Autoantibody Cancer Biomarker: Extracellular Protein Kinase A

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Abstract
In cancer cells, cyclic AMP–dependent protein kinase (PKA) is secreted into the conditioned medium. This PKA, designated as extracellular protein kinase A (ECPKA), is markedly up-regulated in the sera of patients with cancer. The currently available tumor markers are based on the antigen determination method and lack specificity and sensitivity. Here, we present an ECPKA autoantibody detection method for a universal biomarker that detects cancer of various cell types. We tested sera from 295 patients with cancers of various cell types, 155 normal controls, and 55 patients without cancer. The specificity and sensitivity of this autoantibody enzyme immunoassay method were compared with the conventional antigen determination method by receiver-operating characteristic plots. In the sera, the presence of autoantibody directed against ECPKA was highly correlated with cancer. High anti-ECPKA autoantibody titers (frequency, 90%; mean titer, 3.0) were found in the sera of patients with various cancers, whereas low or negative titers (frequency, 12%; mean titer, 1.0) were found in the control group. The receiver-operating characteristic plot showed that autoantibody enzyme immunoassay exhibited 90% sensitivity and 88% specificity, whereas the enzymatic assay exhibited 83% sensitivity and 80% specificity. These results show that the presence of such autoantibodies could serve for cancer diagnosis (1–5). The limitations of the presently available serum tumor markers, based on the antigen determination method, indicate the need for other means of screening (1–5).

Introduction
Early detection and diagnosis are of utmost importance in cancer management. Serum biomarker measurement in body fluid immunoassays has been the most widely used approach, generally of such established tumor-associated markers as CAE, AFP, hCG, PSA, and CA125. The lack of tumor specificity of these markers, however, precludes their general use in cancer screening and diagnosis (1–5). The limitations of the presently available serum tumor markers, based on the antigen determination method, indicate the need for other means of screening (1–5).

In normal mammalian cells, cyclic AMP–dependent protein kinase (PKA) is present strictly intracellularly (6). Intriguingly, however, cancer cells of various types excrete PKA into the conditioned medium (7, 8). This PKA, designated as extracellular PKA (ECPKA) was found to be markedly up-regulated in the serum of patients with cancer (7, 8), and surgical removal of tumors led to a decrease in ECPKA levels in patients (9). Two types of PKA exist, designated type I (PKA-I) and type II (PKA-II; ref. 6); they are distinguished by different regulatory (R) subunits (RI and RII), and they contain a common catalytic (C) subunit (10). Importantly, the ratios of PKA-I to PKA-II change dramatically during cell development, differentiation, and transformation (11). In cancer cells, ECPKA expression was modulated by changing the ratios of the intracellular PKA-I to PKA-II (7), and down-regulation of ECPKA was shown by a decrease in PKA-I and by a mutant Ca lacking the NH₂-terminal myristyl group (7). These results indicate that ECPKA might be a cancer antigen. There is increasing evidence that patients with cancer produce autoantibodies against antigens in their tumors (12–15), suggesting that such autoantibodies could have diagnostic/prognostic value. We speculated that ECPKA excretion might elicit the induction of serum autoantibodies and that the presence of such autoantibodies could serve for cancer detection. For this, we developed a novel enzyme immunoassay (EIA) that measures the anti-IgG autoantibody for ECPKA.

Materials and Methods
We tested 295 serum samples from patients with cancer. The cancers included various cell types: breast (n = 24), cervical (n = 13), colon (n = 40), lung (n = 6), ovarian (n = 20), prostate (n = 35), pancreatic (n = 6), renal cell (n = 60), and rectal (n = 14) carcinomas, melanomas (n = 50), and other carcinomas (n = 19), including bladder, esophageal, gastric, hepatic, pulmonary, and small bowel; and sarcoma, thymoma, liposarcoma, and leiomyosarcoma. As controls, we used normal sera from a blood bank (n = 155) and sera from patients with diseases other than cancer (n = 55; systemic lupus erythematosus, 20; Carney complex, 25; other diseases, 10). The sera used for this autoantibody EIA assay were from existing individual Institutional Review Board–approved, consented samples, and were therefore not drawn from patients for the specific studies presented here. The EIA autoantibody assay was not done to distinguish the site of malignancy but was done in the sera of patients with a wide range of active malignancies. For comparison, the same samples were also analyzed with the conventional PKA assay (antigen determination).

The serum samples were aliquoted (5 µL volume) and kept frozen at −80°C until use. The serum samples were thawed once only before use, and these diluted serum samples were never used twice. The autoantibody levels were stable over 6 months.

EIA method. Anti-ECPKA IgG autoantibodies were measured by solid phase EIA. The plates were coated with 100 µL of diluted antigen (2 µg/mL concentration in PBS) of the purified recombinant human PKA C subunit (7), and were incubated overnight at room temperature. The plates were then washed once with washing buffer [20 mmol/L Hepes, 0.9% NaCl, 30 mmol/L sucrose, 0.1% bovine serum albumin, BSA (pH 7.0)], blocked for 2 hours at room temperature with 100 µL of Blockase (Sero-tec), and washed

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twice with sodium citrate washing solution [50 mmol/L sodium citrate, 0.15 mol/L NaCl, and 0.1% Tween 20 (pH 5.0-5.2)]. We then added 100 μL of 25,000-fold diluted serum samples [dilution buffer: PBS (pH 7.4), 0.25% BSA (fatty acid-free fraction V), and 0.05% Tween 20] and incubated the plates for 1 hour at 37°C. After three washes with sodium citrate solution, we added 100 μL of 20,000-fold diluted anti-human IgG-horseradish peroxidase antibody-enzyme conjugate (Jackson Immunoresearch Laboratories, West Grove, PA), in PBS, 0.09% NaCl, and 1% BSA, incubated the plates for 1 hour at room temperature, then washed them five times in sodium citrate solution and added 100 μL of TMB substrate. The reaction was stopped with 100 μL of 0.45 mol/L H2SO4 reagent and the absorbance was read at 450 nm and recorded on an ELISA reader (microplate reader benchmark; Bio-Rad, Hercules, CA).

**Purification of PKA Co**. The recombinant human PKA Co (1.1 kb) from the OT1529-Co plasmid (7) was infused with pQE31 DNA leading to the production of pQE31-Co (S.H. Hong, Seoul National University, Seoul, Korea). The purified recombinant human Co protein (1 μg/lane) was subjected to 10% SDS-PAGE, and electroblotted onto nitrocellulose membrane. The membrane were blocked, washed, and the blot strips were incubated with patients’ or normal sera diluted in Tween 20 PBS solution, the strips were washed, then incubated with anti-human IgG-horseradish peroxidase conjugate, and immunochemistry was done by enhanced chemiluminescence (Aemersham Pharmacia Biotech, Piscataway, NJ).

**Western blotting analysis of anti-ECPKA autoantibody**. The purified, recombinant human Co protein (1 μg/lane) was subjected to 10% SDS-PAGE, and electroblotted onto nitrocellulose membrane. The membrane were blocked, washed, and the blot strips were incubated with patients’ or normal sera diluted in Tween 20 PBS solution, the strips were washed, then incubated with anti-human IgG-horseradish peroxidase conjugate, and immunochemistry was done by enhanced chemiluminescence (Aemersham Pharmacia Biotech, Piscataway, NJ).

**ECPKA enzymatic assay**. The serum ECPKA activity was measured as previously described (7). One unit of PKA activity was defined as the amount of enzyme that transferred 1 pmol of 32P from [γ-32P]ATP to recovered protein in 1 minute at 37°C in the standard assay system.

**Statistical analysis**. Means, SD, and confidence intervals were used where appropriate. Data on the reproducibility of the ELISA test was analyzed by analytic coefficients of variation at relevant concentrations and for appropriate time intervals; we also measured intraobserver and interobserver variability. $P < 0.05$ were considered significant throughout. Receiver-operating characteristic (ROC) curves (16) were used to calculate cutoff values for optimal sensitivity and specificity.

**Results**

**ECPKA autoantibody detection in the sera of patients with cancer**. Our speculation that ECPKA excretion might elicit the induction of serum autoantibodies led us to develop a novel ELISA method to measure the anti-IgG autoantibody for ECPKA.

We tested the sera of patients with various types of cancer (see Materials and Methods; $n = 295$), controls ($n = 155$), and patients with diseases other than cancer ($n = 55$) for the presence of anti-ECPKA autoantibody using the ELISA method. Anti-ECPKA autoantibody titers were expressed arbitrarily as ratios to the mean absorbance of normal control sera. Compared with the anti-ECPKA autoantibody titers of normal controls, a $>1.3$ ratio was considered positive (Fig. 1).

The titers for anti-ECPKA autoantibody in sera from patients with cancer and from controls are shown in Fig. 1. Both frequency and mean titer of the patients with cancer are significantly higher (frequency, 90%; mean titer, 3.0) than those of the normal controls (frequency, 12%; mean titer, 1.0).

We also examined whether the level of ECPKA autoantibody is detectable as a function of tumor type. The results obtained with different types of tumors were: breast ($n = 20$; mean titer, 3.05), colon ($n = 24$; mean titer, 2.95), lung ($n = 6$; mean titer, 1.95), melanoma ($n = 50$; mean titer, 3.00), ovary ($n = 20$; mean titer, 2.85), pancreas ($n = 6$; mean titer, 3.10), prostate ($n = 30$; mean titer, 2.95), and renal cell ($n = 40$; mean titer, 2.58). These results show that the ECPKA autoantibody is produced, not as a function of specific tumor type, but by various cancer cell types.

The ECPKA autoantibody levels were also evaluated in the sera of patients with diseases other than cancer. Patients with systemic lupus erythematosus, an autoimmune disease that accompanies marked decrease in intracellular PKA (17); Carney complex, an adrenal gland disorder causatively related to mutational loss of the PKA RIα subunit (18); and patients with diseases other than cancer, including pancreatitis, angina, and hypertension, exhibited average ECPKA autoantibody titers of 1.3, 0.8 (Fig. 1), and 1.2 (data not shown), respectively, levels close to that of normal controls (1.0; Fig. 1). These results show that the autoantibody ECPKA is elevated in patients with a wide range of active malignancies of various cell types (see Materials and Methods).

**Comparison between ECPKA autoantibody ELISA and ECPKA enzymatic assay**. We compared the ECPKA ELISA with an ECPKA enzymatic assay that measures antigen (7). The ECPKA enzymatic assay exhibited a significant overlap between patients with cancer ($n = 66$) and normal controls ($n = 66$) in frequency and mean values (patients: frequency, 83%; mean value, 130 mU/mL; normal controls: frequency, 20%; mean value, 60 mU/mL), indicating a lack of sensitivity and specificity (Fig. 2A). A comparison of individual anti-ECPKA autoantibody titers obtained by ELISA with those measured by PKA enzymatic assay showed no correlation between the two assays (Fig. 2B).

The sensitivity and specificity of the autoantibody ELISA (Fig. 1) and those of the ECPKA assay (Fig. 2) were evaluated using ROC (16) plots (Fig. 3). At this point of interaction, the cutoff value for anti-ECPKA autoantibody titer was 1.3, exhibiting the highest sensitivity whereas maintaining a high specificity. The autoantibody ELISA showed 90% sensitivity (95% confidence interval, 0.80-0.92) and 91.4% specificity (95% confidence interval, 0.87-0.95).
88% specificity (95% confidence interval, 0.90-0.95) in discriminating between patients with cancer and the control group (Fig. 3). The enzymatic assay exhibited 83% sensitivity and 80% specificity, with an area under the curve (AUC) of 0.86938 (compared with the autoantibody EIA AUC of 0.93675; Fig. 3). These results show that the ECPKA autoantibody EIA did better than the enzymatic assay in distinguishing between patients with cancer and controls.

Immunologic identification of ECPKA autoantibody. We examined the immunologic identification of ECPKA autoantibody present in the sera of patients with cancer (Fig. 4). Randomly selected patients’ sera exhibited immuno-cross-reactivity toward the purified PKA Cα protein (40 kDa; Fig. 4, strips 5-9), whereas no such immuno-cross-reactivity for Cα protein was observed in normal sera (Fig. 4, strips 10-14). Importantly, the immuno-cross-reactivity for Cα of ECPKA autoantibody in patients’ sera was comparable to those of polyclonal and monoclonal antibodies raised against the Cα protein (Fig. 4, compare strips 1-4 with strips 5-9).

Autoantibody EIA method to detect other cancer antigens. To examine whether the autoantibody detection method of the present study can be extended to other cancer antigens (extracellularly secreted) that produce autoantibodies, an experiment was done using HCT-15 (a multidrug-resistant human colon carcinoma grown in nude mice; National Cancer Institute, Frederick, MD) tumor extracts as a cancer antigen source. These results plotted on a ROC curve showed that the autoantibody EIA had 92% sensitivity (95% confidence interval, 0.80-0.95) and 84% specificity (95% confidence interval, 0.78-0.90) in discriminating between patients with cancer and controls. The observed titers of autoantibodies for cancer antigens in sera from patients with cancer were shown with different types of cancer. The results show that it is possible to use the autoantibody detection method described here for ECPKA to detect other cancer antigens that produce autoantibodies against the antigens.

Discussion

Cancer diagnosis is a serious step in cancer management. Therefore, we vigorously validated the method and carefully evaluated the data (Fig. 1). We measured the level of autoantibodies generated against ECPKA, a cancer antigen recently identified (7, 8), from the growth of various tumors by using serum samples and found that the accuracy and sensitivity of the present method exceeds that of the conventional assays. Our purpose was not to distinguish the stage and site of malignancy. The autoantibody assay was done on sera from active malignancies at various stages and the same samples were assayed with the conventional PKA enzymatic assay (antigen assay) for comparison. The results showed that the autoantibody-EIA assay exhibited better sensitivity and specificity than did the conventional antigen assay.

The ECPKA autoantibody present in patient’s sera was immunologically cross-reactive with the purified preparation of PKA catalytic (Cα) subunit and the cross-reactivity was comparable to that of the polyclonal and monoclonal antibodies raised against the Cα protein (Fig. 4), demonstrating the specificity of the sera autoantibody against ECPKA and the immunologic identity of ECPKA with the Cα subunit of PKA.

The anti-ECPKA autoantibody was elevated almost regardless of the site or cell types of the malignancy; that is, anti-ECPKA...
autoantibody is a measure of malignant transformation in all cells, not specific to one type of cancer. Unlike tests such as CEA, which measures less well-defined antigens and whose serum levels tend to be inconsistent but elevated late in the disease (3), the ECPKA autoantibody test measures the autoantibody of a well-defined cancer antigen, ECPKA, whose serum levels are specifically up-regulated in the sera of patients with cancer and are regulated by the changes in the intracellular levels of PKA-I (7).

In the present study, as negative controls of the ECPKA autoantibody test, we chose sera from patients with Carney complex (spotty skin pigmentation that can accompany multiple endocrine neoplasia; ref. 18) and sera from patients with systemic lupus erythematosus (an autoimmune disease; ref. 17). The absence or low level of PKA-I is closely causatively associated with both of these diseases (17, 18). Our results show that both sera from patients with Carney complex and lupus erythematosus exhibited ECPKA autoantibody titers as low as that of normal controls (Fig. 1), supporting the specificity of the ECPKA autoantibody test for cancer diagnosis.

Among the most important criteria for cancer markers is the ability to distinguish cancer from inflammatory diseases and diseases other than cancer. Because known cancer markers all depend on the measurement of cancer antigens, distinguishing between inflammation and cancer is difficult using these cancer markers. In the present study, we have presented a novel biomarker, autoantibody ECPKA, that could make it possible to distinguish between cancer, inflammation, and diseases other than cancer. We have shown here that the ECPKA autoantibody titers were not elevated in a limited number of patients with pancreatitis, angina, or hypertension. In a recent report (19), autoantibodies against peptides derived from prostate cancer tissue were used for a screening test for prostate cancer. The peptide autoantibodies did better than did the PSA (antigen test) in distinguishing between the group with prostate cancer and the control group, demonstrating the importance of prostate signature autoantibodies for the early detection of prostate cancer.

Importantly, the ECPKA autoantibody detection could provide the retrospective analysis and follow-up of disease extension. In our preliminary studies, the first line serum samples of stage I melanoma, those bled through during 10 years (1985-1996), exhibited the mean ECPKA autoantibody titer of 3.0 (see Results). These data also suggest the possibility of the early diagnosis of ECPKA autoantibody.

A test based on the demonstration of autoantibodies to tumor antigen in the sera of patients, as described here, could be of great importance for early diagnosis. The prolonged time course of carcinogenesis (20) opens the possibility that a very small tumor or a subtle biochemical change in the cell produces a detectable level of antistimulant autoantibodies in response to chemical or viral carcinogens. This could happen well before the released tumor antigen reaches a detectable level. Our results suggest that the autoantibody detection rather than antigen detection would serve for early diagnosis.

We have not tested the ECPKA autoantibody EIA for cancer screening; this requires extension and confirmation in multi-institutional based screening cohorts. It will be important to evaluate whether the ECPKA autoantibody EIA is specifically associated with cancer but not with benign malignancies, inflammations, and other diseases. Although the technique is simple and promising, its ultimate performance in multi-institutional studies must yet be determined.

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References

Autoantibody ECPKA as a Cancer Biomarker

In the article on autoantibody ECPKA as a cancer biomarker in the September 15, 2006 issue of Cancer Research (1), the correct spelling of the seventh author's name is Islam U. Khan.

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