Extracellular Activity of Cyclic AMP–Dependent Protein Kinase as a Biomarker for Human Cancer Detection: Distribution Characteristics in a Normal Population and Cancer Patients

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Abstract

The overexpression of cyclic AMP (cAMP)–dependent protein kinase (PKA) has been reported in patients with cancer, and PKA inhibitors have been tested in clinical trials as a novel cancer therapy. The present study was designed to characterize the population distribution of extracellular activity of cAMP-dependent protein kinase (ECPKA) and its potential value as a biomarker for cancer detection and monitoring of cancer therapy. The population distribution of ECPKA activity was determined in serum samples from a Chinese population consisting of a total of 603 subjects (374 normal healthy volunteers and 229 cancer patients). The serum ECPKA was determined by a validated sensitive radioassay, and its diagnostic values (including positive and negative predictive values) were analyzed. The majority of normal subjects (>70%) have undetectable or very low levels of serum ECPKA. In contrast, the majority of cancer patients (>85%) have high levels of ECPKA. The mean ECPKA activity in the sera of cancer patients was 10.98 units/mL, 5-fold higher than that of the healthy controls (2.15 units/mL; $P < 0.001$). In both normal subjects and cancer patients, gender and age had no significant influence on the serum ECPKA. Among factors considered, logistic analysis revealed that the disease (cancer) is the only factor contributing to the elevation of ECPKA activity in cancer patients. In conclusion, ECPKA may function as a cancer marker for various human cancers and can be used in cancer detection and for monitoring response to therapy with other screening or diagnostic techniques. (Cancer Epidemiol Biomarkers Prev 2007;16(4):789–95)

Introduction

Cyclic AMP (cAMP)–dependent protein kinase (PKA) is involved in cell proliferation, gene induction, metabolism, angiogenesis, ion channel regulation, and apoptosis (1, 2). More importantly, PKA has been implicated in the initiation and progression of numerous cancers and in response to cancer therapy (3-7). There are four regulatory (R) isoforms, two for each isoform of PKA (I or II; refs. 8-11), and the oncogenic potential of PKA depends on which isoform is expressed. The RII subunits are expressed preferentially in normal tissues and have growth-inhibitory effects; the RI subunits stimulate growth and have been implicated in cancer progression, drug resistance, and a poorer prognosis for patients (12-19). PKA RIα is overexpressed in many different types of human tumor tissues and cell lines, including breast (20), colon (15), lung (21), and ovarian (13) carcinomas.

Recently, an excreted, extracellular form of PKA has been found (12). It seems that excretion of the extracellular PKA is increased by type I PKA, which is frequently overexpressed in tumor cells (22, 23). Cancer cells of various types excrete extracellular cAMP-dependent protein kinase (ECPKA), including prostate, bladder, breast, and colon carcinoma cell lines, as well as a lung adenocarcinoma line (12, 24). Additionally, serum samples from patients with a variety of cancers have shown elevated ECPKA activity compared with normal serum samples (12). The ECPKA found in serum is constitutively active and cannot be further activated by addition of cAMP.

As a result of its overexpression in many cancers and its role in cancer progression, PKA has been suggested as a novel molecular target for cancer therapy. Several specific inhibitors of PKA have shown *in vitro* and *in vivo* antitumor activity (25-31). For example, a mixed-backbone antisense oligonucleotide containing both phosphorothioate-modified nucleosides and ribonucleosides showed antitumor effects in animal models of breast, colon, prostate, and lung cancer (32, 33). A trend toward decreased ECPKA was observed in the serum of patients treated with the antisense oligonucleotide (34). Additionally, in cancer cell lines and tumor samples treated with the antisense drug, there was an increase in the expression of genes associated with differentiation and reverse transformation and a decrease in expression of genes associated with proliferation and transformation (35).

Based on these preliminary results demonstrating that ECPKA is overexpressed in the serum of cancer patients and is decreased by treatment targeting PKA, we have undertaken

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Note: H. Wang, M. Li, W. Lin, and W. Wang contributed equally to this work.

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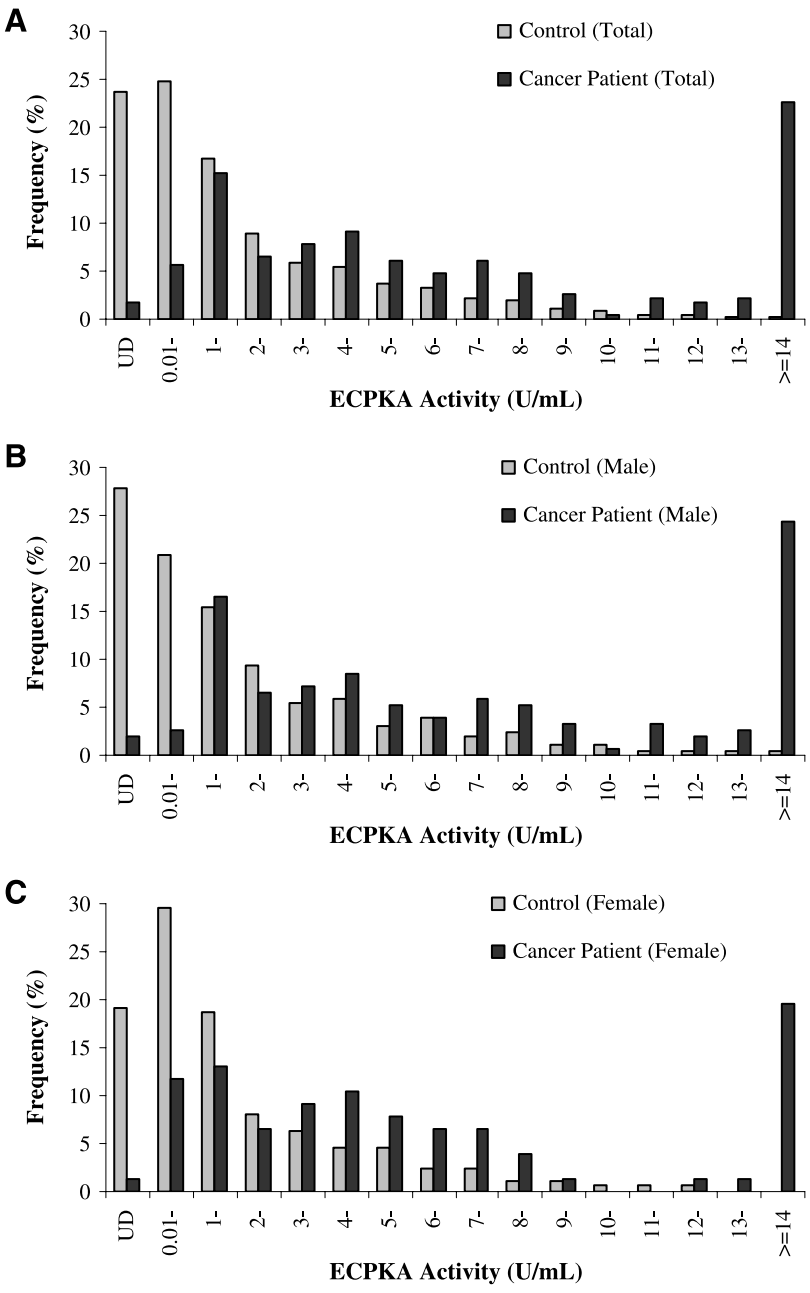

Figure 1. Population distribution of ECPKA in healthy volunteers and cancer patients. The activity of ECPKA (units/mL) was determined for control and cancer patients, and the frequency within the population was calculated. **A.** The frequency of ECPKA activity levels in all patients. **B.** The frequency of ECPKA activity levels in male patients. **C.** The frequency of ECPKA activity levels in female patients. *Light gray columns,* control patients; *dark gray columns,* cancer patients.

a study to characterize the population distribution of ECPKA in the normal population and cancer patients to determine whether ECPKA may serve as a biomarker for cancer detection and monitoring of therapy. To our knowledge, this is the first report demonstrating the value of ECPKA as a biomarker in cancer detection based on a large population study composed of both normal subjects and cancer patients.

Patients and Methods

Study Population. A total of 603 subjects (374 normal healthy volunteers and 229 cancer patients) from the metropolitan area of Nantong city of the People's Republic of China were included in this study. Informed consent was obtained from each subject. The study involving human subjects was approved by the Institutional Review Board of Haimen Cancer Institute. A questionnaire was administered to each participant, collecting basic demographic data as well as disease history and health status. All blood samples were drawn from patients before treatment. Serum was obtained by centrifugation at 2,000 rpm for 15 min at 4°C and stored at −80°C until analysis. The randomized study population included 152 male and 77 female cancer patients and 202 male and 172 female healthy volunteers. Patients diagnosed with various types of cancer were included in this study, including breast (30 patients), colon (29 patients), esophageal (24 patients), gastric (45 patients), hepatic (28 patients), lung (63 patients), and pancreatic (10 patients) cancers. The diagnosis of cancer for each patient was confirmed by pathology analysis. Healthy volunteers were randomly recruited into this study, with a strategy to balance age (similar sample size for each age group) and gender (similar gender distribution at each age group) in the normal population. The healthy controls had no evidence of surgical history or chronic or acute diseases. All healthy controls had no current use of any medication.

ECPKA Assay. The radioassay for ECPKA was a modification of the reported procedure (12). The Affinity Ultrafiltration Separation Assay (AUSA®) cAMP-Dependent Protein Kinase

Assay Kit was used according to the manufacturer's instructions (Transbio Corporation; Baltimore, MD). In brief, the sample was combined with cAMP and reacted with γ-^{32}P-ATP and a biotinylated peptide (Kemptide) specifically designed as a substrate for PKA. After 10 min at 30°C for binding, the reaction was stopped and subjected to centrifugal ultrafiltration with a membrane that allows free γ-^{32}P-ATP to pass through. The purified ^{32}P-peptide product was then evaluated by liquid scintillation spectrometry (LS 6000T A; Beckman, Irvine, CA) and PKA-specific activity was calculated according to the manufacturer's instructions. One unit of ECPKA is defined as the amount of enzyme that transfers 1 pmol of ^{32}P from [γ-^{32}P]ATP to the recovered protein in 1 min at 30°C in the standard assay system. The linear range of the assay was 0 to 250 units/mL. The interday and intraday variations were <5%.

Lactate Dehydrogenase Assay. The activity of lactate dehydrogenase (LDH) was determined using the Lactate Dehydrogenase (LD-L) kit (Sigma Diagnostics Inc., St. Louis, MO). Briefly, after the LDH reagent was prepared (as per the manufacturer's instructions), the sample was added and allowed to incubate for 30 s at 30°C. The absorbance at 340 nm was recorded immediately following this 30 s, and another reading was taken after an additional 60 s of incubation at 30°C to determine the change per minute. The activity was then calculated according to the manufacturer's instructions. The interday and intraday variations were <5%.

Statistical Analysis. The population distribution of ECPKA was analyzed by age, gender, and health/disease status. The overall or stratified distribution of ECPKA activity is presented by sample size, mean, SD, median, and range. A two-sample *t* test was applied to compare the PKA activity between patients and healthy volunteers. ANOVA was used to compare PKA activity among age groups for both cancer patients and healthy volunteers. Pearson's correlation coefficient was also calculated between age and PKA activity for both groups. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of PKA activity versus cancer status were estimated using different cutoff levels (1, 2, 4, 8,

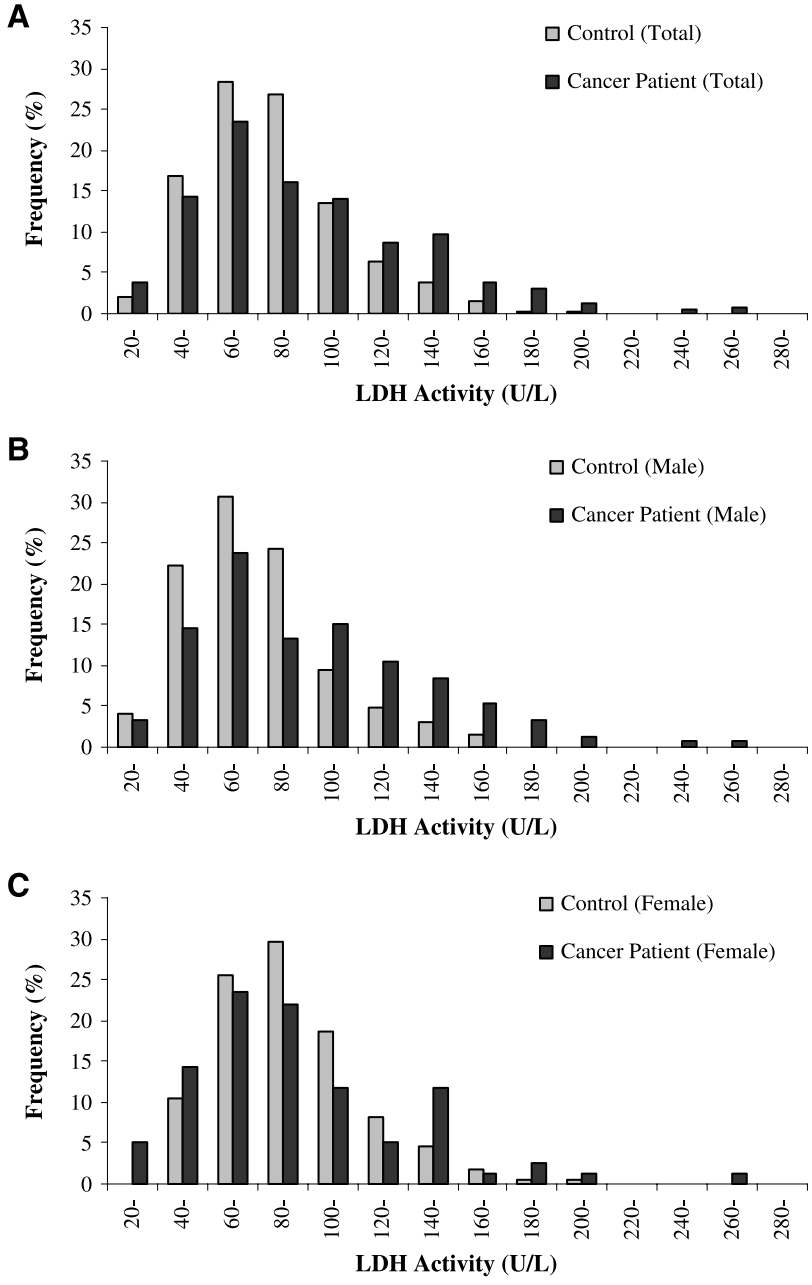

Figure 2. Population distribution of LDH in healthy volunteers and cancer patients. The activity of LDH (units/L) was determined for healthy controls and cancer patients, and the frequency within the population was calculated. Normal LDH levels are between 55 and 170 units/L. **A.** The frequency of LDH activity levels in all patients. **B.** The frequency of LDH activity levels in male patients. **C.** The frequency of LDH activity levels in female patients. *Light gray columns,* control patients; *dark gray columns,* cancer patients.

and 10 units/mL). This was also done for stratification of age group and gender. The correlation between PKA and cancer is presented in a 2 × 2 table using the different cutoff levels of PKA. The χ^2 test was applied to determine if the correlation was statistically significant.

Logistic regression was used to explore the correlation between cancer and PKA activity, adjusted for age and gender; the presence or absence of cancer was defined as the dependent variable. In addition to logistic regression of samples from all of the cancer patients, we also used logistic regression to evaluate each cancer type individually. We used the Hosmer-Lemeshow goodness-of-fit test to check the fitness of the model. The odds ratio between cancer and PKA was derived from logistic regression, and the 95% confidence intervals of the ratios were also determined.

Results

Population Distribution of ECPKA Activity. Serum samples were obtained from the 603 subjects, and the activity of ECPKA was determined by a validated, sensitive radioassay as described above. Although there is variation in the level of ECPKA activity between individuals, the overall levels of ECPKA in cancer patients was significantly higher than in healthy controls (Fig. 1A). The mean ECPKA activity in the sera of cancer patients was 10.98 units/mL, 5-fold higher than that of the healthy controls (2.15 units/mL).

As a quality control measure, the activity of LDH was quantified for each sample. LDH levels are often elevated in the serum of cancer patients, but can also be indicative of hemolysis of blood samples or quality of sample preparation and storage. The distribution of LDH in normal subjects and cancer patients can be found in Fig. 2A. There were no significant differences in population distribution between healthy subjects and cancer patients. Logistic regression analyses indicate that the distribution of ECPKA in subjects with lower LDH activity is similar to that of subjects with expression levels in the reference range, indicating that the LDH levels were not affected by sample preparation or storage. The LDH levels in men and women varied slightly, but without statistical significance (Fig. 2B and C).

The serum levels of both ECPKA and LDH activities were further analyzed by dividing the subjects into various age and gender groups (Fig. 3A-D). Age groups represent 10-year separations (i.e., age group 2 represents ages 20-29), except group 7, which encompasses all patients 70 years and older (range 70-82 years). As would be expected, fewer cancer patients were from age groups 2 and 3 compared with older groups. Among normal controls and cancer patients, age seemed to have no major effects on PKA or LDH activities ($P = 0.4176$, Fig. 3A-D). Because we recruited similar numbers of healthy volunteers in the younger age groups (under 40 years old) in the control population and fewer patients were available in the cancer patient group, the average ages are different between the control and cancer patient groups. However, this difference should not affect our conclusion regarding the significant differences between cancer patients and controls because there are no significant differences in sex- and age-specific PKA levels.

ECPKA Activity in Cancer Patients. To investigate whether there are differences in the serum levels of ECPKA in patients diagnosed with various types of cancer, patients with breast, pancreatic, esophageal, colorectal, lung, gastric, and hepatic cancers were included in the study. Patients from all of the represented cancer types consistently had higher serum PKA activity than healthy controls (Fig. 4A, $P < 0.0001$). Although the controls had a mean ECPKA activity of 2.15 units/mL, the lowest mean ECPKA activity in cancer patients was 6.44 units/mL, an ~3-fold difference (breast and pancreatic cancer patients, $P = 0.0473$ and $P = 0.1750$, respectively). Increases in average ECPKA activity in the sera of esophageal ($P = 0.0015$), colorectal ($P = 0.0004$), and lung cancer patients ($P = 0.0001$) were more than 4- and 5-fold. Even greater differences in activity were seen in sera from patients with gastric cancer ($P = 0.0002$) and hepatomas ($P = 0.0025$), ~6-fold and 8-fold increases, respectively, compared with normal controls. Along with the activity of ECPKA, the activity of LDH was also stratified for the different cancer types. There was no apparent

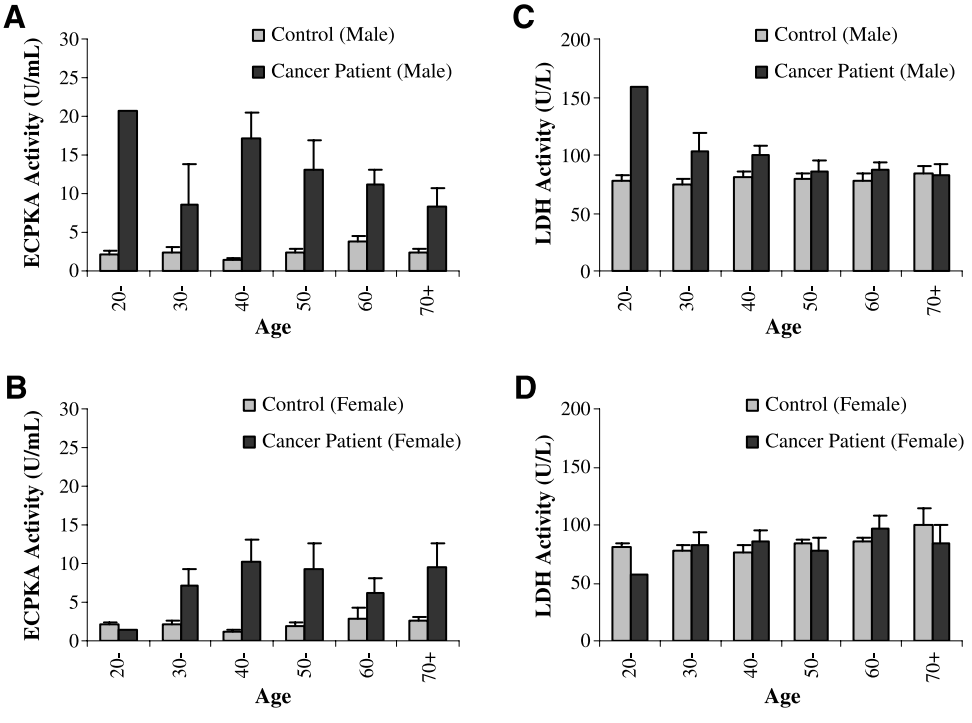

Figure 3. ECPKA and LDH activity in normal and cancer patients, separated by age and gender. **A** and **B,** the ECPKA activity in normal male (**A**) and female (**B**) populations, and male (**A**) and female (**B**) cancer patients was evaluated at different ages. **C** and **D,** the LDH activity in a normal male (**C**) and female (**D**) population, and a population of male (**C**) and female (**D**) cancer patients was evaluated at different ages.

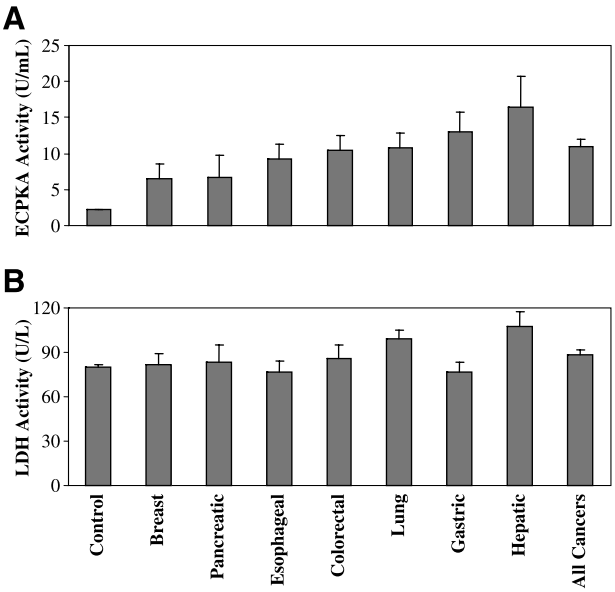

Figure 4. Comparison of mean ECPKA (**A**) and LDH (**B**) activity in healthy volunteers and patients with different cancers. The activity levels of ECPKA and LDH were determined, and the mean activities of the enzymes (**A**) ECPKA (units/mL) and (**B**) LDH (units/L) are represented for control patients, patients within each cancer subtype, and all cancers combined.

correlation between LDH activity and ECPKA in normal controls or cancer patients (Fig. 4B).

Statistical Analysis for ECPKA as a Biomarker. To determine a cutoff point for when ECPKA could be used as a cancer biomarker, different activity levels were used to determine the predictive value of ECPKA expression for revealing the presence of cancer. The activity levels chosen as cutoff points were 1, 2, 4, 8, and 10 units/mL. These divisions were then further stratified by age group and gender. Sensitivity, specificity, PPV, and NPV were calculated for each cutoff level, where sensitivity is the probability of a positive test given the subject has cancer, specificity is the probability of a negative test given the subject is cancer free, PPV is the probability of the presence of cancer given a positive test, and NPV is the probability of the absence of cancer given a negative test. The results can be seen in Table 1. Not surprisingly, when ECPKA activity of 10 units/mL was chosen as the cutoff point, the PPV and NPV were much better than for lower cutoff points. Based upon the sensitivities, specificities, and PPV and NPV, a cutoff of 8 units/mL and above for all cancers combined would be useful for predicting the presence of cancer.

In addition, logistic regression was done for all of the cancers types combined; the results can be seen in Table 2. Logistic regression was also done for each of the cancer subtypes, focusing on lung and gastric cancers (Table 2B). A Hosmer-Lemeshow goodness-of-fit test was then used to test the fit of the model. For individual cancers, ages and genders were combined to increase the number of patients per group. When compared with 2 and 4 units/mL, the cutoff point of 8 units/mL was again the best for patients with gastric, colorectal, esophageal, hepatic, and pancreatic cancers. However, it may be feasible to use a lower cutoff point of 2 or 4 units/mL for breast and lung cancers and possibly pancreatic cancer (Table 2B). If 8 units/mL is chosen as the cutoff point (for all cancers combined), the overall sensitivity is 37%, and the specificity is 95%. This gives a PPV of 81% and a NPV of 71% (Table 1).

Discussion

There is an urgent need for cancer biomarkers to improve cancer detection and monitoring of therapy. Serum biomarkers, such as carcinoembryonic antigen, vascular endothelial growth factor, aberrant DNA or RNA, neuron-specific enolase, cancer antigens (i.e., CA125), cytokeratin fragments (i.e., CYFRA 21-1), and several others, have been identified (36-43). These have been evaluated as biomarkers for diagnosis, prognosis and progression, and treatment efficacy. Singly, none of these molecules have optimal specificity or sensitivity to accurately differentiate between cancer and normal patients because they are expressed by normal tissues or because there is too much variation between patients or between the concentrations in the same patient on different days (36-41). Nevertheless, some of these molecules are being used as end points for therapy and are being evaluated in clinical trials for their predictive value (44, 45). Frequently, a set of biomarkers composed of a combination of molecules involved in a particular cancer is used to increase the predictive power (40, 46). To overcome the interpatient variation in expression, some studies are evaluating these molecules in patients over time (i.e., from early in the diagnosis until the patient goes into remission, is cured, or expires) to better understand the evolution of biomarker expression and to determine individual prognosis (36, 47, 48). This is particularly useful for predicting and indicating the response to therapy.

The most well-known serum biomarker is the prostate-specific antigen (PSA), an androgen-regulated serine protease expressed by both normal and cancerous prostate tissue (49). Since the early 1990s, PSA has been examined as a biomarker

Table 1. Sensitivity, specificity, PPV and NPV based on different ECPKA cutoff points

	N	PKA	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Overall	603	≥1 versus <1	93	49	53	92
		≥2 versus <2	77	66	58	82
		≥4 versus <4	62	81	66	78
		≥8 versus <8	37	95	81	71
		≥10 versus <10	29	98	88	69
Male	354	≥1 versus <1	95	49	58	93
		≥2 versus <2	79	64	62	80
		≥4 versus <4	65	79	70	75
		≥8 versus <8	42	94	83	68
		≥10 versus <10	33	97	89	66
Female	249	≥1 versus <1	87	49	44	89
		≥2 versus <2	74	67	50	85
		≥4 versus <4	57	82	59	81
		≥8 versus <8	27	96	75	75
		≥10 versus <10	22	98	85	74

Table 2. Logistic regression for all subjects (cancer as the dependent variable)

(A) Final logistic regression for all subjects*

PKA	Reference	Odds ratio	95% CI †	*P* value
≥2	<2	5.8	3.9-8.7	<0.0001
≥4	<4	7.2	4.7-11.0	<0.0001
≥8	<8	9.3	5.2-16.6	<0.0001

(B) Logistic regression results for subjects with specific cancer and controls ‡

Tumor	PKA	Reference	Odds ratio	95% CI ‡	*P* value
Gastric cancer (*N* = 45)	≥2	<2	7.0	3.1-15.9	<0.0001
	≥4	<4	7.2	3.5-14.7	<0.0001
	≥8	<8	11.1	4.9-25.3	<0.0001
Lung cancer (*N* = 63)	≥2	<2	6.5	3.3-13	<0.0001
	≥4	<4	7.7	4.1-14.3	<0.0001
	≥8	<8	7.3	3.4-15.4	<0.0001
Breast cancer (*N* = 30)	≥2	<2	5.0	2.0-12.1	0.0004
	≥4	<4	3.8	1.6-8.8	0.0021
	≥8	<8	2.0	0.5-8.6	0.3289
Colorectal cancer (*N* = 29)	≥2	<2	4.5	1.8-11.2	0.0011
	≥4	<4	7.0	3.0-16.3	<0.0001
	≥8	<8	12.0	4.6-31.5	<0.0001
Esophageal cancer (*N* = 24)	≥2	<2	3.6	1.4-9.3	0.0066
	≥4	<4	3.6	1.5-8.7	0.0043
	≥8	<8	8.7	3.2-23.7	<0.0001
Hepatic cancer (*N* = 28)	≥2	<2	4.9	2.0-12.1	0.0005
	≥4	<4	11.1	4.4-27.5	<0.0001
	≥8	<8	26.7	10.5-67.9	<0.0001
Pancreatic cancer (*N* = 10)	≥2	<2	3.6	0.9-14.4	0.0740
	≥4	<4	3.7	1.0-13.7	0.0489
	≥8	<8	4.1	0.8-22.2	0.0999

*Other variables in the logistic regression model: gender, age group.
† Confidence interval for odds ratio.
‡ Other variables in the logistic regression model: gender, age. Due to the small number of cases of specific tumors, we use continuous ''age'' in logistic regression for specific tumors, unlike for the previous table, in which we use ''age groups.''

for prostate cancer (49, 50). As with the markers described above, long-term follow-up is often necessary to determine whether and when a patient should receive treatment (50). The results from ongoing large randomized studies will help pinpoint the efficacy, applications, and ultimate benefits of PSA screening.

PKA is a well-documented oncogene with activity in a variety of cellular processes (3, 26, 27). Therefore, compared with PSA expression which correlates with the size of the prostate, regardless of whether the cells are benign or malignant, ECPKA expression is likely to be a more accurate reflection of the presence of malignant cells. For instance, a study of melanoma patients indicated that the level of ECPKA reflects the status of the tumor, and that following resection of the tumor, ECPKA levels decrease; however, upon relapse, the level again increases (51). There is also evidence that decreasing the expression of or inhibiting the activity of the RIα subunit of PKA can lead to potent antitumor effects (21, 22, 26, 32-34). Our earlier study indicated that ECPKA decreases in cancer patients after treatment with antisense PKA inhibitor (34). Thus, ECPKA may be used both to predict the presence of cancer or its recurrence and to gauge the response of tumors to specific anti-PKA therapy. However, the precise correlation between ECPKA levels and tumor stages was not determined in the present study due to the limited number of cases for each cancer type. Further studies in this field are needed.

A previous study examining the expression of ECPKA in cancer patients found that ECPKA concentrations were 10-fold higher in cancer patients than in healthy controls (7). The results reported here confirm the overexpression of active ECPKA in patients with various types of cancer. In contrast to this previous study, we found only about a 5-fold increase in expression. However, this earlier study was not a population study, nor did it include a sufficient number of control (noncancer) patients (n = 14) for comparison. There was also a lack of detailed statistical analysis. The difference in activity could also be due to the cancer types examined; 40% of the cancer patients examined in the earlier study were melanoma patients, and different cancer types have different levels of expression.

In summary, ECPKA may prove to be of great significance when used either alone or in combination with other technologies for diagnosing and monitoring several kinds of cancer. Combination with other biomarkers, or with imaging techniques such as computed tomography scans, could further improve the utility of ECPKA. Additional long-term and more diverse studies, including other ethnic groups, will be necessary, as will further data analyses. It may prove beneficial to continue evaluating the cutoff point where a diagnosis will be made. A new data set could be used to evaluate the validity of the chosen cutoff point (8 units/mL) and to evaluate whether other cutoff points may be better. Depending on the purpose of the screening, different cutoff values may be beneficial. For example, it may be necessary to use different ECPKA expression cutoff levels to monitor previously diagnosed cancer patients for progression and response to therapy than are used for initial diagnosis. Long-term studies in individual patients tracking changes in ECPKA expression throughout cancer treatment and progression or remission would also be of great interest. A larger study of individual cancer types could also result in information about the correlation between ECPKA and the tumor-node-metastasis stage of tumors. In conclusion, ECPKA may represent a useful diagnostic biomarker for the presence of cancer. ECPKA levels may also be used for various cancers as an end point for therapy and as a rapid and minimally invasive method for examining patients for recurrence.

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