Original Article
CPA4 is a promising diagnostic serum biomarker for pancreatic cancer

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Abstract: CPA4 belongs to a member of the metallocarboxypeptidase family, and its expression in pancreatic cancer samples and clinical significance are still not investigated until now. In this study, we aimed to evaluate the level of CPA4 in pancreatic cancer samples and study its clinical implications as a diagnostic marker for pancreatic cancer. The levels of CPA4 in pancreatic cancer tissues and serum samples were measured by immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA), respectively. Among 150 pancreatic cancer tissues examined, 86.7% (130/150) of cases showed positive staining for CPA4. Clinicopathological relevance analysis showed that CPA4 expression was correlated with advanced clinical stage and lymph node metastasis. Also, we found that the levels of CPA4 in serum samples were significantly high in cases whose expression was also high in paired tissue samples (N=50). In a larger sample set, we found that serum CPA4 in pancreatic cancer patients was significantly higher than for healthy controls (P<0.05). In addition, high serum CPA4 was significantly associated with the TNM stage, Lymph node involvement and distant metastasis. At a cutoff value of 0.3 ng/ml, CPA4 might be a better diagnostic biomarker of pancreatic cancer than CA199. In conclusion, CPA4 overexpression is associated with pancreatic cancer progression, and it might be a potential diagnostic serum marker for pancreatic cancer.

Keywords: CPA4, pancreatic cancer, marker, immunohistochemistry, ELISA

Introduction

Pancreatic cancer (PC) is one of the most lethal malignancies with a 5-year survival rate of only 6% [1]. Because of the asymptomatic nature in early stage, aggressive disease course, and limitations of current detection technologies, fewer than 20% of pancreatic cancer patients are diagnosed with localized resectable disease. Early detection of pancreatic cancer can offer patients the best chances for survival and can increase five-year survival rates from ~5% to 20-30% [2]. Serum-based assays are the most used tests for the early detection of cancer. Although CA199 is the gold-standard serum biomarker for monitoring and evaluating prognosis of pancreatic cancer, it is also elevated in other benign conditions and multiple cancer types with limited sensitivity [3, 4].

Consequently, the discovery of novel biomarkers involved in the diagnosis and progression of pancreatic cancer is of great value.

Carboxypeptidase A4 (CPA4) is a member of the metallocarboxypeptidase family [5].

CPA4 is a zinc-containing exopeptidase that catalyzes the release of carboxy-terminal amino acids. It was proved to be secreted from cells as a soluble proenzyme (pro-CPA4) that is activated by proteolytic cleavage [5]. CPA4 was originally found in a screen of mRNAs up-regulated by sodium butyrate-induced differentiation of cancer cells. There was a relation between CPA4 and prostate cancer aggressiveness [10]. However, the clinical significance of CPA4 expression in pancreatic cancer still remains unclear.

In this study, we firstly demonstrated that CPA4 level was significantly elevated in pancreatic cancer tissues as well as serum samples, and is closely associated with tumor progression. From a clinical standpoint, it might be a potential diagnostic serum marker for pancreatic cancer.

Materials and methods

Clinical samples

All tissue and blood specimens were collected from patients in the Department of Pathology in Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. Patients did not receive any treatment prior to surgery, and signed informed consent forms for sample collection. All tissue samples were taken by experienced surgeons and examined independently by two experienced pathologists. For immunohistochemistry analysis, 150 paraffin-embedded pancreatic cancer tumors and paired adjacent normal pancreatic tissues were randomly obtained from patients during 2008-2012. For ELISA study, preoperative peripheral blood samples were obtained from 100 pancreatic cancer patients (median age at 53.6 with a range of 21 to 77 years) during 2010-2012. Total of 50 specimens of healthy individuals (median age at 47.1 with a range of 21 to 65 years) were donated on a voluntary basis. For all the specimens, clinicopathological information (age, gender, and pathology, differentiation, and TNM stage) was available. The study was approved by the medical ethics committee of Cancer Institute and Hospital, CAMS.

CPA4 immunostaining of formalin-fixed tumor tissues

Formalin-fixed paraffin-embedded normal tissues and tumor tissues were evaluated using standard immunohistochemical staining for CPA4 expression. Tissue microarrays were prepared from archival formalin-fixed, paraffin-embedded tissue blocks. For each tumor, a representative tumor area was carefully selected from a H&E-stain section. Sections 5 μm in thickness were obtained and mounted on positively charged slides for immunohistochemical analysis. Standard avidin-biotin complex peroxidase immunohistochemical staining was performed. Briefly, after deparaffinizationin xylene and graded alcohols, heated antigen retrieval was done in citrate buffer (10 mmol/L pH 6.0) by water-bath kettle heating for 30 min. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide for 10 min. Nonspecific binding was blocked by incubation in 10% normal animal serum for 10 min. Sections were incubated at 4°C for 24 h with a primary antibody for CPA4 (HPA021030, Sigma-Aldrich, 1:300). Expression levels of proteins were scored by malignant/epithelial cells staining intensity and the percentage of immunoreactive cells. Tissues with no staining were rated as 0, with faint staining or moderate to strong staining in 20% of cells as 1, with moderate staining or strong staining in 20% to 40% of cells as 2, and with strong staining in >40% of cells as 3. Pancreatic cancer tissues that registered levels 0 and 1 were defined as negative for expression, whereas samples at levels 2 or 3 were defined as positive.

Measurement of human plasma levels of CPA4

A commercially available ELISA kit (SEF317Hu, Cloud-Clone Corp.) was used to measure CPA4 level. The sensitivities of the immunoassays for CPA4 were 0.156 ng/mL-10 ng/mL respectively. Each serum sample was run in duplicate. Briefly, 100 ml of serum (1:2 dilution) were placed into each well of the ELISA plate and incubated for 45 min at 37°C. The plates were washed four times with buffer and incubated with 100 ml of detection antibody at 37°C for 45 min. After four washes, the plates were incubated with substrate solution for 15 min at room temperature, then the reaction was stopped and the plates were read by a spectrophotometer at wavelength 450 nm. A standard curve was generated with the provided standards and used to calculate the quantity of CPA4 in each serum sample.

Statistical analysis

The SPSS 15 software package (SPSS, Inc., Chicago, IL) was used for statistical analysis. Mean between-group values were compared using the χ^2-test. The independent sample t test was used for ELISA group analysis. The association between the markers and clinicopathologic features was analyzed using χ^2-test or two-sided t-test as appropriate. Receiver operating characteristics (ROC) curves were generated to compare the predictive sensitivity and specificity, and the area under the curve
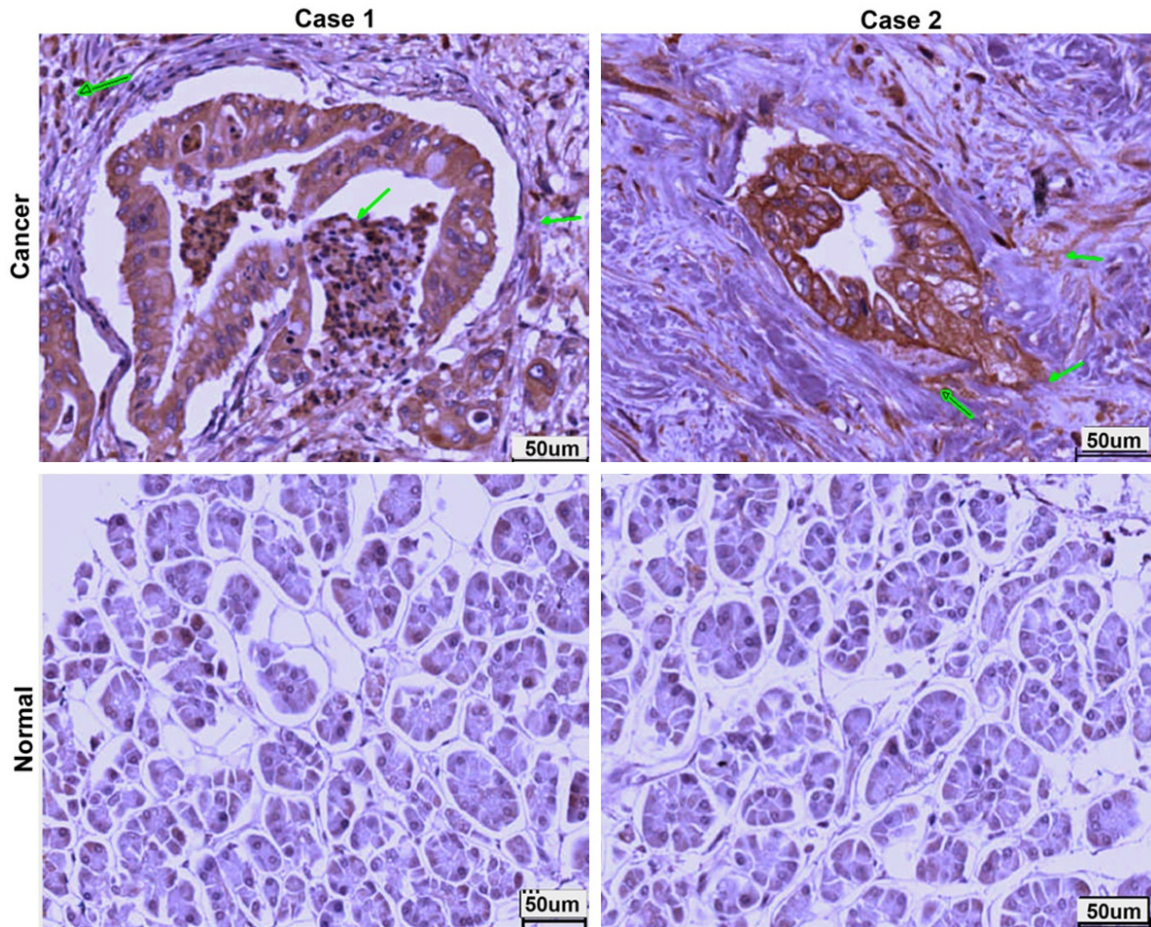
Figure 1. Expression of CPA4 in human primary pancreatic cancer tissues and normal adjacent tissues.

(AUC). All comparisons were two-tailed, and *p* values of <0.05 were considered significant.

Results

CPA4 expression in pancreatic cancer tissues by immunohistochemistry

To determine the level of CPA4 in pancreatic cancer and analyze the clinical significance, we evaluated its expression in a TMA of pancreatic tissues containing 150 pancreatic cancer tissues and adjacent normal tissues. IHC scoring showed that 130 of 150 of the primary lesions exhibited positive staining for CPA4, but there was no detectable staining of CPA4 in the normal tissues. As a secreted protein, we also identified specific expression of CPA4 in interstitial tissues of pancreatic cancer (**Figure 1**). Statistical analysis indicated that CPA4 expression in pancreatic cancer was correlated with Depth of invasion, Lymph Node Metastasis and

Stage (P<0.05). There was no significant association with other clinicopathologic variables (**Table 1**). Taken together, these observations demonstrated that over-expression of CPA4 is significantly associated with pancreatic cancer progression, which can be released into tumor surroundings.

Correlation between CPA4 level in serum and cancer tissues

To investigate the correlation, we harvested the 50 paired pancreatic cancer tissue and serum samples from Cancer Hospital, and evaluated the expression of CPA4. In pancreatic cancer tissues, the expression of CPA4 was high in 27 cancer tissues (27/50, 54%) by IHC. Similarly, 24 cancer serum sample (24/50, 48%) can be detected the existence of CPA4 by ELISA. Furthermore, correlation analysis using Spearman's rho test revealed a significant correlation between CPA4 in tissue and serum samples (Spearman rho =0.887; P<0.01) (**Table 2**).

Table 1. Correlation between CPA4 expression in pancreatic cancer tissues and clinicopathological parameters

	CPA4		
	Negative	Positive	p-value
Sex (Male: Female)	14:8	60:68	0.146
Age	59±4.8	60.2±9.2	0.567
Depth of invasion			0.006*
T1	4	4	
T2	18	112	
T3	0	12	
T4	0	0	
Lymph node involvement			0.000*
N0	22	76	
N1	0	48	
N2	0	0	
Liver metastasis			0.228
M0	22	120	
M1	0	8	
Stage			0.000*
1	22	60	
2	0	60	
3	0	0	
4	0	8	
Grade			0.367
1	0	6	
2	18	88	
3	4	34	

*p-value is statistically significant.

Table 2. Association between CPA4 expression in tissue and in serum of pancreatic cancer patients

		CPA4 (tissue)				p-value
		0	1	2	3	
CPA4 (in serum)	0	7	16	3	0	0.000*
	1	0	0	6	18	

*p-value is statistically significant.

Serum CPA4 in pancreatic cancer patients was significantly higher than that in healthy people

To evaluate the potential of CPA4 as serum pancreatic markers, we examined their levels in sera from pancreatic cancer patients (n=100) and healthy controls (n=50). The serum levels of CPA4 in patients with Pancreatic cancer were significantly greater than those of the healthy group (CPA4: 1.695±2.093 vs 0.123±0.251 ng/mL, P=0.000). ROC curve for serum

CPA4 concentration was constructed to determine the cutoff values. The best cut-off value of CPA4 for distinguishing between pancreatic cancer patients and controls was 0.3 ng/ml, with a sensitivity and specificity of 61% and 90%, respectively (**Figure 2A**). The approximate area under the ROC curve assessing serum CPA4 as a diagnostic tool for the detection of pancreatic cancer against normal controls was 0.783.

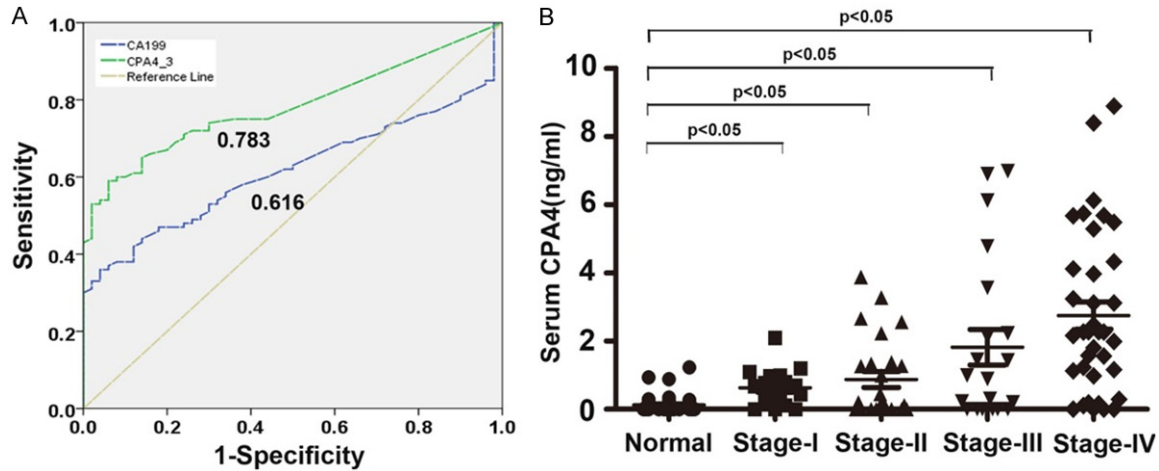*Clinical significance and prognostic value of CPA4 as a serologic biomarker for pancreatic cancer*

The clinical profile of pancreatic patients with a serum CPA4 level above the cut-off level (0.3 ng/ml) is shown in **Table 3**. Serum CPA4 levels were significantly positively correlated with TNM stage (P=0.036), lymph node involvement (P=0.016), and distant metastases (P=0.004). However, no significant correlation was observed between serum CPA4 level and other clinicopathologic parameters, including age (P=0.793), gender (P=0.213) and Depth of invasion (P=0.973). Further analysis showed that a gradual increase in serum levels of CPA4 at different stages of pancreatic cancer was observed (**Figure 2B**). Thus, the elevation of serum CPA4 levels appears to be closely associated with pancreatic cancer progression.

Discussion

Pancreatic cancer is one of the most lethal human cancers with a very low survival rate of 5 years. Most pancreatic cancer patients can not be early diagnosed because of the latent onset and lack of good biomarkers [6]. If pancreatic cancer is localized without invading local structures or metastasis, the survival is better after surgical resection [7]. Therefore, it is necessary to find the biomarkers for early detection of pancreatic cancer to achieve the best clinical outcomes.

Previous studies showed that sodium butyrate up-regulated the mRNA level of CPA4 in androgen-independent prostate cancer cells, which is involved in the Histone Hyperacetylation signaling pathway [8]. Moreover, they demonstrated that CPA4 mRNA level was extremely low in normal prostate tissues. These findings implied that CPA4 may play important roles in tumor progression.

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detect the CPA4 serum level by ELISA. For the first time, we showed that serum levels of CPA4 in patients with pancreatic cancer were significantly greater than those of the healthy group. Moreover, we also found that serum levels of CPA4 were significantly positively correlated with TNM stage (P=0.036), lymph node involvement (P=0.016), and distant metastases (P=0.004). Further analysis showed that a gradual increase in serum levels of CPA4 at different stages of pancreatic cancer was observed. ROC curves demonstrated that the best cut-off value of CPA4 for distinguishing between pancreatic cancer patients and controls was 0.3 ng/mL, with a sensitivity and specificity of 61% and 90%, respectively. The approximate area under the ROC curve assessing serum CPA4 as a diagnostic tool for the detection of pancreatic cancer against normal controls was 0.748.

Conclusions

In summary, CPA4 level was significantly elevated in pancreatic cancer tissues and serum samples, and it might be a potential diagnostic serum marker for pancreatic cancer.

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Disclosure of conflict of interest

None.

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