calibrated to be equivalent to the expression observed with the commercially available anti-EpEX antibody MOC-31 (clone: MOC-31, 1:50, Dako).[13] Calibration relied on increase of EpICD antibody dilution from 1:25 to 1:50, 1:100, 1:250, 1:500 and 1:1000 on whole tissue sections from normal colon mucosa and colorectal cancer patients (figure 1A) to obtain similar staining pattern to that observed with the EpEX antibody. An optimised concentration of the EpICD antibody of 1:100 was then used to stain one TMA series and the second TMA series was stained with the EpEX antibody MOC-31 as described below.

Immunohistochemistry

Immunohistochemistry of tumour samples with the antibody clone MOC-31 directed against the extracellular domain (EpEX) of EpCAM was performed according to manufacturer's instructions. This staining protocol is used routinely in pathology laboratories for evaluation of pathological specimens.[13]

For detection of the C-terminus of the intracellular domain (EpICD) of EpCAM, the mouse monoclonal antibody clone 9-2 (distributed by HVD Life Science GmbH; for more information: http://www.oncotyrol.at/business/produkte/anti-epicd-epcam-c-term/) was designed in our laboratory and generated by Biogenes GmbH as described previously.[12] Briefly, 4 μm thick sections from formalin-fixed paraffin-embedded tissue specimens were stained using the automated immunostainer Leica BOND-MAX™ (Leica Microsystems, Wetzlar, Germany). Leica provides all the solutions used for specific staining, with the exception of the primary antibody clone 9-2 directed against EpICD. The following steps were programmed on the staining machine: dewaxing at 72°C; antigen retrieval with ER2 (epitope retrieval, pH 9) 20 min at 100°C; wash solution; peroxide blocking solution for 5 min at room temperature; wash solution; treatment with the primary antibody 9-2 at a concentration of 1:100 for staining of microarrays; wash solution; postprimary antibody treatment over 8 min at room temperature; wash solution; Leica BOND-MAX Polymer treatment over 8 min at room temperature; wash solution; mixed DAB treatment over 10 min at room temperature; washing with distillate water; counterstaining with HTX over 8 min at room temperature; washing with distillate water; dehydratation in alcohol and xilene and mounting on glass slides with entelan.

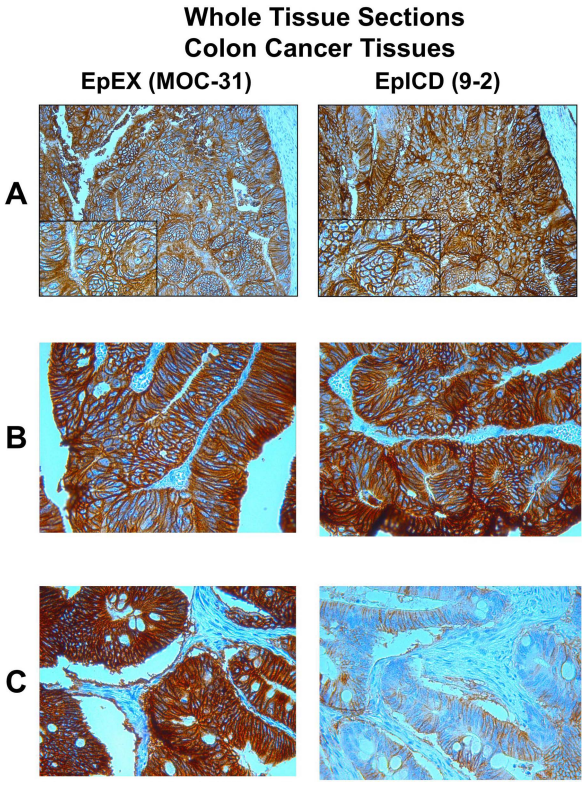

Figure 1 Two tissue sections of the same tumour sample stained with the EpEX antibody MOC-31 and the EpICD antibody 9-2. (A) Calibration of the staining intensity of both antibodies using a colorectal cancer sample. (B) Colon cancer sample with the predominant expression of the full-length variant $EpCAM^{MF}$. (C) Colon cancer sample with the predominant expression of the truncated variant $EpCAM^{MT}$.

Scoring

EpEX and EpICD antigen expression was evaluated by two independent observers (GM and LT) using light microscopy in a blinded fashion. Discordant cases were re-evaluated on a double-headed microscope to achieve a consensus. Antigen expression was defined as the presence of specific staining on surface membranes of tumour cells as described previously.[14 15] Briefly, the expression of EpEX and EpICD was evaluated by calculating a Total Immunostaining Score (TIS) as the product of a Proportion Score (PS) and an Intensity Score (IS). The PS describes the estimated fraction of positive stained tumour cells (0, none; 1, <10%; 2, 10%–50%; 3, 51%–80%; 4, >80%). The IS represents the estimated staining intensity as compared with control cell lines (0, no staining; 1, weak; 2, moderate; 3, strong). The TIS (TIS=PS×IS) ranges from 0 to 12 with only nine possible values (ie, 0, 1, 2, 3, 4, 6, 8, 9 and 12). Using the total immunoreactive score we defined four groups (TIS Group; TISG): no expression (TISG=0, TIS=0); weak expression (TISG=1, TIS 1–4); moderate expression (TISG=2, TIS score=6 and 8); and intense expression (TISG=3, TIS score=9

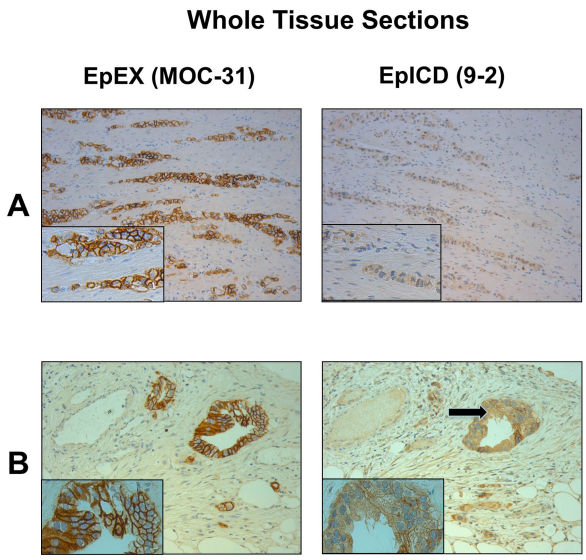

Figure 2 (A) Two tissue sections of the same gastric cancer sample stained with the EpEX antibody MOC-31 and the EpICD antibody 9-2 showing the predominant expression of the truncated variant $EpCAM^{MT}$. (B) Two tissue sections of a pancreatic cancer sample showing the predominant expression of the truncated variant $EpCAM^{MT}$. Note the EpICD expression in the cytoplasm (arrow).

and 12). A predominant expression of the $EpCAM^{MT}$ variant in a tumour sample was defined if membranous staining was present for EpEX and the corresponding tissue showed a reduction in membranous EpICD staining intensity by at least two TISG scoring points ($EpEX^+/EpICD^-$ phenotype). Accordingly, the predominant expression of the $EpCAM^{MF}$ variant in a tumour sample was defined by the presence of positive membranous staining for both epitopes without a significant reduction of EpICD expression (ie, loss of <2 TISG scores; $EpEX^+/EpICD^+$ phenotype). Samples with weak EpEX expression (TISG=1) and no EpICD expression (TISG=0) were classified as $EpCAM^{MF}$. Figure 1 and Figure 2 show representative tumour samples with predominant expression of $EpCAM^{MF}$ and samples with the predominant expression of $EpCAM^{MT}$ variants. Differences between histological subtypes of a single tumour entity, if available, were statistically analysed using the χ^2 test.

RESULTS

Whole tissue sections

To test the suitability and specificity of the 9-2 antibody on formalin-fixed material, a total of 30 whole tissue sections from patients with tumours of the colon, stomach, endometrium, pancreas and ovary were analysed. The known expression status of EpEX staining with the monoclonal antibody MOC-31 of normal colonic epithelium and colon carcinomas served as control. In addition, to validate the results obtained by immunostaining, western blot analysis was performed for EpICD antibody specificity as described previously.[12] In concordance with previous reports,[11 15] a strong homogenous membranous expression of the extracellular domain EpEX of the EpCAM antigen was detected in the majority of the tumour samples. However, some tumours concurrently revealed differences in the expression pattern of the intracellular domain EpICD. Based on these observations, we defined two membrane-bound EpCAM variants: the full-length variant $EpCAM^{MF}$ defined by a similar expression of both EpEX and EpICD and the truncated variant $EpCAM^{MT}$ defined by a significant loss of EpICD expression compared with EpEX expression (figures 1 and 2). No nuclear expression of EpICD was observed in these tumour samples.

TMA analysis

To further evaluate the prevalence of these two EpCAM variants in human solid tumours, expression of EpEX and EpICD was investigated in two TMA series composed of 1564 independent cases. A total of 1564 human tumour and normal tissue specimens were successfully analysed. For most tumour types, representative number of cases (n>30) was available within the array. EpEX/EpICD staining was predominantly membranous, but weak cytoplasmic staining could also be observed in some tumour types. No nuclear expression of EpICD was observed in these tumour samples. For all analyses, only membranous staining was considered (table 1). As described previously, mesenchymal tissue and tumours as well as tumours of neural origin were EpCAM-negative. As expected, colon cancer demonstrated the most frequent (>90%) expression of EpEX followed by tumours of the female genital tract (table 1). To further evaluate the frequency of loss of EpICD in EpEX positive tissues, a detailed analysis for the subgroup of EpEX-positive tissue samples (n=713) was performed (table 2).

Expression of $EpCAM^{MF}$ and $EpCAM^{MT}$ variants in human tissues

In total, 713 tumour samples were EpEX-positive and were further evaluated. Figure 3 shows gastric cancer samples expressing prevalently the $EpCAM^{MF}$ variant (figure 3A) as well as a tumour expressing the $EpCAM^{MT}$ variant (figure 3B), respectively. Altogether, the majority (73, 9%) of tissues analysed demonstrated a predominant expression of the $EpCAM^{MF}$ variant. However, the ratio between $EpCAM^{MF}$ and $EpCAM^{MT}$ changed significantly dependent on the tumour type. The prevalence of $EpCAM^{MF}$ and $EpCAM^{MT}$ expression in the most frequent EpEX-positive human malignancies is outlined in figure 4. We identified endometrial and bladder cancer as tumours with the highest proportion (>50%) of cases expressing the truncated EpCAM variant. Moreover, we observed tumour entities (ie, stomach, pancreatic, colorectal and oesophageal cancer) with an intermediate proportion (33%–50%) and tumours with a low proportion of expression of the $EpCAM^{MT}$ variant (<33%; ie, tumours of the lung, ovary, gallbladder, breast, prostate and well-differentiated neuroendocrine carcinomas), respectively. A detailed analysis of all tissue samples is shown in table 2. Taken together, these results support the view of a widespread loss of membranous EpICD expression in human epithelial carcinomas resulting in different degrees of the expression of the $EpCAM^{MT}$ and $EpCAM^{MF}$ variants. Of note, adenomas and adenolymphomas but not cylindromas of the parotid gland also showed a high prevalence of expression of the $EpCAM^{MT}$ variant (68%). Intriguingly, the expression of the $EpCAM^{MT}$ variant was observed in 3 (8.1%) of 37 samples of low-grade dysplasia (table 2). In contrast, $EpCAM^{MT}$ expression was observed in 14 (17.5%) out of 66 tissue samples of high-grade dysplasia and 11 (33.3%) out of 22 colon carcinomas (p=0.024). Finally, the $EpCAM^{MT}$ variants appear to be particularly frequent in bronchoalveolar carcinoma of the lung (80%, p=0.034).

DISCUSSION

In this study, we investigated for the first time the membranous expression pattern of two EpCAM variants in a large cohort

Table 1 EpEX and EpICD expression defined by MOC-31 and 9-2 antibodies in human malignancies

	EpEX expression				EpICD expression			
	Number of tissue samples analysed (%)							
Tumour tissue	**Negative**	**Weak**	**Moderate**	**Strong**	**Negative**	**Weak**	**Moderate**	**Strong**
Pancreatic cancer (n=28)	8 (28.6)	10 (35.7)	7 (25.0)	3 (10.7)	20 (71.4)	6 (21.4)	2 (7.1)	0 (0)
Prostate cancer (n=45)	3 (6.7)	19 (42.2)	16 (35.6)	7 (15.5)	17 (37.9)	10 (22.2)	11 (24.4)	7 (15.5)
Oesophageal cancer (n=33)	18 (54.5)	8 (24.2)	6 (18.2)	1 (3.0)	30 (90.9)	2 (6.1)	1 (3.0)	0 (0)
Mesothelioma (n=17)	13 (76.5)	4 (23.5)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)	0 (0)
Gallbladder cancer (n=36)	10 (27.8)	14 (38.9)	8 (22.2)	4 (11.1)	17 (47.2)	12 (33.3)	6 (16.7)	1 (2.8)
Cervical cancer (n=13)	8 (61.5)	3 (23.1)	2 (15.4)	0 (0)	10 (76.9)	3 (23.1)	0 (0)	0 (0)
Laryngeal cancer (n=16)	11 (68.8)	3 (18.8)	1 (6.2)	1 (6.2)	10 (62.6)	4 (25.0)	1 (6.2)	1 (6.2)
Hepatocellular carcinoma (n=60)	54 (90.0)	5 (8.3)	1 (1.7)	0 (0)	59 (98.3)	1 (1.7)	0 (0)	0 (0)
Colon								
Low-grade dysplasia (n=38)	1 (2.6)	0 (0)	0 (0)	37 (97.4)	0 (0)	3 (7.9)	14 (36.8)	21 (55.3)
High-grade dysplasia (n=81)	1 (1.2)	0 (0)	1 (1.2)	79 (97.6)	2 (2.4)	12 (14.8)	40 (49.4)	27 (33.4)
Adenocarcinoma (n=33)	0 (0)	0 (0)	3 (9.1)	30 (90.9)	1 (3.0)	13 (39.4)	10 (30.3)	9 (27.3)
Stomach								
Intestinal cancer (n=36)	1 (2.8)	9 (25.0)	6 (16.6)	20 (55.6)	20 (55.6)	6 (16.6)	3 (8.4)	7 (19.4)
Diffuse cancer (n=12)	3 (25.0)	4 (33.3)	3 (25.0)	2 (16.7)	10 (83.4)	1 (8.3)	1 (8.3)	0 (0)
Mixed histology (n=3)	0 (0)	1 (33.3)	1 (33.3)	1 (33.4)	3 (100)	0 (0)	0 (0)	0 (0)
Small intestine cancer (n=14)	0 (0)	3 (21.4)	4 (28.6)	7 (50.0)	6 (42.9)	2 (14.3)	1 (7.1)	5 (35.7)
Endometrium								
Endometrioid cancer (n=38)	6 (15.8)	7 (18.4)	8 (21.1)	17 (44.7)	29 (76.3)	5 (13.2)	4 (10.5)	0 (0)
Serous cancer (n=25)	1 (4.0)	0 (0)	11 (44.0)	13 (52.0)	10 (40.0)	7 (28.0)	5 (20.0)	3 (12.0)
Urinary bladder cancer (n=48)	25 (52.1)	7 (14.6)	10 (20.8)	6 (12.5)	42 (87.4)	3 (6.3)	0 (0)	3 (6.3)
Pheochromocytoma (n=13)	13 (100)	0 (0)	0 (0)	0 (0)	13 (100)	0 (0)	0 (0)	0 (0)
Paraganglioma (n=5)	5 (100)	0 (0)	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)
Lung								
Adenocarcinoma (n=50)	1 (2.0)	12 (24.0)	24 (48.0)	13 (26.0)	17 (34.0)	18 (36.0)	13 (26.0)	2 (4.0)
Bronchoalveolar carcinoma (n=6)	1 (16.6)	1 (16.7)	1 (16.7)	3 (50.0)	3 (50.0)	3 (50.0)	0 (0)	0 (0)
Squamous cell carcinoma (n=20)	2 (10.0)	4 (20.0)	10 (50.0)	4 (20.0)	8 (40.0)	9 (45.0)	1 (5.0)	2 (10.0)
Large cell carcinoma (n=11)	3 (27.3)	2 (18.2)	5 (45.5)	1 (9.0)	4 (36.3)	2 (18.2)	5 (45.5)	0 (0)
SCLC (n=33)	9 (27.3)	10 (30.3)	10 (30.3)	4 (12.1)	13 (39.4)	14 (42.4)	4 (12.1)	2 (6.1)
Ovarian								
Endometrioid cancer (n=27)	0 (0)	4 (14.8)	13 (48.2)	10 (37.0)	3 (11.1)	12 (44.5)	10 (37.0)	2 (7.4)
Serous cancer (n=27)	0 (0)	6 (22.2)	13 (48.1)	8 (29.7)	8 (29.7)	6 (22.2)	9 (33.3)	4 (14.8)
Mucinous cancer (n=11)	1 (9.1)	1 (9.1)	5 (45.4)	4 (36.4)	2 (18.2)	2 (18.2)	5 (45.4)	2 (18.2)
Breast								
Ductal cancer (n=19)	8 (42.1)	8 (42.1)	2 (10.5)	1 (5.3)	9 (47.4)	7 (36.8)	3 (15.8)	0 (0)
Mucinous cancer (n=7)	0 (0)	6 (85.7)	1 (14.3)	0 (0)	2 (28.6)	5 (71.4)	0 (0)	0 (0)
Tubular cancer (n=7)	4 (57.1)	3 (42.9)	0 (0)	0 (0)	6 (85.7)	1 (14.3)	0 (0)	0 (0)
Medullary cancer (n=38)	7 (18.4)	21 (55.3)	7 (18.4)	3 (7.9)	18 (47.4)	14 (36.8)	4 (10.5)	2 (5.3)
Lobular cancer (n=18)	12 (66.7)	5 (27.8)	1 (5.5)	0 (0)	17 (94.5)	1 (5.5)	0 (0)	0 (0)
Parotid gland								
Pleomorphic adenoma (n=31)	17 (54.8)	6 (19.4)	8 (25.8)	0 (0)	27 (87.1)	3 (9.7)	1 (3.2)	0 (0)
Adenolymphoma (n=21)	4 (19.0)	2 (9.5)	12 (57.2)	3 (14.3)	14 (66.6)	6 (28.6)	1 (4.8)	0 (0)
Cylindroma (n=27)	16 (59.3)	9 (33.3)	2 (7.4)	0 (0)	21 (77.8)	6 (22.2)	0 (0)	0 (0)
Well-differentiated NET (n=26)	3 (11.5)	6 (23.1)	5 (19.2)	12 (46.2)	3 (11.5)	7 (26.9)	6 (23.1)	10 (38.5)
Thymoma (n=34)	25 (73.5)	8 (23.6)	1 (2.9)	0 (0)	34 (100)	0 (0)	0 (0)	0 (0)

Expression in lymphomas, soft tissue sarcomas and brain tumours are shown in the online supplementary table.

including the major human malignancies by using two monoclonal antibodies targeting the ectodomain and endodomain of EpCAM. For the question addressed, we used the high-throughput technique of a multi-tissue array to minimise batch-to-batch variability and make the analyses most comparable. The application and validation of the microarray technology for biomarker assays compared with whole tissue sections have been sufficiently proven.[16] Our data provide strong evidence that a different degree of expression of two membranous EpCAM variants occurs during carcinogenesis in the majority of epithelial cancers. Activation of EpCAM through cleavage of its intracellular domain EpICD seems to play an important role in the neoplastic transformation.

The regulated intramembrane proteolysis process occurs for an increasing number of membrane proteins and was first described for EpCAM by Maetzel *et al*[3] who demonstrated that the cytoplasmic domain EpICD is cleaved by the two proteases, ADAM17 and γ-secretase, producing a 5 kDa intracellular

Table 2 Expression of membranous full-length ($EpCAM^{MF}$) EpCAM and the truncated variant ($EpCAM^{MT}$) in EpEX-positive tumours

	$EpCAM^{MF}$		$EpCAM^{MT}$		
Tumour entity	**n**	**%**	**n**	**%**	χ^2
Pancreatic cancer (n=20)	13	65	7	35	NA
Prostate cancer (n=42)	41	97.6	1	2.4	NA
Oesophageal cancer (n=15)	10	66.7	5	33.3	NA
Mesothelioma (n=4)	4	100	0	0	NA
Gallbladder cancer (n=26)	22	84.6	4	15.4	NA
Cervical cancer (n=5)	4	80	1	20	NA
Laryngeal cancer (n=5)	5	100	0	0	NA
Hepatocellular carcinoma (n=6)	5	83.3	1	16.7	NA
Colon					
Low-grade dysplasia (n=37)	34	91.9	3	8.1	0.024
High-grade dysplasia (n=80)	66	82.5	14	17.5	
Adenocarcinoma (n=33)	22	66.7	11	33.3	
Stomach					
Intestinal type cancer (n=35)	19	54.3	16	45.7	0.180
Diffuse cancer (n=9)	6	66.7	3	33.3	
Mixed histology (n=3)	2	33.3	1	66.7	
Small intestine cancer (n=14)	9	64.3	5	35.7	NA
Endometrium					
Endometrioid carcinoma (n=32)	12	37.5	20	62.5	0.961
Serous carcinoma (n=24)	11	45.8	13	54.2	
Urinary bladder cancer (n=23)	11	47.8	12	52.2	NA
Lung					
Adenocarcinoma (n=49)	38	77.6	11	22.3	0.034
Bronchoalveolar carcinoma (n=5)	1	20	4	80	
Squamous cell carcinoma (n=18)	14	77.8	4	22.2	
Large cell carcinoma (n=8)	7	87.5	1	12.5	
SCLC (n=24)	20	83.3	4	16.7	
Ovarian					
Endometrioid cancer (n=27)	20	74.1	7	25.9	0.180
Serous cancer (n=27)	21	77.8	6	22.2	
Mucinous cancer (n=10)	10	100	0	0	
Breast					
Ductal carcinoma (n=11)	9	81.8	2	18.2	0.815
Mucinous carcinoma (n=7)	7	100	0	0	
Tubular carcinoma (n=3)	3	100	0	0	
Medullary carcinoma (n=31)	28	90.3	3	9.7	
Lobular carcinoma (n=6)	5	83.3	1	16.7	
Parotid gland					
Pleomorphic adenoma (n=14)	6	42.9	8	57.1	0.001
Adenolymphoma (n=17)	4	23.5	13	76.5	
Cylindroma (n=11)	10	90.9	1	9.1	
Well-differentiated NET (n=23)	19	82.6	4	17.4	NA
Thymoma (n=9)	8	88.9	1	10.1	NA
Total (n=713)	526	73.8	187	26.2	

NA, Not applicable.

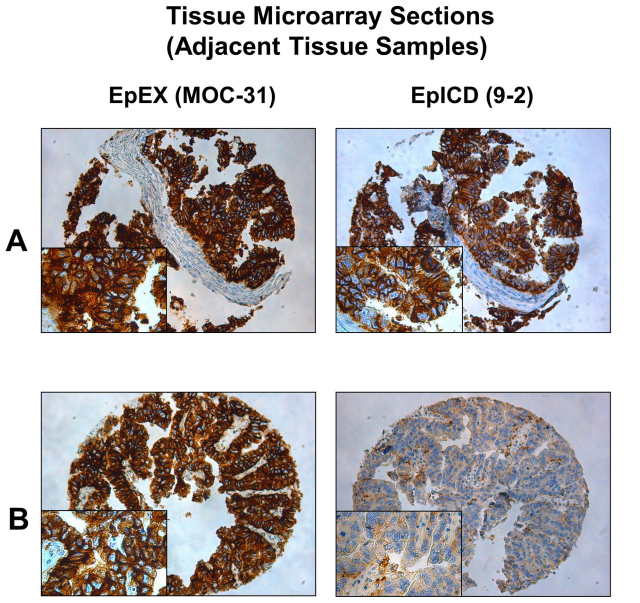

Figure 3 Staining of the EpEX antibody antibody MOC-31 and the EpICD antibody 9-2 using two tissue microarray (TMA) sections of the same series. (A) Two punches of the same gastric cancer sample with predominant $EpCAM^{MF}$ expression. (B) Two punches of the same gastric cancer sample with predominant $EpCAM^{MT}$ expression. Respective tissue samples (A and B) were situated in adjacent positions on the TMA.

fragment with oncogenic potential. Since then, several studies reported significant alterations in the levels of the intracellular and extracellular domains of EpCAM as well as the accumulation of different EpCAM fragments within cellular compartments.[17–19] Only recently, Schnell *et al*[10] could demonstrate that EpCAM can be cleaved at multiple positions within its ectodomain resulting in various N-terminal proteolytic fragments. Importantly, in the present study the monoclonal antibody MOC-31 was used, which recognises the most N-terminal located epitope of the EpEX antigen thus detecting the full-length EpEX domain as described by Schnell *et al*. We postulate that combining MOC-31 with the EpICD antibody 9-2 detects the two most predominant variants of EpCAM in human cancer tissues.

A reciprocal loss of membrane EpEX but an increased nuclear and cytoplasmic accumulation of EpICD was first described in aggressive thyroid cancer depending on the histological subtype and differentiation grade of tumour cells.[20] In papillary thyroid microcarcinoma, nuclear translocation of EpICD as well as loss of membranous EpEX was found to correlate with metastatic disease. In addition, the same authors could provide evidence that accumulation of nuclear and cytoplasmic EpICD is a frequent event in other epithelial cancers.[9] Moreover, concurrent high EpICD and EpEX membrane expression was observed in some cancer cells suggesting the expression of the full-length protein ($EpCAM^{MF}$) in a subset of tumours. Similar results have been reported by Lin *et al*[18] who demonstrated an increased nuclear EpICD expression in colon cancer compared with normal tissue. Increased release of EpEX enhanced EpICD cleavage resulting in activation of reprogramming factors and epithelial-mesenchymal transition genes suggesting that EpICD participates in tumour initiation and progression. However, they could not detect a homogenous expression of soluble EpICD in cytoplasm or nucleus in any tumour cells assuming that cleavage of EpICD might be a dynamic process. We also could not detect a precise nuclear EpICD staining by using immunohistochemistry in the present study confirming the data by Schnell *et al*.[10]

The prognostic value of EpCAM is still a matter of debate. While we and others could demonstrate a poor clinical outcome in patients with tumours harbouring EpCAM overexpression,[14 21–25] there is evidence for the same antigen to correlate with good prognosis in a variety of tumour entities.[26 27] Our group could show that EpCAM can act both as tumour suppressor and tumour promotor[28 29] in breast cancer cell lines and the

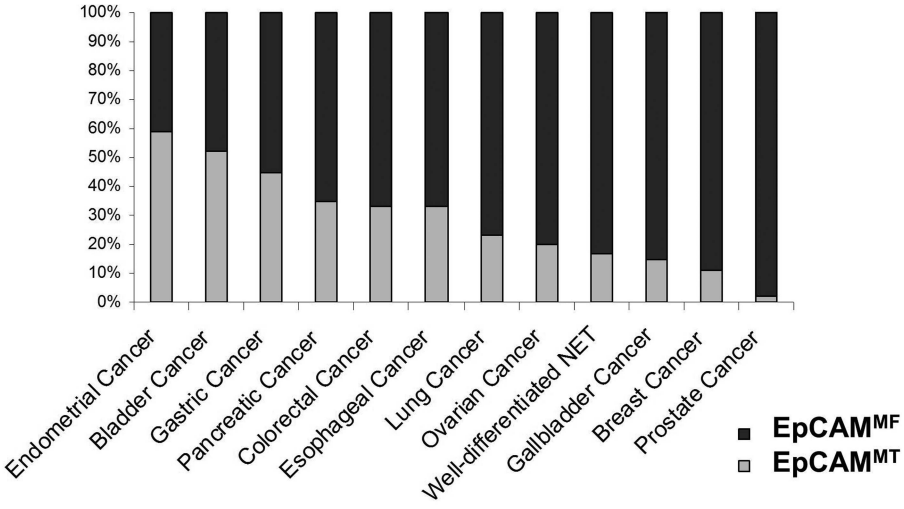

Figure 4 Expression of $EpCAM^{MF}$ and $EpCAM^{MT}$ in most frequent EpEX-positive human malignancies. Endometrial and bladder cancer demonstrated the highest proportion (>50%) of cases expressing the truncated EpCAM variant. Carcinomas of the stomach, pancreas, colon, rectum and oesophagus revealed an intermediate proportion (33%–50%) and tumours of the lung, ovary, gallbladder, breast, prostate and well-differentiated neuroendocrine carcinomas exhibited a low proportion (<33%) of expression of the $EpCAM^{MT}$ variant.

dual role of EpCAM was confirmed by a recent study in breast cancer patients.[30] Only recently, Eichelberg *et al*[31] reported a positive EpCAM status to be significantly associated with a prolonged overall survival in renal cell carcinoma. Moreover, the prognostic value of cytoplasmic accumulation of EpCAM was demonstrated in patients with node positive breast cancer.[17] However, none of the studies mentioned above considered the importance of incorporating different EpCAM variants within their assays. Notably, in pancreatic cancer we could show that loss of membranous EpICD expression predicts poor prognosis in patients with tumours harbouring the $EpCAM^{MT}$ variant[12] suggesting a different biological function of these variants.

Most colorectal carcinomas undergo neoplastic progression through the adenoma–carcinoma sequence accompanied by an accumulation of successive genetic alterations.[32 33] A focal loss of membranous EpEX expression was observed at the invasive margin in colorectal cancer, which was significantly associated with a higher extend of tumour budding and a more invasive phenotype.[34] In our study, the majority of adenomas with low-grade dysplasia showed a predominant $EpCAM^{MF}$ expression, whereas a significant higher expression of the $EpCAM^{MT}$ variant was observed in high-grade dysplasia and invasive colon cancer specimens. Further studies on colorectal cancer samples are necessary to confirm these results. Moreover, subgroup analyses of different lung cancer types are necessary to clarify if differential expression of $EpCAM^{MT}$ variant in bronchoalveolar carcinoma is confirmed. Our preliminary results from EpCAM mRNA analyses in endometrial carcinoma rather suggest post-translational modification than EpCAM mutation in sporadic tumour development (data not shown). Additionally, the data from Lin *et al*[18] emphasise the tumour-initiating abilities of EpCAM in colon cancer. We speculate that changes in expression of membranous EpCAM variants are associated with malignant transformation and contribute to the development in a subset of colon cancers. However, benign lesions of the parotid gland such as adenomas and adenolymphomas also showed a high prevalence of expression of the $EpCAM^{MT}$ variant. Future studies are necessary to clarify if EpCAM plays an important role in the development of these entities.

In conclusion, we show for the first time the differential expression of $EpCAM^{MF}$ and $EpCAM^{MT}$ variants in epithelial tumours. Additional studies investigating membranous expression of both EpEX and EpICD as well as the prognostic and predictive value of EpCAM variants might help to better elucidate the clinical relevance of EpCAM in carcinogenesis. Finally, a better understanding of the biological role of EpCAM will hopefully help to develop more effective anti-EpCAM strategies.

Take home messages

- At least two membranous variants of epithelial cell adhesion molecule (EpCAM) ($EpCAM^{MT}$ or $EpCAM^{MF}$) are differentially expressed in human malignancies.
- Malignancies with the highest degree of the truncated $EpCAM^{MT}$ variant are endometrial, urothelial, gastric, colorectal and pancreatic cancers.
- Future studies evaluating the predictive and prognostic role of $EpCAM^{MT}$ and $EpCAM^{MF}$ variants with respect to treatment response to EpCAM-specific antibodies (catumaxomab, adecatumumab, etc) are necessary.

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Competing interests GS has served as consultant and advisory board member for Fresenius Biotech.

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