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Detection of Serum Anti-Extracellular Protein Kinase a Autoantibodies as a Potential Tumor Marker

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Abstract

In previous studies, it has been discovered that cancer cells not only overexpress regulatory subunit I (RI)/protein kinase type I (PKA-I) but also secrete outside the cell an extracellular form of PKA (ECPKA) and that the ECPKA secretion detected in patients' serum is obviously greater than that found in non-cancer patients or healthy subjects. We now found that ECPKA elicits the formation of serum autoantibodies that can serve as a cancer diagnostic and prognostic marker. To measure the presence of anti-ECPKA autoantibody in the human sera, basic methodology for ECPKA assay was established an enzyme-linked immunosorbent assay (ELISA). We obtained serum samples from 199 patients with different types of cancer, and also obtained 31 serum samples to compare with ECPKA concentrations from non-cancer patients and 119 normal volunteers. Compared with normal or non-cancer patient sera, we found that the frequency of anti-ECPKA autoantibody was significantly higher in cancer patients (88%) than in those without cancer (17%). Furthermore the presence of anti-ECPKA autoantibodies in the serum of cancer patients was highly correlated with the site of metastasis. The immunoassay developed for anti-ECPKA antibodies is highly sensitive and specific. Therefore, this discovery of an autoantibody-based cancer diagnostic may have serious clinical application and may become an important advance over current technology.

Keywords: *Extracellular protein kinase A (ECPKA), Tumor marker, Diagnosis*

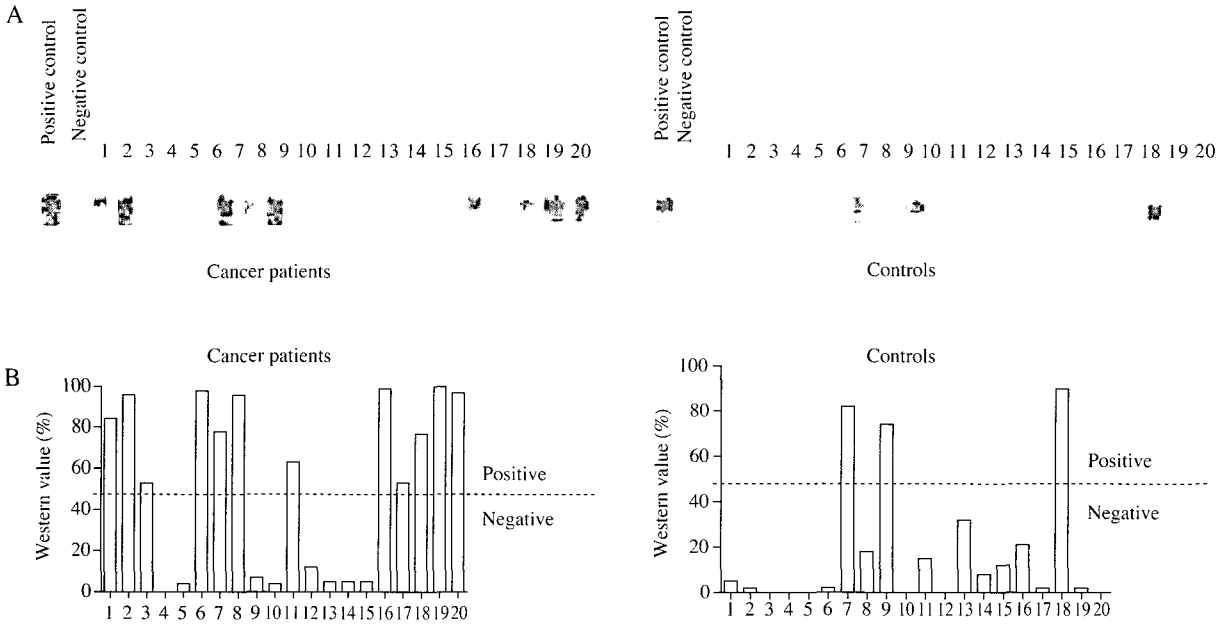
There is increasing evidence for an immune response to cancer in human, demonstrated in part by the identification of autoantibodies against a number of intracellular and surface antigens in patients with different types of cancer[1-3]. The finding that patients with cancer produce autoantibodies against antigens in their tumors[2, 4-8] suggests that such autoantibodies could have diagnostic and prognostic value[2,6,9,10]. One of the most extensively studied cancer-associated antigens is p53, a tumor suppressor protein[11]. Mutant forms of the p53 protein elicit anti-p53 antibodies in 30 to 40 percent of patients with various types of cancer[12], and since then there have been numerous reports confirming and extending this finding[13]. Although factors leading to the production of such autoantibodies are not completely understood, it has been proposed that autoantibodies might be used as reporters identifying aberrant cellular mechanisms in tumorigenesis[14].

Recently, the identification of test that identifies autoantibodies to the tumor markers, p53, Huntingtin Interacting Protein-1 (HIP1), Alpha-FetoProtein (AFP), Carcinoembryonic Antigen (CEA), Cabohydrate Antigen (CA 19-9) and Koc provided hope that use of cytoplasmic tumor markers in addition to secreted antigens could lead to serum screening tests[9,10,12]. The proposed reason for the formation of autoantibodies is that upon turnover of tumor cells, tumor antigens are shed into the circulation at low levels inducing an immune response. Immunoreactivity to other tumor antigens has been described in various cancer patients, but the formation of these autoantibodies did not show high sensitivities[10].

It has been shown in a recent report that cancer cells of various cell types excrete PKA into the conditioned medium. The action of cAMP through the activation of cAMP dependent protein kinase (PKA) in the regulation of various cellular functions, such as metabolism, secretion, cell proliferation, differentiation, and gene induction, is well studied[15-18]. The PKA holoenzyme is composed of two genetically distinct subunits, the catalytic (C) and regulatory (R) subunits, forming a tetrameric holoenzyme R_2C_2[19]. This holoenzyme can release two free catalytically active catalytic subunits under presence of cAMP.

Type I (PKA-I) and type II (PKA-II), which are distinguished by different regulatory subunits (R subunits), RI and RII, as they share a common catalytic subunit (C subunit). Through biochemical studies and gene cloning, four isoforms of the R subunits, RI_α, RI_β, RII_α, and RII_β, have been identified[20,21]. Importantly, the ratios of PKA-I to PKA-II can change dramatically during cell development, differentiation, and transformation. Changes in cyclic adenosine 3′, 5′ monophosphate (cAMP) level mediated phosphorylation have various biological effects on basic cellular functions involved in the regulation of cell growth, metabolism and differentiation, and gene expression[22]. The cAMP -dependent protein kinase (PKA) has been known receptors of cAMP, linking the membrane bound hormone sensitive adenylyl cyclase system and the physiological response of the cell. The free C subunit in addition to phosphorylating proteins in cytoplasm, can migrate to the nucleus where it presumably plays a role in the regulation of gene expression[19]. Overexpression of the C_α or RI_α subunit of PKA upregulates ECPKA expression. Conversely, ECPKA expression is downregulated by overexpression of the RII_β subunit, which eliminates PKA-I and upregulates PKA-II, and it reverts the transformed phenotype. In the serum of cancer patients, ECPKA expression is upregulated compared with normal serum.

In the present study, we have discovered that patients with various cancers produce autoantibodies to anti-ECPKA more frequently than control individuals and



C

Scores	Cancer patients (N=199)	Controls (N=119)
Positive	142*	12
Negative	57	107

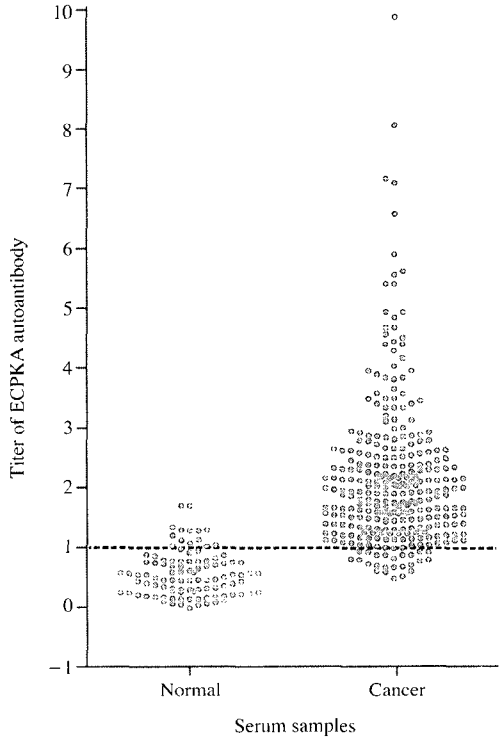
*The frequency of positive test in cancer patients was statistically different than in control patients ($p \leq 0.001$, Specificity (90%), Sensitivity (71%))

Fig. 1. Cancer patients have a specific humoral response to ECPKA overexpression. A, representative immunoblot of 40 sera assayed for reactivity to PKA. Twenty of the 199 biopsy-proven cancer patients and 20 of the 119 control individuals. Equal aliquots of all of the 318 serum samples were analyzed by immunoblot in at least two independent experiments and contained reference positive and negative controls (Positive and Negative lanes, respectively). B, bands were scanned from the developed blots and converted to grayscale values using Adobe Photoshop. Normalized grayscale were converted to percentage of the positive control (Positive lane). Samples with band intensity of $\geq 50\%$ of the positive control were given a positive score (above the dotted line). A negative score was given to samples $< 50\%$ of positive control (below the dotted line). C, distribution of the values between cancer and the control individuals was significantly different ($P < 0.001$, one-way ANOVA). Specificity of the test was 90% and calculated as those control samples with a negative test (107/107+12) and sensitivity was 71% and calculated as the percent of patient samples with a positive test (159/159+40).

that the sera presence of autoantibody directed against ECPKA is highly correlative of cancer. We hypothesized that ECPKA autoantibody formation could yield a useful early diagnosis as a tumor marker for various cancers. Using both immunoblot and ELISA test, we have found that the sensitivity and specificity of this novel tumor marker, has the exciting potential to surpass those of the other antigen test.

Results of screen using sera from various cancer patients and controls (n=20 for each) are shown in Fig. 1A. Ultimately, the sera from 199 cancer patients and 119 normal controls sera were screened by Western blot. The blots were analyzed by measuring the grayscale values of the reactive bands (Fig. 1A, arrows) and quantitated as a percent of the reference positive control (Fig. 1B). A positive score was assigned to bands with a value of $\geq 50\%$ of the positive control, whereas those bands $< 50\%$ of the positive control received a negative score. This cutoff was chosen because it yielded the highest values for specificity and sensitivity, as analyzed from ROC curves created from a randomly chosen subset of the cancer patients and normal controls subjects. All serum samples were validated for autoantibodies to PKA by this immunoblot analysis. PKA antibodies were significantly more frequent in serum from cancer patients compared with normal control ($P < 0.001$), and 142 of 199 (71%) cancer patient sera received a positive score compared with 12 of 119 (10%) of the normal control sera (Fig. 1C).

A



B

Scores	Cancer patients (N=199)	Controls (N=119)
Positive	148*	17
Negative	51	102

*The frequency of positive test in cancer patients was statistically different than in control patients ($p \leq 0.001$. Specificity (85%). Sensitivity (74%))

Fig. 2. Detection of ECPKA humoral response in human cancer patients by ELISA. A, average relative absorbances (ELISA values) and their standard deviations are shown for 62 cancer patient and 90 control sera. A relative absorbance of $1 > 1.0$ (above the dotted line) was considered positive. B, numbers of positive cancer and control sera. The specificity of the test was 90% and the sensitivity was 71%. The difference between cancer patients and controls was significant ($P < 0.01$, one-way ANOVA).

For confirmation of our Western blot results and the development of an additional clinical assay, and ELISA to monitor PKA immune response was developed. ELISA plates were coated with human cAMP dependent Protein Kinase A catalytic subunit, and sera from patients with different cancer types or normal controls were assayed. The measured absorbance was converted to values relative to negative reference controls and duplicate samples were averaged. Figure 2A shows the average relative absorbencies for all of the cancer patient sera and 60 of the control sera. A relative absorbance that was greater than the negative control (ELISA value, > 1) was considered a positive score. The cutoff for this test was, like the Western blot test, determined by using ROC curves on a subset of the patient sera and determining where the ELISA values yielded the highest specificity and sensitivity. All available serum samples were then tested for PKA antibodies by ELISA. The ELISA test results in similar values for specificity and sensitivity as the Western blot analysis.

Discussion

In previous studies, it has been discovered that cancer cells not only overexpress RI/PKA-I but also secrete outside the cell an extracellular form of PKA (ECPKA) and that the ECPKA secretion detected in patients' serum is obviously greater than that found in non-cancer patients or healthy subjects. The free C subunit of PKA is excreted in a conditioned medium of various cancer cells and in serum of cancer patients[24]. ECPKA expression was up-regulated 3 to 10 fold as compared with normal serum. Importantly, this study provided the means of modulating ECPKA expression by changing expression of the intracellular

PKA-I and PKA-II. Namely, overexpression of RI_{α} in the expression vector, which up-regulates intracellular PKA-I, can markedly up-regulate ECPKA expression. Overexpression of RII_{β}, which downregulates PKA-I in the cell and reverts the transformed phenotype, downregulates ECPKA, and a mutation in the C_{α} gene that prevents myristylation allows intracellular PKA upregulation but blocks the ECPKA increase, suggesting that the N-terminal myristyl group of C_{α} is required for ECPKA expression[25].

In the present study, it was reviewed evidence that links the expression of PKA signaling with the natural history of breast cancer;[23] breast cancers overexpression of PKA in comparison with normal breast;[24] RI:RII ratios are significantly higher in specimens of normal breast that demonstrate increased proliferation;[25] although the RI : RII ratio varies greatly among different breast cancers, those with high RI : RII are associated with poor prognosis in terms of early disease recurrence and death following primary treatment[26]. Thus, it is relevant that increased expression of PKA could change sensitivity to estrogens and antiestrogens in cancer cells; in model systems of breast cancers[24]. These and other studies[27] provide the molecular proof of the initial hypothsis: two isoforms of the regulatory subunits (RI and RII) of cAMP dependent protein kinase that bind cAMP are inversely expressed during ontogeny and cell differentiation. Together, these cAMP-binding receptor proteins might regulate the growth of normal cells and their differentiation into nondividing states. Cancer cells can also be made to differentiate and stop growing when the functional balance of these receptor proteins is restored by treatment with site-selective cAMP analogs or with an antisense oligodeoxynucleotide[18].

The humoral immune responses to tumor-associated autoantigens show clearly that antibodies can be useful reagents to identify aberrant cellular mechanisms involved in tumorigenesis. Just as many rheumatic autoantibodies have been important reagents to help in identifying molecules involved in pre-mRNA splicing, cell division, and DNA replication[28], cancer autoantibodies might be anticipated to provide some insights into pathways associated with cell transformation, particularly the post-transcriptional regulation of growth factors and cytokines. Many tumor-associated autoantigens have already been identified[29]. As more are found, it will be important, but not always straightforward[30], to determine which ones are directly related to tumorigenesis.

Success in the treatment of cancer is influenced a great deal by early detection. Clearly, the immune system is capable of sensing at least some tumor-associated dysregulated cell functions before many standard clinical tests for cancer detection. Might certain autoantibodies therefore be added to the array of diagnostic tests for cancer or even for early detection, as now appears to be possible in tests for the characteristic antibodies that presage type 1 diabetes[31]. Although genetic abnormalities of p53 have been shown to be present in up to 50% of all cancers[32], the occurrence of autoantibodies is in the range of 15-20 percent[12]. Other uses of the knowledge brought by autoantibodies regarding cellular molecules involved in tumorigenesis include cancer therapy aimed at restoring normal function or at abrogating harmful effects caused by dysregulation of certain cellular functions. Efforts in these directions are underway, including development of cancer vaccines with p53 as the target[33]. Other major tumor-associated autoantigens could be reasonable targets for cancer therapy. In clinical trials associated with the development of such therapy, it may prove efficient to select subjects with detectable autoantibodies, using the immune response as an indication of which patients have the abnormal cellular mechanisms being targeted for treatment.

The search for new biomarkers for use in the diagnosis of cancer entails characterizing one or a few proteins produced by cancer cells and establishing assays with high specificity and sensitivity for cancer or defining a cancer fingerprint on the basis of a larger set of proteins, many of which are known only as unique peaks on a mass spectrogram. We expect that this new approach will yield some serum proteins or protein patterns with specificity for a particular cancer type and others that common to all cancers. This method, however, are still best at detecting tumors that are large enough to be diagnosed clinically. To make a difference, especially in improving the outcome of treatment, screening methods must be able to detect very early tumors, even premalignant lesions. This means that the tests must have adequate sensitivity and specificity to detect very low levels of cancer proteins. There is no detection instrument that rivals the sensitivity and specificity of the immune system. Hence, one promising approach to the early detection of cancer is to look not for cancer but for the immune response to cancer. There is clear evidence that the immune system, in addition to defending us against pathogens, is also on guard against other threats, including cancer[14]. Many tumor antigens that are currently targets for therapy have been identified with the use of the patient's own anti-cancer antibodies or T cells[4,14,34].

Use of the immunoassay exhibits markedly high anti-ECPKA antibody titers in cancer patients but

low or non-existent titers in normal individual controls. This ECPKA autoantibody-based immunoassay method provides an important diagnostic procedure applicable for the detection of cancers of various cell types. These observations indicate that this phenomenon of ECPKA expression can provide an innovative approach to cancer diagnosis and prognosis.

Methods

Serum Preparation

We obtained serum samples from 199 patients with different types of cancer, including 14 with colorectal cancer, 18 with bladder cancer, 20 with head and neck cancer, 19 with liver cancer, 33 with lung cancer, 24 with lymphoma, 15 with gastric cancer, and 56 with other cancers. We also obtained 40 serum samples to compare with ECPKA concentrations from non cancer patients and 119 normal volunteers from Korea University Medical Centers (Seoul, Korea). Serum from normal healthy individuals was also included as a negative control. All sera were stored in aliquots at −70°C.

Immunoblot Analysis of Anti-ECPKA Antibodies in Human Serum

Human c-AMP dependent Protein Kinase A catalytic subunit (20 μg) was separated on a 10% preparative gel, transferred to nitrocellulose, and blocked overnight at 4°C in TBS with 5% milk and 5% donkey serum (blocking solution). A Miniblotter 28-dual unit system (Immunetics, Inc., Cambridge, MA) was used to make 25 incubation chambers for serum samples, diluted 1 : 50 in 1 : 10 blocking solution. Membranes were incubated with the serum samples for 2 hours at room temperature and wash with TBST. For analysis of human sera, a donkey anti-human biotin conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used at a 1 : 50,000 dilution in 1 : 10 blocking solution for 1 hours. After washing with TBST, HRP-conjugated streptavidin was incubated with the blots (1 : 25,000 dilution in 1 : 10 blocking solution) for 1 hours, and the blots were subjected to a final wash. Super-signal ECL (Pierce, Rockford, IL) was used to develop the HRP.

ELISA Assay (ELISA Test for Anti-ECPKA Autoantibodies)

To measure the presence of anti-ECPKA autoantibody in the human sera, basic methodology for ECPKA assay was carried out by conventional procedures. For this purpose, human cAMP dependent Protein Kinase A catalytic subunit (Sigma, catalog no. C8482) was diluted in PBS to final concentration of 10 μg/mL, and 100 μg were pipetted into each well to coat microtiter plate (KOMA biotech INC., catalog no. K0331011). Incubation were carried out 2 hours at room temperature, and wells were washed 4 times with wash buffer (20 mM HEPES, 0.9% NaCl, 30 mM Sucrose, 0.1% BSA, pH 7.0) and then incubated with blocking buffer (Serotec, cat no. BUF029) for 2 hours at room temperature. After the next washing step with washing solution (500 mM Na-Citrate, 1.5 M NaCl, 1% Tween 20, pH 5.0-5.2), 100 μL of the control and test sera which was diluted 1 : 25,000 in sample diluent (0.25% BSA, 0.05% Tween 20 in PBS) were added to each well and the plates were incubated for 2 hours at room temperature. After washing, we added Peroxidase-conjugated AffiniPure Donkey Anti-Human IgG (Jackson ImmunoResearch), diluted 1 : 20,000 in sample diluent. After 1 hour incubation at room temperature, the plates were washed and the enzymatic reaction was performed by using a substrate solution. The absorbance of each serum sample was measured at 450 nm by an MR600 microplate reader (Molecular Devices, Sunnyvale, CA). Assays were performed in duplicate, and error bars indicate the range.

Statistical Analysis

Data were expressed as mean ± SD (median, [range]). Differences in absorbance between groups were tested for statistical significance by SPSS. A two-tailed $P < 0.05$ denoted the presence of a statistically significant difference.

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