



# The diagnostic value of extracellular protein kinase A (ECPKA) in serum for gastric and colorectal cancer

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**Background:** For the biomarkers of cancer, many chromosomal and genetic alterations have been examined as possible. However, some tumors do not display a clear molecular and genetic signature. While there are some cellular processes regulated by second messenger intracellular pathways indeed involved in carcinogenesis. The first intracellular second messenger was described as cyclic adenosine 3',5'-monophosphate (cAMP), and cAMP-dependent protein kinase (PKA) play a crucial role in several biological processes; The dysregulation of PKA-mediated signaling in several types of cancer should be investigated. More interesting, the alpha catalytic subunit of PKA (PKA $\alpha$ ) could be secreted into the conditioned medium by different types of cancer cells, and it also existed in the serum of some cancer patients, defined as extracellular protein kinase A (ECPKA).

**Methods:** The levels of serum PKA $\alpha$  from healthy people, gastric cancer and colon cancer patients were detected by ELISA kits. Western blotting was used to detect the expression of PKA $\alpha$  in cancer tissue and the adjacent mucosa. Mann-Whitney test was applied to analyze the patients' characteristics and serum PKA $\alpha$ . ROC analyses were performed to further evaluate the utility of PKA $\alpha$  in cancer diagnosis. The correlation of serum PKA $\alpha$  and T stage, age, and tumor markers were analyzed by Spearman rank and Pearson correlation analysis, respectively.

**Results:** There were significant differences of PKA $\alpha$  in serum between the volunteers and the gastric cancers ( $P < 0.01$ ), but not the colorectal cancers ( $P > 0.05$ ). ROC analyses evaluated the utility of PKA $\alpha$  for gastric cancer with 61.90% sensitivities and 87.50% specificities. The serum PKA $\alpha$  was correlated with tumor marker CA50, while there was no significant difference of PKA $\alpha$  expression between the gastric/colorectal cancer tissue and the adjacent mucosa.

**Conclusions:** The above results implied that PKA $\alpha$  levels might be a potential biomarker for the early screening of gastric cancers. Moreover, further research is still needed to investigate the role of secreted PKA $\alpha$  and the regulatory mechanism in tumor progression.

**Keywords:** PKA $\alpha$ ; serum; gastric cancer; colon cancer

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## Introduction

Gastrointestinal cancer, comprising a diverse group of tumors with varying clinical course and prognosis, is one of the most common cancer-related death worldwide due to the high incidence and overall poor outcome. For example, gastric cancer (GC) represents the fifth most common malignancy and remains the third leading cause of cancer mortality with widely varying incidence worldwide, while colorectal cancer (CRC) is the third most common cancer in men and the second most common in women (1). Advances over the last decade in molecular diagnostics in GC and CRC have a benefit to improve our understanding of the complex mechanisms in the development and progression (2). However, the low rate of early diagnosis means that most patients have the advanced-stage disease at diagnosis and so the best surgical window is missed, leading that the 5-year survival rate of patients is extremely low. So, early prediction/detection plays a vital role in improving their prognosis and optimal treatment. Although there is much progress in the cancer diagnosis due to endoscopic ultrasonography application, the early detection of serum biomarkers still has its advantages in patient diagnosis, monitoring, and therapeutic management, compared with some other screening programs (3). It is essential to discover new biomarkers of gastric cancer that could give predictive information about the response to therapy and finally improve the therapeutic outcomes (4). Currently, some serum biomarkers have been identified, including vascular endothelial growth factor, aberrant DNA or RNA, carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), cancer antigens (i.e., CA125), most of them have already used in the clinic, such as CEA, CA125, and AFP. Unfortunately, the ideal specific biomarker for the prediction of gastrointestinal cancer in the early period remains devoid. Protein Kinase A (PKA), also known as a cAMP-dependent protein kinase, is a serine/threonine kinase that is responsible for phosphorylating a wide range of downstream substrates, the extent of which depends on cellular localization (5-7). PKA is inactive heterotetramers of four regulatory (R) subunits, including RI $\alpha$ , RI $\beta$ , RII $\alpha$  and RII $\beta$ , and three catalytic (C) subunits, including C $\alpha$ , C $\beta$ , and C $\gamma$  (8). Each R subunit contains a flexible inhibitory segment that limits catalytic activity by blocking access to the active site of the C subunit (8,9). The binding of cAMP to the regulatory subunit leads to the dissociation and activation of the catalytic subunits and thus releases the C subunits to phosphorylate downstream cytoplasmic and nuclear targets (10,11). One main target in nuclear is

the transcription factor cAMP response binding protein (CREB) (12).

PKA is considered as an essential regulator of several physiological processes (13,14), including ion channels, enzymes catalyzing biosynthesis, metabolism, transmembrane receptors, and other signaling mediator proteins (9,15). More importantly, although PKA is not traditionally considered as an oncogene, dysregulation of PKA signaling is implicated in tumorigenesis and progression of numerous cancers (16). For example, an extracellular form of PKA has been detected in the conditioned medium and serum from several cancer patients, including prostate, renal, bladder, breast, lung, melanoma, lymphomas, and adrenal carcinomas (11,16-19). More recently, it was reported that upregulation transcripts of PKA catalytic subunit alpha (*PKAC $\alpha$* ) have also been detected in breast cancer, specifically in patients resistant to trastuzumab (20); elevated *PKAC $\alpha$*  transcript levels in tissues can also lead to fibrolamellar hepatocellular carcinoma (21). Many studies have indicated that PKA is expressed in various types of cancers; however, the relationship between its expression pattern and disease prognosis remains unclear. Besides, recently, some reports showed that free catalytic subunit of PKA (PKAC) was found in the peripheral blood of cancer patients and secreted by some cancer cells (11,17). This form PKA is active, designated as extracellular protein kinase A (ECPKA) (22). About the role of ECPKA in cancer, there are some different views. Some reports showed that the released PKAC from conditioned media of cultured cancer cells could inhibit angiogenesis (23). And some others found that the ECPKA expression in serum of cancer patients is up-regulated as compared with normal serum (11). So, it is imperative to further elucidate the role of ECPKA in cancer. In this study, we examined the level of *PKAC $\alpha$*  in serum and tissue of gastric and colon cancer people and investigated the relationship between *PKAC $\alpha$*  expression and clinicopathological factors in the diagnosis and prediction of gastric and colon cancer patients.

We present the following article in accordance with the REMARK reporting checklist (available at <http://dx.doi.org/10.21037/tcr-20-764>).

## Methods

### Reagents

*PKAC $\alpha$*  ELISA kit was obtained from Cloud-Clone-Corp (Katy, TX, USA). RIPA buffer was from Proteintech (Rosemont, IL, USA). Antibodies against *PKAC $\alpha$*  and

$\beta$ -actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Electrochemiluminescence (ECL) reagents were purchased from Millipore (Billerica, MA, USA).

### *Serum samples*

Serum samples were collected from regular healthy volunteers (n=24), patients with gastric cancer (n=42), and colorectal cancer (n=20) from the Affiliated Hospital of Jiangsu University in Zhenjiang of China. Serum was obtained by centrifugation at 2,000 rpm for 20 min at 4 °C and stored at -80 °C until use. The diagnosis of cancer for each patient was confirmed by pathology analysis. All the patients didn't receive any treatment. Healthy volunteers, without evidence of surgical history, chronic/acute diseases, or current use of any medication, were randomly recruited into this study. All patients were consent informed. The study involving human subjects was approved by the Human Ethics Committee of School of Medicine, Jiangsu University.

### *Resected specimens*

The surgically resected specimens from patients who underwent curative surgery (24 gastric cancer and 13 colon cancer) were obtained from the Affiliated Hospital of Jiangsu University in Zhenjiang of China. All patients were consent informed. Resected specimens were stored in liquid nitrogen until analysis. The study involving human subjects was approved by the Medical Ethics Committee of Jiangsu University.

### *ELISA assay*

The serum samples were detected according to the PKAC $\alpha$  ELISA kit manufacturer's specifications and read at 450 nm immediately. And the BCA Protein Quantification Kit (Sigma-Aldrich, MO, USA) was applied for determining the total concentration of proteins in the tissue lysis. The data were obtained from three independent experiments.

### *Tissue lysis*

The cancer tissue and adjacent mucosa were cut into smaller pieces while keeping them on ice and transferred into a homogenizer and then homogenized thoroughly with

RIPA buffer plus protease inhibitors on ice. Centrifuged at 12,000  $\times$ g for 20 minutes at 4 °C to pellet cell debris, and then transferred the supernatant to a fresh microfuge tube. Samples were frozen at -80 °C until used.

### *Western blotting*

Equal amounts of protein lysis were separated by 8–10% SDS-PAGE gels electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking by 5% non-fat dried milk, the membranes were incubated with specific primary antibodies against PKAC $\alpha$  or  $\beta$ -actin overnight at 4 °C. After washing 3 times with TBS/T, the horseradish peroxidase-labeled (HRP) secondary antibodies were incubated for 1 h at room temperature. The bands were detected with ECL reagents.

### *Statistical analysis*

The population expression of PKAC $\alpha$  was analyzed with age, gender, metastasis, and some tumor makers. Two groups were compared by *t*-test for normal distribution and Wilcoxon Rank Sum Test for non-normal distribution. Differences among normally distributed data of three or more groups were analyzed by one-way ANOVA, following by the Bonferroni post hoc test. The relationship of serum PKAC $\alpha$ , gender, liver metastasis, and lymphatic metastasis was analyzed by the Mann-Whitney test. The rank correlation of serum PKAC $\alpha$  and T stage, age, and tumor markers were analyzed by Spearman rank correlation and Pearson correlation. SPSS 24.0 software and GraphPad Prism 8.0 were applied to assay all the above data, and  $P < 0.05$  was considered significant for all tests.

## **Results**

### *PKAC $\alpha$ in serum of the cancer patient is higher than healthy people*

To investigate whether there are differences in the serum levels of PKAC $\alpha$  between patients with gastric, colorectal cancers and volunteers, the serum of patients was collected and detected by ELISA. The data were first analyzed by Kolmogorov-Smirnov (K-S) test, and the results showed that  $Z=0.127$ ,  $P=0.002$ , indicating that level of PKAC $\alpha$  in serum and were abnormal distributions (*Table 1*), so the data were further analyzed by Kruskal-Wallis H test,

and multiple comparisons of PKAC $\alpha$  in serum between the three groups were analyzed by Bonferroni correction. The results showed that the levels of serum PKAC $\alpha$  were higher in patients with colon and gastric cancer (258.75 $\pm$ 90.20 and 288.31 $\pm$ 177.67 pg/mL, respectively) compared with the volunteers (191.08 $\pm$ 73.44 pg/mL) (Figure 1A, Tables 1,2). There were significant differences between the volunteers and the gastric cancers (P<0.01), but no difference between the volunteers and the colorectal cancers (P>0.05). ROC analyses were performed to further evaluate the utility of PKAC $\alpha$  as a potential cancer biomarker (Figure 1B,C). For gastric cancer, the sensitivities and specificities of PKAC $\alpha$  was 61.90% and 87.50%, at the cutoff value of 253.92 pg/mL, with an AUC value of 0.727 (Z=3.637, P<0.001). The above results

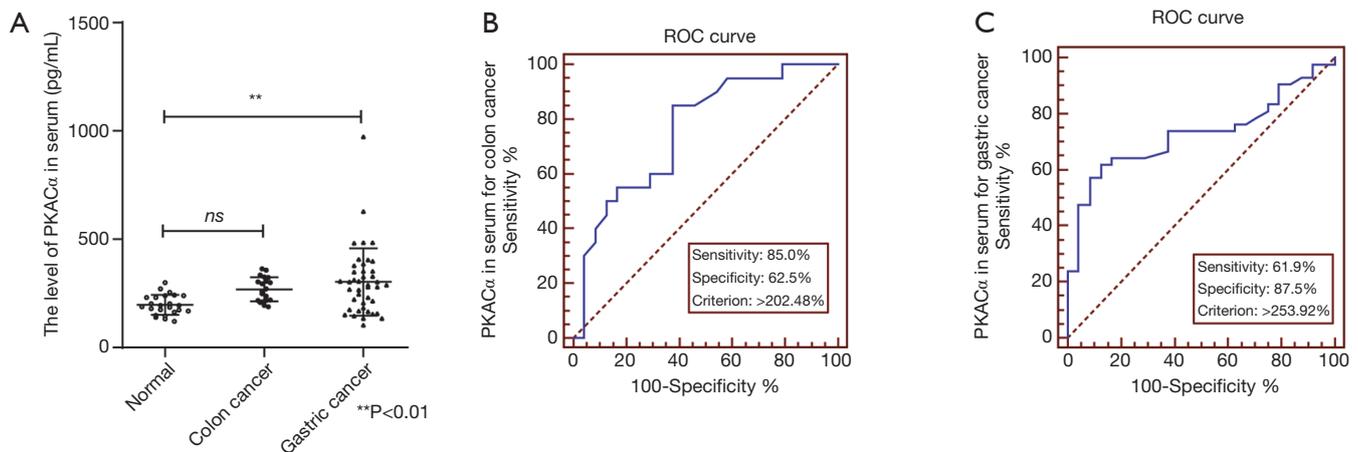
implied that specific PKAC $\alpha$  levels might be a potential biomarker for gastric cancer diagnosis while it is not suitable for the diagnosis of colorectal cancer.

**Association between serum PKAC $\alpha$  and clinicopathological factors**

To investigate whether there is a relationship between serum PKAC $\alpha$  and clinicopathological factors. Mann-Whitney test was applied to analyze the patients' characteristics and serum PKAC $\alpha$ . Also, the results in Table 3 showed that the differences of gender, liver metastasis, and lymphatic metastasis were not significantly co-related with the difference of the levels of serum PKAC $\alpha$ . Besides, the analysis of Spearman rank correlation was applied to analyze the relationship of PKAC $\alpha$  in serum with T stage, age, and there was no association between PKAC $\alpha$  and T stage (r=-0.231, P=0.094) or age (r=0.129, P=0.322). And the Pearson Correlation analysis was applied for the relationship of PKAC $\alpha$  and tumor markers (including CA50, CA199,

**Table 1** The assay by Kolmogorov-Smirnov normality test

Index	Z	P
PKAC $\alpha$ in serum	0.127	0.002



**Figure 1** Analysis of PKAC $\alpha$  level in human serum from normal subjects, colon cancer patients, and gastric cancer patients. Reaction mixtures containing human sera from normal subjects (n=24); patients with colorectal (n=20) or gastric (n=42) cancer were applied to the ELISA arrays. (A) Distributions of specific PKAC $\alpha$  levels. The levels represent the median  $\pm$  inter-quartile range (M $\pm$ Q) of PKAC $\alpha$  level. \*\*P<0.01 versus Normal; ns, not significant. (B,C) ROC plots of PKAC $\alpha$  level in colon cancer and gastric cancer. ROC, receiver operator characteristic curve.

**Table 2** Descriptive statistics and Kruskal-Wallis H test for the serum PKAC $\alpha$  in different individuals

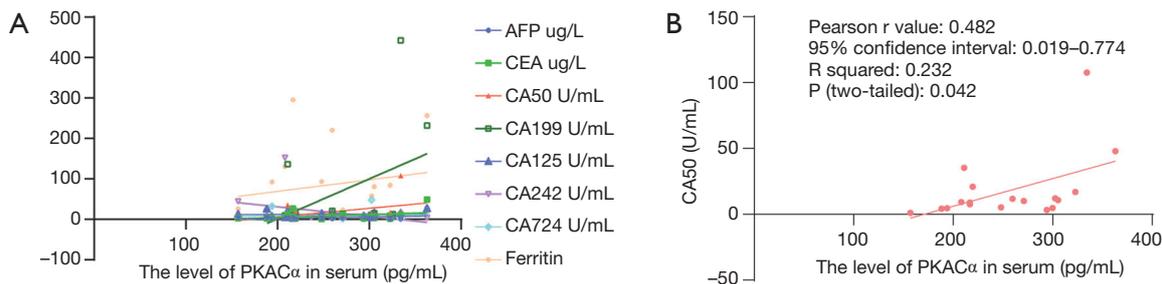
Index	Normal	Colon cancer	Gastric cancer	H	P
PKAC $\alpha$ , M (Q)	191.08 (73.44)	258.75 (90.20)	288.31 (177.67)	11.786	0.002

M, median; Q, inter-quartile range.

**Table 3** Clinicopathological characteristics of the study group and association with PKAC $\alpha$  expression

Characteristic	PKAC $\alpha$ , M (Q)	Z	P
Gender		-1.593	0.111
Male	257.48 (232.65)		
Female	293.98 (195.83)		
Liver metastasis		-1.818	0.069
None	270.16 (168.74)		
Yes	166.91 (63.51)		
Lymphatic metastasis		-0.615	0.539
None	236.75 (134.38)		
Yes	302.63 (139.64)		

M, median; Q, inter-quartile range.



**Figure 2** The correlation of serum PKAC $\alpha$  and tumor markers. (A) Pearson correlation analysis was applied for the relationship of PKAC $\alpha$  and tumor markers (including CA50, CA199, AFP, CEA, CA125, CA242, CA724, and Ferritin). (B) There is a significance Correlation between serum PKAC $\alpha$  and CA50 by Pearson correlation analysis ( $r=0.482$ ,  $P<0.05$ ).

AFP, CEA, CA125, CA242, CA724, and Ferritin). The result in *Figure 2* showed that PKAC $\alpha$  in serum was positively-correlated with CA50 (Pearson  $r=0.482$ ,  $P=0.042$ ), but was not associated with other factors or tumor markers, including AFP, CA199, CEA, CA125, CA242, CA724, and Ferritin.

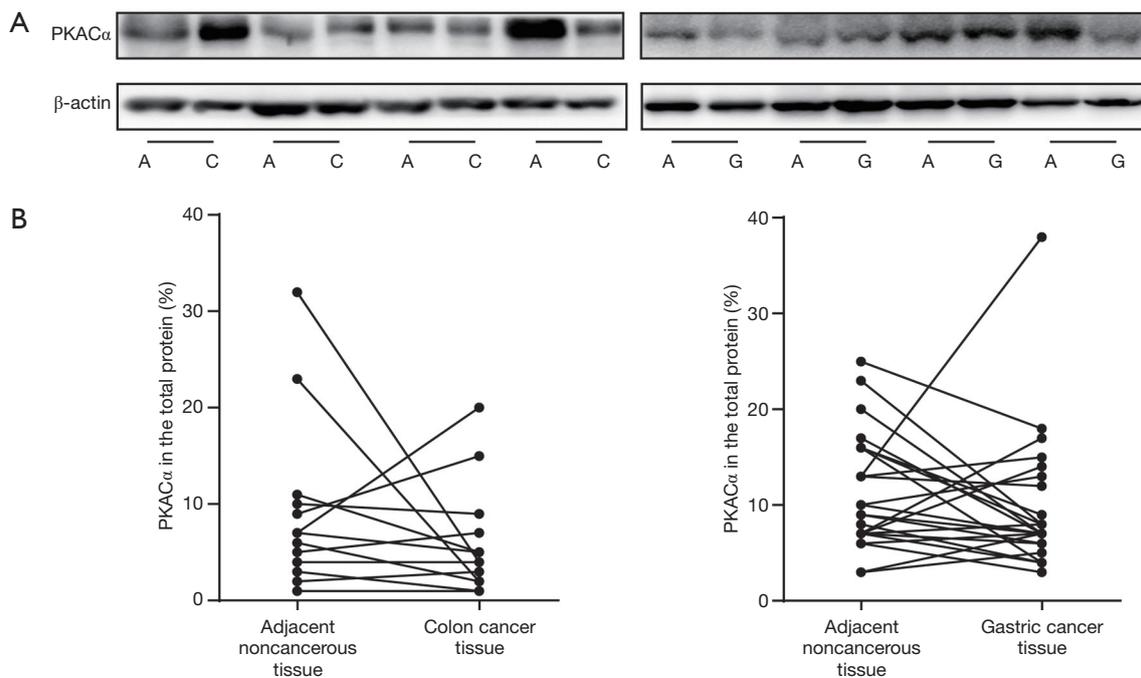
#### The PKAC $\alpha$ expression in cancer tissue and the adjacent mucosa

The expression of PKAC $\alpha$  in colon cancer tissue ( $n=13$ ), gastric cancer ( $n=24$ ) tissue and the adjacent mucosa were also detected. The western blotting and ELISA results (*Figure 3* and *Table 4*) showed that in the colon cancer patients ( $n=13$ ), there were 5 cases, the PKAC $\alpha$  expression in cancer tissue is higher than that in the adjacent noncancerous tissues, while 8 cases, no higher. But there was no significant difference of PKAC $\alpha$  expression between

the colon cancer tissue and the adjacent mucosa ( $P>0.05$ ). In gastric cancer patients ( $n=24$ ), there are 8 cases, the PKAC $\alpha$  expression in cancer tissue is higher than that in the adjacent noncancerous tissues, while 16 cases, no higher. Similarly, PKAC $\alpha$  expressed in the gastric cancer tissue and the adjacent mucosa with no significant difference ( $P>0.05$ ).

#### Discussion

Malignancies of the gastrointestinal tract, such as CRC, GC, and pancreatic carcinoma, are among the most common human cancers (1,24), and each kind of them has specific clinical features (24,25). Usually, the conventional therapies for these malignancies include radiotherapy, chemotherapy, and target therapies. For example, a standard first-line therapy for advanced CRC is the combination of chemotherapy and EGFR inhibitor or VEGFR inhibitor (26). Although many chemotherapeutic agents and combinations



**Figure 3** The PKAC $\alpha$  expression in cancer tissue and the adjacent mucosa. (A) The tissue from cancer and the adjacent mucosa were harvested, lysed, and then detected by Western blotting; (B) ELISA kits were applied for detecting the level of PKAC $\alpha$  in colon cancer tissue, gastric cancer tissue, and the adjacent mucosa. The results are representative of three independent experiments. A, adjacent noncancerous tissue; C, colon cancer tissue; G, gastric cancer tissue.

**Table 4** Descriptive statistics of PKAC $\alpha$  in cancer tissue and the adjacent mucosa

Groups	PKAC $\alpha$ in the total protein (%) (mean $\pm$ SEM)		t value	P value
	The adjacent mucosa	The cancer tissue		
PKAC $\alpha$ in gastric cancer	11.29 $\pm$ 01.22	9.75 $\pm$ 1.49	0.9027	0.3760
PKAC $\alpha$ in colon cancer	9.23 $\pm$ 2.45	6.00 $\pm$ 1.58	1.0940	0.2954

SEM, standard error of mean.

are available, the overall prognosis of patients with advanced gastric and colorectal cancer remains poor (27), and the clinical outcomes of the patients are still not encouraging with a low rate of 5 years survival (28). Some of the reason is due to the concealed early symptoms and lack of sensitive diagnostic biomarkers. So, the prognosis of gastric and colorectal cancer patients can be greatly improved by early detection or prediction of the metastatic potential of the primary tumor. Although there is much progress in the cancer diagnosis due to endoscopic ultrasonography application, the early detection of serum biomarkers still has its advantages in patient diagnosis, monitoring, and therapeutic management, compared with some other

clinical screening examination (3). Currently, some serum biomarkers have been used in the clinic, while the ideal biomarker for gastric and colorectal cancer in the early period is not yet identified.

Recent molecular studies showed that some kinase inhibitors were taken as targeted therapies in gastric or colon tumors (25). So far, there are 518 kinases confirmed in human (25). The elucidation of the first kinase signaling cascade involving PKA in the 1960s marked a milestone (29), followed by the breakthrough discovery that protein kinases can act as oncogenes, which won the 1989 Nobel Prize in Physiology (30). The function of the intracellular PKA in cell survival and migration has been widely

studied (31). Recently, there was indeed an intriguing phenomenon (11,16,17,19,23) that the release of PKAC $\alpha$  into the extracellular environment, including the serum of cancer patients. This form of PKA does not require activation by cAMP, which is distinct from the intracellular tetrameric holoenzyme, and the release of ECPKA into the extracellular milieu is not due to cell lysis (16,17,22). Though the function and mechanism of the secreted kinase are poorly known, it is clear that there are marked differences between ECPKA and intracellular PKA (23).

In our study, there were significant differences between the volunteers and the gastric cancers, and the ROC analyses also implied that specific PKAC $\alpha$  levels might be a potential biomarker for the gastric cancer diagnosis. Moreover, the release of ECPKA in peripheral blood has been further confirmed in other reports from various cancer patients, including breast, colon, cervical, lung, prostate, pancreatic, ovarian, and rectal carcinomas, and surgical removal of tumors led to a decrease in ECPKA levels in patients (11,16,19,22). More recently, there is increasing evidence that patients with cancer produce autoantibodies against antigens in their tumors, such as c-myc, p53, and ECPKA (22,32,33). The autoantibody against ECPKA has been detected in the serum of many patients by an autoantibody enzyme immunoassay (34). These results indicated that extracellular PKA might serve as a cancer antigen for diagnosis and prediction of cancer. However, in the gastric and colon cancer tissue, the expression of PKAC $\alpha$  is not always higher or lower than the adjacent mucosa, and there was no significant difference in this research. One reason is that the number of patients is insufficient and the pathological stage is inconsistent. The second, PKA is a heterotetramer. No data show whether a complete or partial tetramer is secreted into the extracellular milieu, however, in the present work, only the catalytic  $\alpha$  subunit was detected. In mammalian cells, there are two types of PKA (PKA-I and PKA-II). It is known that there are four R isoforms of PKA, with this common catalytic (C) subunit (22), and this PKA-RI and RII showed a different function on the tumor (16). The RII subunits, expressed preferentially in healthy tissues, play a growth-inhibitory role; the RI subunits, promote growth and have been implicated in cancer progression (16). And, the ratios of the intracellular PKA-I to PKA-II could regulate the secretion of ECPKA, a decrease in PKA-I by a mutant would down-regulate the ECPKA (11), while overexpression of PKA-I leads to the secretion of ECPKA (35). So, the oncogenic potential of PKA depends on which isoform is expressed, and it is

difficult to confirm the relationship of PKAC $\alpha$  in tumor tissues and its relation with the tumor stage. Of course, there are more questions needed to address, for example, the discrepancy of PKAC $\alpha$  between cancer patients and patients with benign disease, such as polyps and ulcers should be evaluated. Besides, more attention should pay to the phenomenon and mechanism of PKAC $\alpha$  secretion.

## Conclusions

The ECPKA in the serum of gastric cancers was higher than the healthy people. ROC analyses evaluated the utility of PKAC $\alpha$  for colorectal cancer and gastric cancer with higher sensitivities and specificities. Collectively, ECPKA may prove to be of great significance when used either alone or in combination with other biomarkers (such as CEA, CA199, etc.) or technologies for diagnosing and monitoring cancer. And targeting PKA, a kinase regulating cell metabolism, may provide a new way for tumor treatment.

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## Footnote

*Reporting Checklist:* The authors have completed the REMARK reporting checklist. Available at <http://dx.doi.org/10.21037/tcr-20-764>.

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study involving

human subjects was approved by the Human Ethics Committee of School of Medicine, Jiangsu University. All patients were consent informed.

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