IVYSPRING INTERNATIONAL PUBLISHER

Journal of Cancer

2017; 8(4): 657-664. doi: 10.7150/jca.16200

Research Paper

The Prognostic Impact of the Carcinoembryonic Antigen in Ampullary Cancer – A Retrospective Single Center Study

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Received: 2016.05.17; Accepted: 2016.09.04; Published: 2017.02.25

Abstract

Background: Carcinoembryonic antigen cell adhesion molecule (CEA) is a commonly immunohistochemically used antibody in pathological routine diagnostics with an overexpression in different cancers. We aimed to examine the immunohistochemically detectable CEA level in ampullary cancer and to correlate it with clinico-pathological data.

Methods: Shot-gun proteomics revealed CEA in undifferentiated ampullary cancer cell lines. Next, tumor tissue of 40 ampullary cancers of a retrospective single center cohort of 40 patients was stained immunohistochemically for CEA; CEA expression was determined and correlated with clinico-pathological data.

Results: Thirty-six patient specimens were included in statistical analysis. CEA expression and lymph node ratio (LNR) were the only independent predictors of overall survival in multivariate analysis.

Conclusion: To our knowledge, cell line and patient cohorts are the largest and characterized cohorts examined for CEA so far. Hereby, CEA expression in ampullary cancer cells permits an estimation of outcome and suggests an opportunity for individualized CEA-directed therapy. Further trials with larger cohorts are needed to verify our results and to integrate CEA immunohistochemistry into clinical routine.

Key words: CEA, ampullary cancer, carcinoembryonic antigen.

Introduction

The Ampulla of Vater is a complex anatomic structure formed by the confluence of the pancreatic duct, the common bile duct and duodenal mucosa [1, 2]. Tumors arising in this region show a mixture of histophathological patterns including intestinal, pancreaticobiliary or mixed differentiation [3-5]. Among all gastrointestinal neoplasms, carcinomas of the Ampulla of Vater (AMPAC) are diagnosed in about 0.5 % of cases [6]. Prognostic factors for overall survival include nodal status [7], resection margin

status (R), pancreatic head infiltration [8] and tumor size [9]. The family of the CEAs was discovered in 1965 by Gold et al. [10]. Physiologically they are expressed in fetal gut, liver, and pancreas between the second and sixth months of gestation, with intriguing re-appearance in cell dedifferentiation [11]. In the process of cell differentiation, members of the CEAs are down-regulated and their physiological expression is confined to the apical region of epithelial cells in most parts of the gastrointestinal tract [12]. Many human tumor tissues display overexpression of CEA (e.g., stomach, colon, rectum, pancreas, lung and cervix) [13] and some tumors show elevated CEA serum levels. For example in pancreatic cancer, a combination of serum levels of CEA and CA19-9 allows specific diagnosis [14]. In gastric cancer, high CEA serum levels are associated with poor prognosis [15]. Furthermore in non-small cell lung cancer (NSCLC) elevated CEA levels are associated with circulating tumor cells [16].

Cell-surface localization of CEA is mediated by a carboxy-terminal glycosylphosphatidylinositol (GPI) anchor [17]. At present the expression of CEA is frequently used for the immunohistochemical evaluation of normal and malignant tumorous tissue. However, increasing evidence supports that CEA is also functionally involved in tumor biology. In general, CEA is postulated to play an important regulatory role in apoptosis [18]. It plays an important role in mediating cell-cell/extracellular matrix contacts and thus inhibits anoikis, which is an apoptosis subtype that is triggered by perturbed cell-matrix interactions [19].

Based on the continuing interest in CEA, we have investigated CEA expression in an AMPAC patient cohort with detailed clinico-pathological annotation.

Materials and Methods

Ethics statement

The analyses were performed according to the guidelines of the Declaration of Helsinki. The study was approved by the Ethics Committee of the Medical University Freiburg, (ref 13/11). Before study inclusion, patient data were anonymized.

Patients and tumor tissue

Patients, who were primarily treated by surgery for AMPAC between 2007 and 2011 at the Clinic for General and Visceral Surgery, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Germany, were included in the study cohort. No perioperative deaths have been observed. Histopathological workup was performed at the Institute for Surgical Pathology, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Germany. For the current study, all histological samples from the tumor were revalidated independently by two experienced pathologists (PB, ST). Postoperative adjuvant Gemcitabine based therapy was conducted, if patients were resected R1 or AJCC/UICC Stage Grouping was 2a or higher. Clinical data from the database of the Clinic for General and Visceral Surgery, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Germany, were reviewed by BK and JH for correctness. 40 patients were initially included. For the study one patient fell out due to inadequate tumor material, three patients (resection margin positive (R1)) were excluded due to the little case number.

Standard workup – resection specimens

For all AMPAC specimens included into this study, a standardized workup for gross examination was performed as described previously [20]. Briefly, all specimens were transferred for frozen section to the Institute of Surgical Pathology, Medical Center-University of Freiburg, Germany; and prior the examination by experienced pathologists tumor masses were measured; staging specific parameters (e.g. tumorsize, histological WHO type, tumor grade, UICC classification (pTNM)), status of the resection margins, presence or absence of lymphangiosis or hemangiosis carcinomatosa and perineural invasion were documented.

Proteomics

Mass spectrometry based proteomic analysis of the AMPAC cell line SNU478 was previously reported [21]. For the present work, we additionally determined relative protein expression levels using the iBAQ method [22].

Histological Subtype

According to Albores-Saavedra et al. [23] the tumors were classified into adenocarcinoma with intestinal-type, mixed-type, pancreaticobiliary-type and undifferentiated growth pattern.

CEA immunohistochemistry and evaluation

For immunohistochemical analysis the histological slides were pretreated for 15 minutes with Dako PTLink with EnVision™ FLEX Target Retrieval Solution, High pH (Dako DM827). Thereafter followed a five minutes treatment with EnVision™ FLEX Peroxidase-Blocking Reagent (Dako SM801) and incubation with ready-to-use primary antibody (Carcinoembryonic Antigen (CEA) Clone II-7 (Dako IR622) for 20 minutes. Visualization was done with HRP-conjugated secondary antibody and DAB

chromogen according to the manufacturer's instructions (EnVision™ FLEX /HRP (Dako SM802) and EnVision™ FLEX DAB+ Chromogen (Dako DM827) 1/51 in EnVision™ FLEX Substrate Buffer (Dako SM803)). Sections were counterstained with hemalaun for one minute, dehydrated in an ascending alcohol concentration and covered with Xylol and Coverslipping Film (Tissue-Tek^R 4770).

CEA expression was quantified by expression intensity (0 to 3) and percentage of CEA-positive tumor cells in vision fields of 200 fold magnifications by two experienced pathologists, blinded for patient data and clinical outcome. For semi-quantitative analyses, CEA expression intensity and expression percentage were multiplied and normalized according to the overall mean.

Statistical analysis

For statistical calculations IBM SPSS Statistics Version 21 (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) was used. Survival data was analyzed according to the Kaplan-Meier method and Logrank test. For univariate analyses Spearman Chi squared and Kruskal-Wallis tests were used. For multivariate significance, clinico-pathological predictors were tested in a Cox proportional hazards model. Significance level was set to p=0.05. All statistical tests were performed two-sided.

Results

Proteomic Analysis Indicates Abundant CEA Expression in an Ampullary Cancer Cell Line

We have recently investigated the proteome composition of five different AMPAC cell lines, namely AMP7, AVC1, RCB1280, SNU869 and SNU478 to better understand their suitability as *in vitro* model systems for the investigation of AMPAC [21]. For the present work, we employed the iBAQ method [22] for all five aforementioned cell lines to determine relative protein abundances. As highlighted in Fig. 1, CEA was only identified in SNU478 cell and is among the top 50 % of all identified proteins ranked according to their abundance. This finding further substantiates AMPAC-associated expression of CEAs and characterizes SNU478 cells as a potential *in vitro* model system for putative investigations on functional roles of CEAs. The elevated expression levels of CEAs in SNU478 correspond to its partially dedifferentiated status.

Baseline parameters

36 patients with AMPAC were included. Mean age was 64 years. Patients received a pylorus preserving pancreaticoduodenectomy (PPPD), a Whipple operation or a total pancreatectomy. Mean tumor size was 20 mm. According to the current UICC [24] / AJCC [25]-Classification patients were staged as T1, T2, T3 and T4 for tumor extent, N0 and N1 for local nodal status and M0 and M1 for distant metastases. Furthermore, patients were grouped into UICC/AJJC into Stage IA (pT1, pN0), IB (pT2, pN0, pM0), IIA (pT3, pN0, pM0), IIB (pT1-3, pN1, pM0), III (pT4, pN0/1, pM0) and IV (pT1-4, pN0/1, pM1). Most tumors were moderately differentiated, some tumors were poorly differentiated and respectively one tumor was well and one undifferentiated. More details are presented in Table 1.

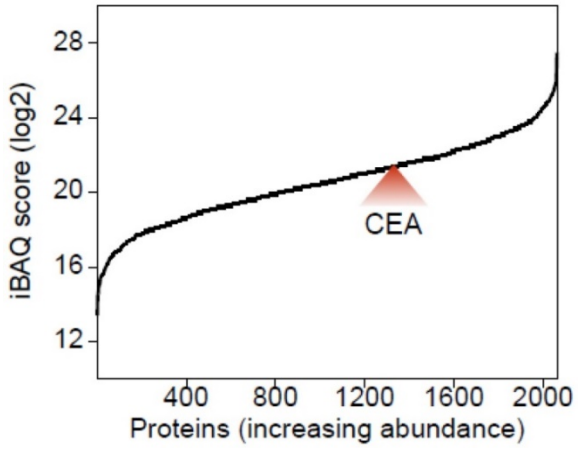

Figure 1: CEA expression level in the SNU478 AMPAC cell line across all identified proteins. Using the MaxQuant implemented iBAQ score, the average abundance (log_2 transformed) of all proteins was plotted from the least to the most abundant protein.

Histological subtyping

Using conventional histology, 18 tumors (50.0 %) with an intestinal-type, two tumors (5.6 %) with a mixed-type, 12 tumors (33.3 %) with a pancreaticobiliary-type and four tumors (11.2 %) with an undifferentiated growth pattern were identified.

CEA immunohistochemistry

CEA expression was analyzed in 36 patients with AMPAC. A completely negative reaction for CEA was not observed in any tumor. Weak staining intensity (Fig. 2A) was seen in 14 tumors (39.2 %), moderate staining intensity (Fig. 2B) in 14 tumors (39.2 %) and strong staining intensity (Fig. 2C) in eight tumors (22.4 %). Quantitatively, the tumor with the lowest CEA positivity expressed CEA in 5% of all tumor cells. The highest detected percentage of CEA positive tumor cells was 95 %. All tumors demonstrated a mixed cytoplasmatic and membranous staining. A nuclear CEA expression was not detectable.

Table 1. Multivariate analysis (included basement parameters): CEA ratio and LNR as multivariate prognostic relevant parameters of ampullary cancer (NR – not reached; NI – not included; e – excluded; HR – Hazard Ratio, CI – Confidence Interval).

Parameters	Condition	n	Events (deaths)	Mean survival (month)	Log Rank p	Cox p	HR
All patients		36	10	73			
Age	< mean	18	6	80	0.499	NI	
	> mean	18	4	78			
Sex	female	16	4	77	0.969	NI	
	male	20	6	81			
Operation	PPPD	31	7	86	0.078	e	
	Whipple	4	2	54			
	Total PE	1	1	22			
T-Group	T 1/2	19	5	84	0.483	NI	
	T 3/4	17	5	70			
N-Status	N0	15	2	97	0.105	e	
	N1	21	8	67			
LNR	< mean	21	2	103	**0.003**	0.004	**7.766 (CI 1.630 - 37.012)**
	> mean	15	8	50			
M	M0	34	9	81	0.303	NI	
	M1	2	1	25			
L	L0	20	3	98	**0.030**	e	
	L1	16	7	53			
V	V0	34	9	80	0.618	NI	
	V1	2	1	61			
Pn	Pn0	24	6	86	0.276	NI	
	Pn1	12	4	34			
G	low	25	5	91	0.150	e	
	high	11	5	59			
AJCC Stage Group	Stage 1A	2	0	NR	0.763	NI	
	Stage 1B	10	2	NR			
	Stage 2A	1	0	NR			
	Stage 2B	17	6	NR			
	Stage 3	4	1	38			
	Stage 4	2	1	7			
Tumorsize	< mean	16	4	85	0.67	e	
	> mean	18	6	70			
Subtype	Intestinal	18	3	90	0.331	NI	
	Mixed	2	1	74			
	PB	12	5	60			
	Undiff.	4	1	53			
Subtype- Group	Intestinal	18	3	90	0.123	e	
	Non-Intest	18	7	67			
CEA intensity	low	14	1	82	**<0.001**	e	
	medium	14	5	76			
	high	8	4	23			
CEA %-intensity	< mean	19	2	94	**0.009**	0.018	**5.280 (CI 1.114 – 25.023)**
	> mean	17	8	57			

N-Status: nodal status; LNR: lymph node ratio; M: distant metastasis; L: lymphangiosis carcinomatosa; V: haemangiosis carcinomatosa; Pn: perineural invasion; G: grading.

Univariate analyses

Classical pathological parameters including LNR (p=0.003) and lymphangiosis carcinomatosa (p=0.03) demonstrated a significant correlation with overall survival in univariate analysis. Grouped histological tumor differentiation (intestinal vs. non-intestinal) (p=0.123), surgical intervention (p=0.078), N- (N0 vs. N1) (p=0.105) stage classification revealed statistical trends for survival. The remaining parameters patient´s age and gender, tumor size, T- Group (T1/2 vs. 3/4), AJCC Stage Group (I-IV) and histological tumor differentiation (intestinal, mixed, pancreaticobiliary and undifferentiated) had no statistical effect on overall survival.

The mean of the CEA expression intensity and CEA expression product (percentage multiplied with intensity thereof the mean) were significant predictors of survival (p= < 0.001 and p= 0.009).

To analyze the CEA expression pattern in the different histologic subtypes of AMPAC, two-sided Chi squared and Kruskal-Wallis tests were performed (Table 2). Hereby only statistical trends were identified for CEA expression in the pancreatobiliary type (83% moderate to high staining intensity) compared to intestinal type AMPAC (89% weak to moderate staining intensity).

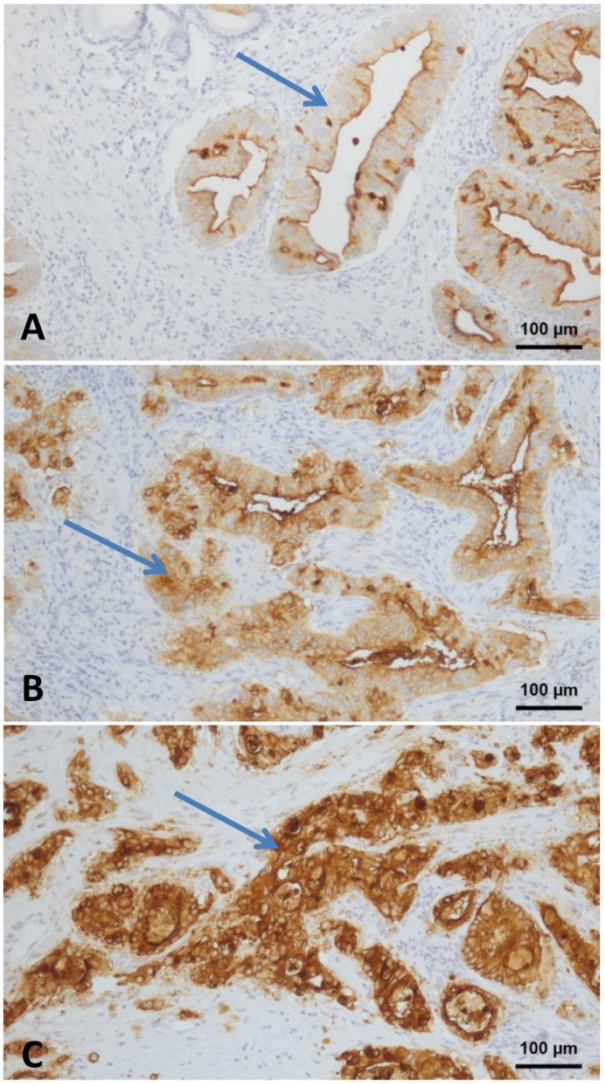

Figure 2: CEA Expression in ampullary cancer: A - weak staining intensity; B - moderate staining intensity; C - strong staining intensity (Arrow: positive tumor cells). All images taken at 100 fold magnification from ampullary cancer specimen.

Table 2. CEA expression pattern in histological subtypes of ampullary adenocarcinoma. p values derived from two-sided Chi squared and Kruskal-Wallis test.

		histologic subtype								
		intestinal		mixed		pancreato-biliary		poorly differentiated		p
		n / median	% / range	n / median	% / range	n / median	% / range	n / median	% / range	
n		18		2		12		4		-
CEA percent		45	5-95	60	60-60	60	5-85	63	15-95	0.930
CEA intensity	negative	0	0%	0	0%	0	0%	0	0%	0.082
	weak	10	56%	0	0%	2	17%	2	50%	
	moderate	6	33%	2	100%	6	50%	0	0%	
	strong	2	11%	0	0%	4	33%	2	50%	
CEA product	high	12	67%	0	0%	5	42%	2	50%	0.237
	low	6	33%	2	100%	7	58%	2	50%	

N-Status: nodal status; LNR: lymph node ratio; M: distant metastasis; L: lymphangiosis carcinomatosa; V: haemangiosis carcinomatosa; Pn: perineural invasion; G: grading.

Multivariate analysis

For multivariate survival analysis, all variables displaying significant correlations and trends ($p < 0.15$) were included in a Cox proportional hazards model with forward selection and backward elimination. Only CEA expression product ($p=0.018$) and LNR ($p=0.004$) were independent predictors of survival after resection. In backward elimination only CEA expression intensity persisted. More details are presented in Table 1.

Discussion

Numerous studies concentrate on parameters influencing the outcome of AMPAC. We identified expression level of CEA and LNR as independent prognostic factors for overall survival in patients with AMPAC.

Comparable to our results, Tol et al. recently presented LNR as an independent prognostic factor in AMPAC [26]. Kohler et al. had previously added histological tumor subtype, local tumor spread and lymph node metastases as independent prognostic factors [27]. Our univariate analysis has revealed similar results, in addition to the histological subtype (intestinal versus non-intestinal, previously published [20]) we found that LNR and CEA are significant survival predictors in univariate analysis. Nevertheless, no statistical significances but trends between CEA and the histological subtype revealing a higher CEA expression in the pancreatibiliary subtype in AMPAC was noted and is in concordance with reduced survival. Interestingly, in multivariate analysis only CEA expression and LNR were independent. Contrary to our results, Lowe et al. had postulated perineural invasion as a more significant prognostic factor regarding survival time than histological subtype [28]. Schueneman et al. had found pancreaticobiliary subtype, perineural infiltration and patient age to be independently correlated with overall survival in a cohort of 154 AMPAC patients [29]. For periampullary carcinomas as a whole, Westgaard et al. had demonstrated that histological subtype is an independent prognostic factor [30], supporting our results that a pancreaticobiliary differentiation predicts poor prognosis.

For pathologists, CEA is a well-established and frequently used immunohistochemical antibody in routine diagnostics. Many human solid cancers overexpress CEA (e.g., stomach, colon, rectum, pancreas, lung and cervix) [13]. Consequently, the suitability of CEA for the immunopathological evaluation of tumors is controversially discussed. Alapat et al. found in medullary thyroid carcinoma a positive immunohistochemical staining for CEA combined with normal CEA serum levels [31]. In colorectal carcinoma the CEA expression correlates especially in combination with elevated serum CEA levels significantly with patients overall survival [32]. In pancreatic and AMPAC Blackman et al. revealed that CEA expression showed strong cytoplasmic positmpivity while in normal and adenomatous tissues they identified CEA positivity mostly along glycocalyceal cell borders [33]. Furthermore in NSCLC could be shown in 2012 that tumor CEA level was confirmed to be an independent prognostic factor in multivariate analysis [34].

In a study with 23 carcinomas (15 duodenal adenocarcinomas and eight AMPAC), Zhu et al. had previously analyzed the impact of CEA, EMA, p53 and TGF-alpha expression regarding patient survival time, tumor stage or histological grade without statistically significant correlation [35]. They have analyzed a number of eight AMPAC regarding immunohistochemical CEA expression, so we speculate that their number of sample was too small to reach statistical significance. In a larger cohort comprising 24 patients, Kamisawa et al. were able to prove the impact of CEA on survival in univariate but not in multivariate analyses [36]. Nevertheless, the findings of Kamisawa et al. further support our

results. At this point it should be highlighted, that our cohort comprises 36 ampullary cancers, which is to our knowledge the largest ampullary carcinoma cohort analysed regarding CEA expression in literature thus far. There are many studies regarding CEA serum levels in patients as a tumor marker in different solid cancers [37, 38]. In this context Kim et al. found in their analyses of 104 ampullary cancers a multivariate significant influence of a CEA serum level > 5 ng/ml and disease recurrence. In univariate analyses especially the CEA serum level of > 5 ng/ml of the intestinal subtype was adversely correlated with disease free survival. [39]. But immunhistochemical analyses and correlation with clinico-pathological data are rare. Batge et al. postulate in 1986 that pancreatic "duct type" carcinomas, in contrast to "non duct type" tumors and "normal ducts", are distinguished by the presence of a CEA related epitope [40]. In 1991 Yamaguchi et al analysed CEA expression in pancreatoduodenal carcinomas but were not able to demonstrate a prognostic relevance [41]. Recently published data for pancreatic cancer demonstrated a positive correlation between CEA overexpression and lymph node status as well as distant metastases and showed a decreased overall survival in univariate analysis. Nevertheless, in multivariate analyses, CEA also failed to reach statistical significance [42]. Obviously our immunohistological study had to deal with the difficulty of tumor heterogeneity and sampling error like other studies. Because of our standardized gross examination and the product of the percentage of positive tumor cells and the staining intensity used for our analyses, a possible bias was minimized.

Biologically, a possible explanation for the positive correlation between CEA expression and tumor aggressiveness was postulated by Ilantzis et al. Their results support the model of a direct influence of CEA onto colon carcinogenesis by inhibiting colonocyte differentiation [43]. Ordoñez et al. showed a prolonged survival of colonocytes without cell-basement membrane adhesion in case of CEA overexpression, compared to mature colonocytes [44]. Furthermore Ilantzis et al. showed that deregulated overexpression of CEA blocks cellular polarization, disrupts tissue architecture and blocks differentiation in cell lines and in vivo [45]. Under physiological circumstances, detached cells undergo anoikis, which can be prohibited by CEA overexpression [19].

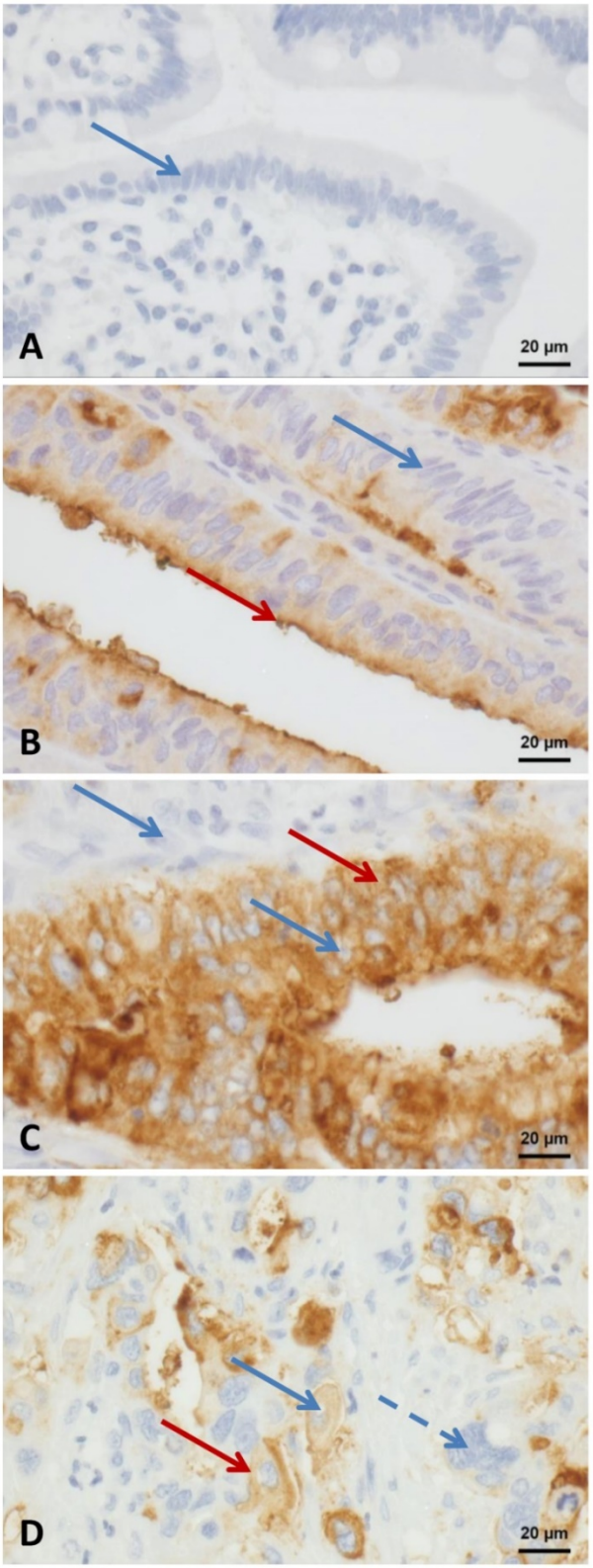

Figure 3: CEA Expression A: negative control – duodenal mucosa; B: tumor with a predominantly apical positivity – red arrow apical positivity; C: tumor with a predominantly cytoplasmatic positivity – red arrow cytoplasmatic positivity; D positive and negative tumor cells - blue arrow dotted: negative tumor cell, Red arrow: membranous and cytoplasmatic positivity; A-D blue arrows negative nuclei. All images taken at 400 fold magnification.

Furthermore, our results have the capability gaining therapeutically relevant importance because of the bispecific T-cell engager MEDI-565 (MT111). The CEA/CD3-Bispecific Antibody MEDI-565 (MT111) binds CEA positive tumor cells and develops cytotoxicity against these tumor cells *in vitro*. *In vivo*, MT111 inhibits growing of colon carcinoma, which was recently supported by a clinical phase I study [46]. Thereby, CEA could be a potential target for MT111 and lengthen patient survival time of patients suffering from ampullary carcinoma with CEA overexpression. Unfortunately CEA serum levels of the patients from the cohort were not available in this study. Hence, comparing CEA serum levels to the immunohistochemically detected CEA expression in the tumor tissue was not possible.

Conclusion

Our findings highlight CEA as a multivariate significant prognosticator in a group of ampullary carcinomas. Although AMPAC typically feature a favorable prognosis because of their early clinical symptoms compared to other pancreatic tumors, the CEA expression may be of value for the detection of cases with a relatively poor prognosis and a specific individual therapeutic need and option. The low incidence of ampullary cancer and the consecutive small patient cohort, even in a high-throughput medical center, is a legitimate limitation. Multicentric prospective clinical trials comprising larger cohorts are the logical consequence for verifying our results and to integrate CEA into clinical routine diagnostics.

Acknowledgement

O.S. is supported by grants of the Deutsche Forschungsgemeinschaft (DFG) (SCHI 871/2 and SCHI 871/5, SCHI 871/6, GR 1748/6, and INST 39/900-1) and the SFB850 (Project B8), a starting grant of the European Research Council (Programme "Ideas" - Call identifier: ERC-2011- StG 282111-ProteaSys), and the Excellence Initiative of the German Federal and State Governments (EXC 294, BIOSS).

Z.W.L is funded by Marie Curie IIF fellowship (Call Identifier: PIIF-GA-2012-329622 GlycoMarker).

The article processing charge was funded by the German Research Foundation (DFG) and the Albert Ludwigs University Freiburg in the funding programme Open Access Publishing.

Competing Interests

The authors have declared that no competing interest exists.

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