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# Relationship between phosphorylation of sperm-specific antigen and prognosis of lung adenocarcinoma

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ABSTRACT

Lung cancer is generally considered as a highly malignant cancer. A major challenge for the management of lung adenocarcinoma patients is to predict the clinical course of the disease after resection. We analyzed the different levels of phosphorylation of proteins in lung adenocarcinoma tissues between a poor prognosis (PP) group, in which six patients exhibited recurrence within five years after surgery, and a good prognosis (GP) group, in which seven patients did not exhibit recurrence within five years after surgery. We found that phosphorylation at Ser92 of the sperm-specific antigen 2 (SSFA2) [phospho-SSFA2(pS92)] was stimulated in the PP group. Using samples from a total of 46 patients, we investigated the utility of phospho-SSFA2(pS92) to discriminate patients of GP and PP groups, with multiple reaction monitoring (MRM) mass spectrometry. Consequently, we confirmed that the PP group had significantly elevated phospho-SSFA2(pS92) levels. Additionally, no expression of SSFA2 recognized in the normal lung tissues. From these results, we demonstrate that phospho-SSFA2 (pS92) is related to the prognosis of early resected lung adenocarcinomas. Therefore, we suggest that phosphorylation of this protein indicates its role as a potential biomarker and new therapeutic target.

*Biological significance:* Lung adenocarcinoma patients often experience a high rate of recurrence after surgery. It is important to discover biomarkers for prognostic prediction and therapeutic targets for treatment of early-stage lung adenocarcinoma. In this study, using tissue samples obtained from patients with lung adenocarcinoma that had been stored for five years at −80 °C, we identified 13 unique phosphorylated peptides, which were differentially expressed between poor and good prognosis groups. We confirmed that phosphorylation at Ser92 of the sperm-specific antigen 2 (SSFA2)[phospho-SSFA2 (pS92)], was related to poor prognosis. Our study demonstrates that prognostic prediction of early-stage lung adenocarcinoma is possible, and suggests new therapeutic targets for its treatment.



## 1. Introduction

Good prognosis can generally be ensured by early detection of lung adenocarcinoma and resection of cancer tissues. However, even if early lung adenocarcinoma is aggressively treated, 20% patients die within five years because of relapse and metastasis [1,2]. Because such treatments are unnecessary for 80% patients who undergo early resection, it is essential to urgently develop biomarkers which permit prompt diagnosis of patients with a high risk of relapse, who require a multitude of postoperative therapies after surgery. Simultaneously, it is imperative that more efficient postoperative adjuvant therapies are developed.

Recently, using proteomic approaches, it has become possible to analyze the post-translational modifications of disease-associated proteins more comprehensively and accurately [3–8]. This includes phosphorylation of proteins. There are many reports describing the relationship between abnormal phosphorylation and diseases [9–13]. In these studies, the abnormal phosphorylation has been postulated as a potential biomarker for diagnosis. However, there is not enough information on abnormal phosphorylation in relation to disease prognosis. If a relationship between abnormal phosphorylation and prognosis can be established, it may be possible to predict prognosis using phosphorylation as a biomarker.

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Previously, we used a transforming growth factor-β (TGF-β)–induced epithelial-mesenchymal transition (EMT) model to quantitatively identify tyrosine- phosphorylation of protein during the course of EMT, in relation to malignant characteristics of lung adenocarcinoma cells. From this analysis, we found that phosphorylation at Tyr1404 of tensin-1(TNS1), Tyr1234 of hepatocyte growth factor receptor (*c*-Met), and Tyr516 of neuro tropic tyrosine kinase receptor (TrkC) was stimulated by TGF-β treatment. Phosphorylation of these proteins was observed only in the poor prognosis (PP) patient group. From these data, we concluded that phosphorylation of these proteins was related to disease prognosis, particularly for patients in the early stages (Ia and Ib) of lung adenocarcinoma [8]. However, there are no reports on phosphorylation of serine or threonine in relation to the prognosis of lung adenocarcinoma.

In the present study, we identified a lung adenocarcinoma prognosis-related protein sperm-specific antigen 2 (SSFA2) which was phosphorylated at the Ser92 residue. The level of phosphorylation was high in the PP patient group. We suggest that this protein can be used as a marker to discriminate the GP and PP patients.

## 2. Materials and methods

### 2.1. Tissue samples

Samples were collected with informed consent and the Kanagawa Cancer Center and Yokohama City University research ethics board approval (Supplementary Table S1). We obtained frozen cancer tissue samples from 59 patients at the early stages (Ia and Ib) of lung adenocarcinoma. Patients who exhibited recurrence within five years after extirpation were included in the PP group (23 patients), whereas patients who did not exhibit recurrence within five years after surgery were included in the good prognosis (GP) group (36 patients). Tissues were stored at −80 °C before use.

### 2.2. Protein extraction

Tissue samples were cut into small pieces (about 1 $mm^3$) and washed three times with PBS buffer containing a protease inhibitor cocktail (Roche, Switzerland). Tissue pieces were homogenized in 50 mM ammonium bicarbonate, 8 M urea, and 4% (*w*/*v*) sodium deoxycholate with a Protease Inhibitor Mix (GE Healthcare, Piscataway, NJ, USA) and Phosphatase Inhibitor Cocktail 2,3 (Sigma, Madison, WI, USA), using a Sample Grinding Kit (GE Healthcare). The homogenate was then sonicated 10 times (1-s intervals) using a UR-21P (TOMY, Tokyo, Japan), and incubated for 30 min at room temperature with gentle shaking. The homogenate was centrifuged at 15,000*g* for 15 min at 4 °C, and the supernatant was collected as the protein extract.

### 2.3. Preparation of phosphopeptides

Protein extracts were reduced with 10 mM DTT at room temperature for 45 min and alkylated using 10 mM iodoacetamide at room temperature for 30 min. One hundred micrograms of the extracted protein was diluted four times with 50 mM ammonium bicarbonate to a final concentration of 2 M urea and measured using a NanoDrop 2000c (Thermo Fisher Scientific, Bremen, Germany). Trypsin (Sigma) was added to the reduced and alkylated samples at a protein to enzyme ratio of 20:1 (*w*/w), followed by incubation at 37 °C for 16 h. Sodium deoxycholate, including the peptide solution, was removed by the phase-transfer surfactant (PTS) method [14]. Peptides were desalted using C18 Stage Tips [15]. Phosphopeptides were concentrated using Titansphere $TiO_2$ bulk beads (GL Sciences, Tokyo, Japan) [16]. The concentrated phosphopeptides were then desalted using C18 Stage Tips.

### 2.4. Shotgun LC–MS/MS analysis

An UltiMate® 3000 LC system (Thermo Fisher Scientific, Bremen, Germany) coupled to a LTQ Orbitrap Velos (Thermo Fisher Scientific) was used. Before injecting into the mass spectrometer, phosphorylated peptides were loaded online in a reverse-phase pre-column (C18 Pepmap column, LC Packings) and resolved on a nanoscale C18 Pepmap™ capillary column (75 μm i.d. × 15 cm, LC Packings) with a gradient of acetonitrile/0.1% (v/v) formic acid. This was performed at a flow rate of 300 nL/min. The mobile phases were A (2% acetonitrile, 98% water, and 0.1% formic acid) and B (95% acetonitrile, 5% water, and 0.1% formic acid). A gradient condition with a flow rate of 300 nL/min was employed; 2–33% B over 120 min and subsequently increased to 95% B during the next 10 min. The general mass-spectrometric conditions were as follows: *m*/*z* range, 350–1200 *m*/*z*; spray voltage, 1.8 kV; capillary temperature, 250 °C; normalized collision energy, 35.0%; isolation width, 2 *m*/*z*; activation time, 10 ms; activation Q, 0.25; dynamic exclusion, 180 s; resolution, 60,000; and data-dependent mode, the 15 most intense ions in the full-scan mass spectrum.

### 2.5. Label-free protein relative quantitation analysis

Label-free relative peptide quantitation was performed using the Progenesis LC–MS data analysis software (version 4.1, Nonlinear Dynamics, Durham, NC, USA). The data evaluated by Progenesis LC–MS (Nonlinear Dynamics) were searched against *Homo sapiens* entries (20,233 entries) in the Swiss-Prot database (January 2013) using the MASCOT software, version 2.4.1 (Matrix Science, London, UK). Charges of +2, +3, or +4 were selected, and peptide mass tolerance was ±5 ppm. Variable modifications were phosphorylation of serine, threonine, or tyrosine, carbamidomethylation of cysteine, and oxidation of methionine. Fragment mass tolerance was ±0.5 Da, with a 1% overall peptide false discovery rate (FDR), and trypsin specificity was applied with a maximum of two missed cleavages. Peptides shown to be upregulated in the PP group were extracted using the following parameters in Progenesis LC–MS: max fold change >1.5; $p < 0.05$; and peptide ion score >60 as the cutoff.

### 2.6. Absolute quantitation by MRM analysis

The level of phospho-SSFA2(pS92) and total peptide levels of SSFA2(S92) were analyzed by absolute quantitation using MRM MS. We purified phosphopeptides from 100 μg of protein and analyzed this by MRM MS. Furthermore, 10 μg of each extracted protein were treated with alkaline phosphatase (CIAP, Takara, Tokyo, Japan) and were then digested with trypsin to measure the amounts of dephosphorylated peptide by MRM MS. Two μg of dephosphorylated peptides were analyzed using MRM MS. We confirmed dephosphorylation by western blot analysis (Supplementary Fig. S1).

An Eksigent cHiPLC nanoflex system (AB Sciex, Foster City, CA, USA) coupled to a TripleTOF 5600 (AB Sciex) was used. We used a direct injection mode with the Eksigent cHiPLC nanoflex system (AB Sciex), and the column oven temperature was set at 50 °C. Before injecting into the mass spectrometer, phosphorylated peptides were loaded online and resolved on a nanoscale Chrom XP C18-CL (75 μm id × 15 cm) (AB Sciex), with a gradient of acetonitrile/0.1% (v/v) formic acid, at a flow rate of 300 nL/min. The mobile phases were A (2% acetonitrile, 98% water, and 0.1% formic acid) and B (80% acetonitrile, 20% water, and 0.1% formic acid). A gradient condition with flow rate of 300 nL/min was employed; 5–42% B over 42 min and this was subsequently increased to 100% B during the next 7 min. To analyze dephosphorylated peptides, a gradient condition with flow rate of 300 nL/min was employed; 15–35% B in 42 min and this was subsequently increased to 100% B during the next 7 min. The mass-spectrometric conditions were as follows: *m*/*z* range, 100–1600 *m*/*z*;