Kinase Activity of Protein Kinase Cα in Serum as a Diagnostic Biomarker of Human Lung Cancer

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Abstract. *Background: Recently, we reported on the existence of activated protein kinase Cα (PKCα) in blood and the possibility for its use in cancer diagnosis. Materials and Methods: In the present study, serum samples collected from patients with different lung cancer types (small-cell cancer, adenocarcinoma, and anaplastic cancer) were phosphorylated with a PKCα-specific peptide substrate and the phosphorylation ratio was detected by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Results: When 13 patient serum samples were phosphorylated with peptide substrates, phosphorylated peaks were obtained in eight samples. However, no peak associated with the phosphorylated peptide was observed using serum samples obtained from 10 healthy persons. Moreover, broadly used cancer biomarkers (progastrin-releasing peptide, carcino-embryonic antigen, and cytokeratin-19 fragment) were identified in eight samples among the 13 samples studied. Conclusion: These results suggest that serum activated PKCα is a reliable biomarker, applicable to lung cancer diagnosis.*

The use of cancer biomarkers in body fluids (*e.g.* blood, urine, and saliva) has several advantages, such as easier sampling and handling, less pain in patients, and non-invasive detection. Several biomarkers, such as antigens, soluble proteins, metabolites and genes, are broadly used for the diagnosis of cancer and for monitoring the recurrence of cancer after surgical resection, or for evaluating the effect of radiation or anticancer drug therapies (1, 2).

Cancer cells have numerous signal transduction pathways that respond to the extracellular signals (ligands) required to regulate downstream gene expression. Among the signal transduction pathways, phosphorylation of the target proteins by protein kinases and proteolytic cleavage by proteases play a key role in cancer cell motility, differentiation, proliferation, and survival (1, 2).

Protein kinase C (PKC) isozymes, which are phospholipid-dependent serine/threonine kinases, play key roles in differentiation, growth, and survival of cancer cells. PKC isozymes are classified into three subfamilies, based on their structural and activational characteristics: conventional or classic PKCs (α, βI, βII, and γ), novel or non-classic PKCs (δ, ε, η, and θ) and atypical PKCs (ζ, ι, and λ). The activation of classic PKCs requires diacylglycerol (DAG) as an activator and phosphatidylserine (PS) and Ca^{2+} as activation co-factors. The non-classic PKCs are regulated by DAG and PS, but do not require Ca^{2+} for activation. In the case of atypical PKCs, their activity is stimulated only by PS, and not by DAG and Ca^{2+} (3, 4).

Among PKC isozymes, PKCα participates in differentiation, growth, and survival of cancer cells. PKCα is hyperactivated in several cancer cell lines and tissues, but has negligible activity in normal cells and tissues (3, 4). Recently, our group found that activated PKCα existing in the blood is a useful biomarker for cancer diagnosis. The level of PKCα was significantly increased in blood samples of cancer-bearing mice compared with that in normal mice (5).

In the present study, serum samples prepared from patients were studied for cancer diagnosis. Each sample was reacted with a PKCα-specific peptide substrate (Alphatomega) (6) and the phosphorylation ratio was detected by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS).

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Table I. *Demographic and clinical characteristics of patients and detection of phosphorylated peak in samples.*

Case	Cancer Type (histological type)	Age, years	Gender	Clinical stage[a]	Biomakers[b]	Phosphorylated peak detected[d]
1	Adenocarcinoma	57	M	4	CEA	○
2	Adenocarcinoma	70	F	4	CEA	×
3	Adenocarcinoma	58	M	4	CEA	○
4	Adenocarcinoma	68	M	4	CEA	×
5	Anaplastic cancer	72	F	4	CEA	○
6	Anaplastic cancer	54	M	4	CYFRA 21-1	○
7	Small-cell cancer	69	M	4	ProGRP	○
8	Small-cell cancer	61	M	4	ProGRP	○
9	Small-cell cancer	63	M	3A	-[c]	×
10	Small-cell cancer	67	M	4	-	○
11	Small-cell cancer	55	M	4	-	×
12	Small-cell cancer	71	M	4	-	○
13	Small-cell cancer	49	M	4	-	×

[a]Clinical stage was classified according to the tumor, node, metastasis (TNM) staging system (11). [b]CEA, carcinoembryonic antigen; ProGRP, progastrin-releasing peptide; CYFRA 21-1, cytokeratin-19 fragment. [c]–, Not detected. [d]○, Phosphorylated peak detected; ×, no phosphorylated peak detected.

Materials and Methods

Peptide synthesis. A peptide substrate (FKKQGSFAKKK) was synthesized and purified, as described previously (5, 6). The purity of the synthetic peptide was identified by high-performance liquid chromatography and MALDI-TOF MS, and the peptide with >95% purity was used for the phosphorylation reaction.

MALDI-TOF MS analysis. α-Cyano-4-hydroxycinnamic acid matrix (10 mg/ml) was prepared in 50% water/acetonitrile and 0.1% trifluoroacetic acid. The matrix and serum samples collected from patients and healthy persons were mixed at a ratio of 20:1. A total volume of 1 μl of the analyte/matrix mixture was then applied to the MALDI plate and then allowed to dry to induce crystallization.

Analyses were conducted using a Voyager DE RP BioSpectrometry Workstation (Applied Biosystems, Framingham, MA, USA) in positive ion reflectron mode. All spectra were analyzed using the Data Explorer software (Applied Biosystems). The phosphorylation ratio is defined as the ion intensity ratio of phosphorylated to unphosphorylated material, and was calculated using the formula: [phosphorylated peptide intensity/(phosphorylated peptide intensity + non-phosphorylated peptide intensity) ×100].

Phosphorylation of peptide substrate with serum sample. Patient blood samples (200-500 μl) were collected at the Kyushu University Hospital between June 1 and June 25, 2010 using sterile tubes that contain no additive, heparin, or ethylenediamine tetraacetic acid. Blood samples from 10 healthy persons were obtained as normal controls. Blood samples were centrifuged (3,000 rpm) for 10 min after maintaining them at room temperature for 30 min. The supernatant (serum) was used for the phosphorylation reaction with the peptide substrate. The total protein concentration was determined using the method of Bradford (Coomassie Brilliant Blue G-250 reagent; BIO-RAD Lab., Hercules, CA, USA) and detected by absorbance at 595 nm. The phosphorylation reaction of the peptide substrate was carried out in 30 μl buffer (20 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, and 100 μM ATP) containing 30 μM of the synthetic peptide and serum (2 mg/ml of protein). After incubation for 60 minutes at 37˚C, the sample was analyzed by MALDI-TOF MS. Triplicate samples were prepared and each was analyzed twice.

Results

Serum samples from 13 patients with different lung cancer types (small-cell cancer, adenocarcinoma, and anaplastic cancer) were prepared in order to determine the existence of activated PKCα in the serum of these patients and its possibility for use in cancer diagnosis. Serum samples collected from 10 healthy persons were used as controls. Broadly used cancer biomarkers [progastrin-releasing peptide (ProGRP), carcinoembryonic antigen (CEA), and cytokeratin-19 fragment (CYFRA 21-1)] were identified in eight samples out of the 13 samples. When the serum samples were phosphorylated with PKCα-specific peptide, phosphorylated peaks were obtained in eight of the 13 samples (Table I; Figures 1 and 2). The peak associated with the phosphorylated peptide showed an increase in the *m/z* value of 80 Da (Figure 1). However, no peak associated with the phosphorylated peptide was observed in the serum samples of 10 healthy persons (Figure 1).

Discussion

Several lung cancer biomarkers have been reported and used for lung cancer diagnosis. ProGRP, produced by lung cancer, such as small-cell lung carcinoma has high stability in blood and is used as a biomarker for lung cancer diagnosis (7, 8). Serum CEA is a glycoprotein involved in normal glandular and mucosal cells and its serum levels increase in several

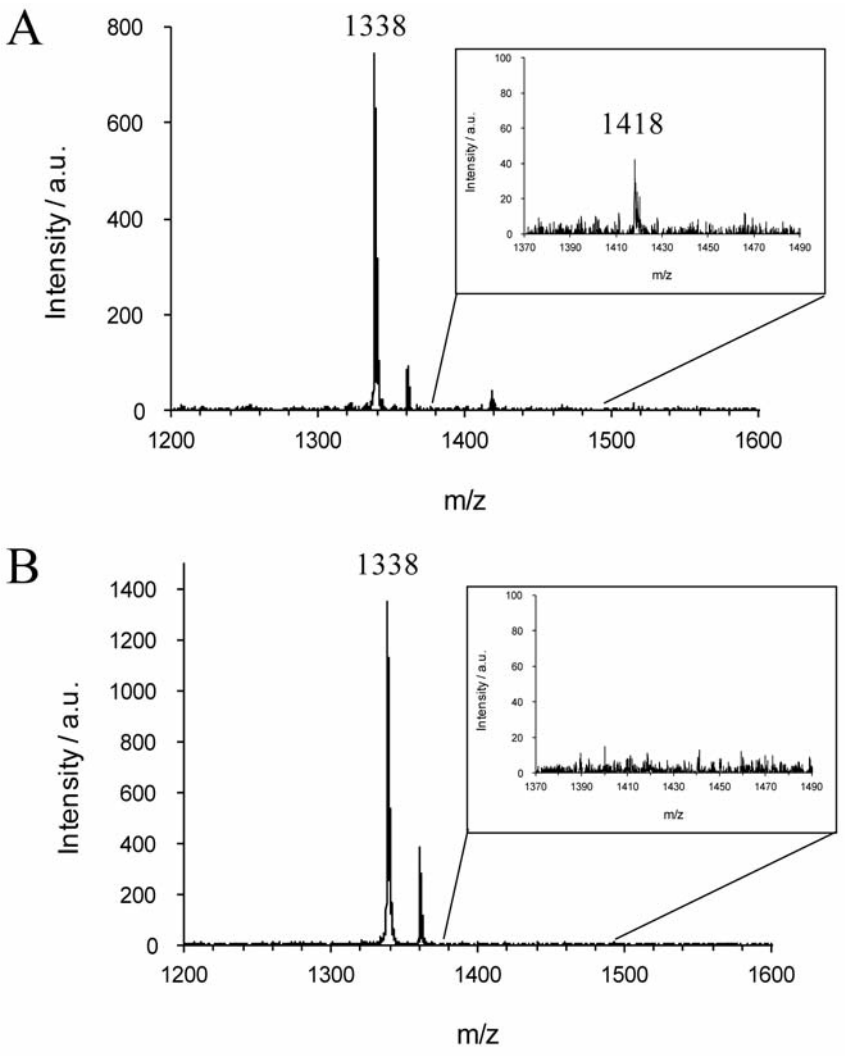

Figure 1. *Typical matrix-assisted laser desorption/ionization-time-of-flight mass spectrometric spectra obtained from the phosphorylation reaction of peptide substrate with serum sample of (A) patient or (B) healthy person.*

lines of cancer cells (*e.g.* lung, gastric, colonic, and breast), and thus it is used as a tumor biomarker for lung cancer diagnosis (7-10). Moreover, serum CYFRA 21-1 levels are also used as an indicator for lung cancer diagnosis (7-10). An increase in serum levels of these biomarkers has been associated with tumor progression and low survival rates. In spite of several useful clinical reports for these biomarkers, the diagnostic accuracy for lung cancer is different from research group to research group, and differs depending on the type and the stage of lung cancer (7-9). Thus, the combination of these biomarkers is encouraged to increase the diagnostic accuracy of lung cancer (8, 9).

In the present study, we detected the presence of phosphorylated peptide in eight serum samples collected from a group of 13 patients with lung cancer after the

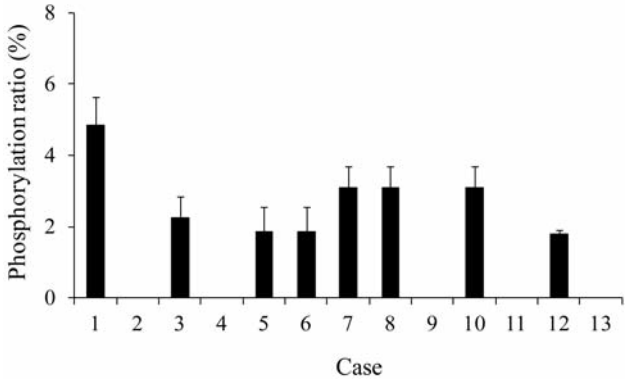

Figure 2. *Phosphorylation ratio is altered after reaction with peptide substrate of serum samples. Data are means±standard deviation of three independent experiments.*

phosphorylation reaction. However, no detection of phosphorylated peptide was detected in the serum samples taken from 10 healthy persons. The diagnostic accuracy for lung cancer by serum PKCα was not lower compared with that of other biomarkers (ProGRP, CEA, and CYFRA 21-1). Thus, our study suggests that serum activated PKCα may be a good biomarker applicable to cancer diagnosis using small samples of patient serum. In spite of these positive results, however, there are two limitations regarding the present study. The first limitation is relatively few cases and the second limitation is that patients with early-stage (stage 1 and 2) lung cancer are not contained.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

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References

1 Hanash SM, Pitter SJ and Faca VM: Mining the plasma proteome for cancer biomarkers. Nature *452*: 571-579, 2008.
2 Martin KJ, Foumier MW, Reddy GP and Pardee AB: A need for basic research on fluid-based early detection biomarkers. Cancer Res *70*: 5203-206, 2010.
3 O'Brian CA, Chu F, Bornmann WG and Maxwell DS: Protein kinase Cα and ε small-molecule targeted therapeutics: A new roadmap to two holy grails in drug discovery? Expert Rev Anticancer Ther *6*: 175-186, 2006.
4 Mackay HJ and Twelves CJ: Targeting the protein kinase C family: Are we there yet? Nat Rev Cancer *7*: 554-562, 2007.
5 Kang JH, Asai D, Toita R, Kitazaki H and Katayama Y: Plasma protein kinase C (PKC) as a biomarker for the diagnosis of cancers. Carcinogenesis *30*: 1927-1931, 2009.
6 Kang JH, Asai D, Yamada S, Toita R, Oishi J, Mori T, Niidome T and Katayama Y: A short peptide is a protein kinase C (PKC)α-specific substrate. Proteomics *8*: 2006-2011, 2008.
7 Schneider J, Philipp M, Velcovsky HG, Morr H and Katz N: Pro-gastrin-releasing peptide (ProGRP), neuron-specific enolase (NSE), carcinoembryonic antigen (CEA) and cytokeratin 19-fragments (CYFRA 21-1) in patients with lung cancer in comparison to other lung diseases. Anticancer Res *23*: 885-893, 2003.
8 Molina R, Augé JM, Bosch X, Esudero JM, Viñolas N, Marrades R, Ramírez J, Carcereny E and Filella X: Usefulness of serum tumor markers, including progastrin-releasing peptide, in patients with lung cancer: correlation with histology. Tumour Biol *30*: 121-129, 2009.
9 Barlési F, Gimenez C, Torre JP, Doddoli C, Mancini J, Greillier L, Roux F and Klieisbauer JP: Prognostic value of combination of CYFRA 21-1, CEA and NSE in patients with advanced non-small cell lung cancer. Respir Med *98*: 357-362, 2004.
10 Huang WW, Tsao SM, Lai CL, Su CC and Tseng CE: Diagnostic value of HER-2/neu, CYFRA 21-1, and carcinoembryonic antigen levels in malignant pleural effusions of lung adenocarcinoma. Pathology *42*: 224-228, 2010.
11 Goldstraw P: The 7th edition of TNM in lung cancer: What now? J Thorac Oncol *4*: 671-673, 2009.

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