

# Phosphorylation and Activation of Cell Division Cycle Associated 5 by Mitogen-Activated Protein Kinase Play a Crucial Role in Human Lung Carcinogenesis

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## Abstract

We analyzed the gene expression profiles of clinical lung carcinomas using a cDNA microarray containing 27,648 genes or expressed sequence tags, and identified *CDCA5* (cell division cycle associated 5) to be up-regulated in the majority of lung cancers. Tumor tissue microarray analysis of 262 non-small cell lung cancer patients revealed that *CDCA5* positivity was an independent prognostic factor for lung cancer patients. Suppression of *CDCA5* expression with siRNAs inhibited the growth of lung cancer cells; concordantly, induction of exogenous expression of *CDCA5* conferred growth-promoting activity in mammalian cells. We also found that extracellular signal-regulated kinase (ERK) kinase phosphorylated *CDCA5* at Ser79 and Ser209 *in vivo*. Exogenous expression of phospho-mimicking *CDCA5* protein whose Ser209 residue was replaced with glutamine acid further enhanced the growth of cancer cells. In addition, functional inhibition of the interaction between *CDCA5* and ERK kinase by a cell-permeable peptide corresponding to a 20-amino-acid sequence part of *CDCA5*, which included the Ser209 phosphorylation site by ERK, significantly reduced phosphorylation of *CDCA5* and resulted in growth suppression of lung cancer cells. Our data suggest that transactivation of *CDCA5* and its phosphorylation at Ser209 by ERK play an important role in lung cancer proliferation, and that the selective suppression of the ERK-*CDCA5* pathway could be a promising strategy for cancer therapy. *Cancer Res*; 70(13); 5337–47. ©2010 AACR.

## Introduction

Lung cancer is the leading cause of cancer-related death worldwide, as 1.3 million patients die annually (1). Despite the recent development of surgical techniques combined with various treatment modalities, such as radiotherapy and chemotherapy, the overall 5-year survival rate of lung cancer is still only 15% (1). Newly developed cytotoxic agents have emerged to offer multiple therapeutic options for patients with advanced non-small cell lung cancers (NSCLC); however, each of the new regimens can provide only modest survival benefits compared with conventional platinum-based therapies (2). Although many genetic alterations in-

involved in the development and/or progression of lung cancer have been reported, the molecular mechanism is not fully elucidated (3). Hence, novel therapeutic strategies based on the precise molecular pathology of lung cancer, such as the development of molecular-targeted agents, nucleic acid drugs, and antibodies, as well as cancer vaccines, are eagerly awaited.

Systematic analysis of expression levels of thousands of genes on a cDNA microarray is an effective approach for identifying molecules involved in carcinogenic pathways (4); some of the upregulated genes or their products may become candidate targets for the development of novel anti-cancer drugs and tumor biomarkers. To isolate such molecules, we have been analyzing genome-wide expression profiles of 101 lung cancers, using enriched populations of tumor cells prepared by laser microdissection (5–9). To verify the biological and clinicopathologic significance of the respective gene products, we have established a screening system by a combination of tumor tissue microarray analysis of clinical lung cancer materials with RNA interference (RNAi) technique (10–36). In the course of those systematic studies, we found that *CDCA5* (cell division cycle associated 5; alias Sororin) was overexpressed in a great majority of lung cancers.

*CDCA5* was initially identified as a substrate of anaphase-promoting complex and as a regulator of sister chromatid cohesion in HeLa and mouse myeloma Nso-1 cells (37, 38).

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CDCA5 protein is degraded through anaphase-promoting complex-dependent ubiquitination in the G<sub>1</sub> phase, and is required for stable binding of cohesion to chromatid (38). Despite these biological studies in a limited number of cell lines, there has been no report describing the significance of the activation of CDCA5 in human carcinogenesis and its potential as a diagnostic and therapeutic target.

We here report that CDCA5 is indispensable for growth of cancer cells and is possibly involved in the extracellular signal-regulated kinase (ERK) pathway, and that targeting the CDCA5 and/or the ERK-CDCA5 pathway is likely to be a promising strategy for the development of new molecularly targeted drugs for cancer treatment.

## Materials and Methods

### Cell lines and clinical samples

Twenty-three human lung cancer cell lines used in this study included 19 NSCLC cell lines (A427, A549, NCI-H1373, LC319, PC-14, PC-3, PC-9, NCI-H1666, NCI-H1781, NCI-H647, NCI-H226, NCI-H1703, NCI-H2170, NCI-H520, LU61, RERF-LC-AI, SK-MES-1, EBC-1, and LX1) and 4 small-cell lung cancer cell lines (SCLC; DMS114, DMS273, SBC-3, and SBC-5; Supplementary Table S1). All cells were grown in monolayers in appropriate medium supplemented with 10% fetal calf serum (FCS) and were maintained at 37°C in an atmosphere of humidified air with 5% CO<sub>2</sub>. Human airway epithelial cells SAEC (Cambrex Bio Science, Inc.) were also included in the panel of the cells used in this study. Primary lung cancer tissue samples had been obtained earlier with informed consent, as reported elsewhere (5, 9, 11). All tumors were staged based on the pathologic tumor-node-metastasis (pTNM) classification of the International Union Against Cancer (39). Formalin-fixed primary NSCLCs and adjacent normal lung tissue samples used for immunostaining on tissue microarrays were obtained from 262 patients undergoing curative surgery at Hokkaido University and its affiliated hospitals (Sapporo, Japan; please see Supplementary Table S2). To be eligible for this study, tumor samples were selected from patients who fulfilled all of the following criteria: (a) patients with primary NSCLC with histologically confirmed stage (only pT<sub>1</sub> to pT<sub>3</sub>, pN<sub>0</sub> to pN<sub>2</sub>, and pM<sub>0</sub>); (b) patients who underwent curative surgery but did not receive any preoperative treatment; (c) NSCLC patients positive for lymph node metastasis (pN<sub>1</sub>, pN<sub>2</sub>) who were treated with platinum-based adjuvant chemotherapies after surgery, and patients with pN<sub>0</sub> who did not receive adjuvant chemotherapies; and (d) patients whose clinical follow-up data were available. This study and the use of all clinical materials mentioned were approved by individual institutional ethics committees.

### Semiquantitative reverse transcriptase-PCR

We prepared appropriate dilutions of each single-stranded cDNA prepared from mRNAs of clinical lung cancer samples, using the level of β-actin (*ACTB*) transcript as a quantitative control. The primer sets for amplification were as follows: *ACTB*-F (5'-GAGGTGATAGCATTGCTTTCG-3') and *ACTB*-R (5'-CAAGTCAGTGTACAGGTAAGC-3'); *CDCA5*-F1 (5'-CGC-

CAGAGACTTGAAATGT-3') and *CDCA5*-R1 (5'-GTTTCT-GTTTCTCGGGTGGT-3'). All reactions involved initial denaturation at 95°C for 5 minutes followed by 22 (for *ACTB*) or 30 (for *CDCA5*) cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds on a GeneAmp PCR system 9700 (Applied Biosystems).

### Northern blot analysis

Human multiple tissue blots (23 normal tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocyte, stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland, bone marrow; BD Biosciences Clontech) were hybridized with a <sup>32</sup>P-labeled PCR product of *CDCA5* cDNA. The partial-length cDNA of *CDCA5* was prepared by reverse transcriptase-PCR (RT-PCR) using primers *CDCA5*-F2 (5'-GCTTGTAAGTCTCGAAAGTT-3') and *CDCA5*-R2 (5'-ATCTCAACTCTGCATCATCTGGT-3'). Pre-hybridization, hybridization, and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at -80°C for 7 days.

### Anti-CDCA5 antibody

Plasmids expressing full-length CDCA5 that contained His-tagged epitopes at their NH<sub>2</sub> termini were prepared using pET28 vector (Novagen) and primers *CDCA5*-F3 (5'-CCG-GAATTCATGTCTGGGAGGCGAACGCG-3') and *CDCA5*-R3 (5'-CCGCTCGAGTTCACCAGGAGATCAAAGTCTGCTC-3'). Recombinant proteins were expressed in *Escherichia coli* BL21 codon-plus strain (Stratagene) and purified using Ni-NTA (Qiagen) according to the supplier's protocol. The protein was inoculated into rabbits; immune sera were purified on affinity columns according to standard methods. Affinity-purified anti-CDCA5 antibodies were used for Western blotting as well as immunocytochemical and immunohistochemical studies. We confirmed that the antibody was specific to CDCA5 on Western blots using lysates from cell lines that had been transfected with CDCA5 expression vector as well as those from lung cancer cell lines that endogenously expressed CDCA5.

### Western blotting

Cells were lysed in lysis buffer: 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.5% deoxycholate-Na, 0.1% SDS, plus protease inhibitor (Protease Inhibitor Cocktail Set III; Calbiochem). We used an enhanced chemiluminescence Western blotting analysis system (GE Healthcare Biosciences), as described previously (11).

### Immunocytochemical analysis

Cultured cells were washed twice with PBS(-), fixed in 4%paraformaldehyde solution for 30 minutes at 37°C, and then rendered permeable with PBS(-) containing 0.1% Triton X-100 for 3 minutes. Before the primary antibody reaction, cells were covered with blocking solution [3% bovine serum albumin in PBS(-)] for 10 minutes to block nonspecific antibody binding. After cells were incubated with

antibodies to human CDCA5 (generated to recombinant CDCA5; please see above), Alexa Fluor 488 goat anti-rabbit secondary antibody (Molecular Probes) was added to detect endogenous CDCA5. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Antibody-stained cells were viewed with a laser-confocal microscope (TSC SP2 AOBs; Leica Microsystems).

#### Immunohistochemistry and tissue microarray analysis

To investigate the significance of CDCA5 expression in clinical NSCLCs, we stained tissue sections using ENVISION+ kit/horseradish peroxidase (HRP; DakoCytomation). Affinity-purified anti-CDCA5 antibody (generated to recombinant CDCA5; please see above) was added after blocking endogenous peroxidase and proteins, and each section was incubated with HRP-labeled anti-rabbit IgG as the secondary antibody. Substrate-chromogen was added, and the specimens were counterstained with hematoxylin. Tumor tissue microarrays were constructed as published previously, using formalin-fixed NSCLCs (40–42). Tissue areas for sampling were selected based on visual alignment with the corresponding H&E-stained sections on slides. Three, four, or five tissue cores (diameter, 0.6 mm; height, 3–4 mm) taken from donor tumor blocks were placed into recipient paraffin blocks using a tissue microarrayer (Beecher Instruments). A core of normal tissue was punched from each case. Five-micrometer sections of the resulting microarray block were used for immunohistochemical analysis. Three independent investigators semiquantitatively assessed CDCA5 positivity without prior knowledge of clinicopathologic data. Because the intensity of staining within each tumor tissue core was mostly homogenous, the intensity of CDCA5 staining in the nucleus and cytoplasm was evaluated by recording as negative (no appreciable staining in tumor cells) or positive (brown staining appreciable in the nucleus and cytoplasm of tumor cells). Cases were accepted as positive if two or more reviewers independently defined them as such.

#### Statistical analysis

Statistical analyses were done using the StatView statistical program (SAS). We used contingency tables to correlate clinicopathologic variables, such as age, gender, and pTNM stage, with the positivity of CDCA5 protein determined by tissue microarray analysis. Tumor-specific survival curves were calculated from the date of surgery to the time of death related to NSCLC, or to the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and for CDCA5 expression; differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were performed with the Cox proportional hazard regression model to determine associations between clinicopathologic variables and cancer-related mortality. First, we analyzed associations between death and possible prognostic factors, including age, gender, smoking, histologic type, pT classification, and pN classification, taking into consideration one factor at a time. Second, multivariate Cox analysis was applied on backward (stepwise) procedures that always forced strong CDCA5 ex-

pression into the model, along with any and all variables that satisfied an entry level of a *P* value <0.05. As the model continued to add factors, independent factors did not exceed an exit level of *P* < 0.05. A two-tailed *P* value of <0.05 was considered statistically significant.

#### RNAi assay

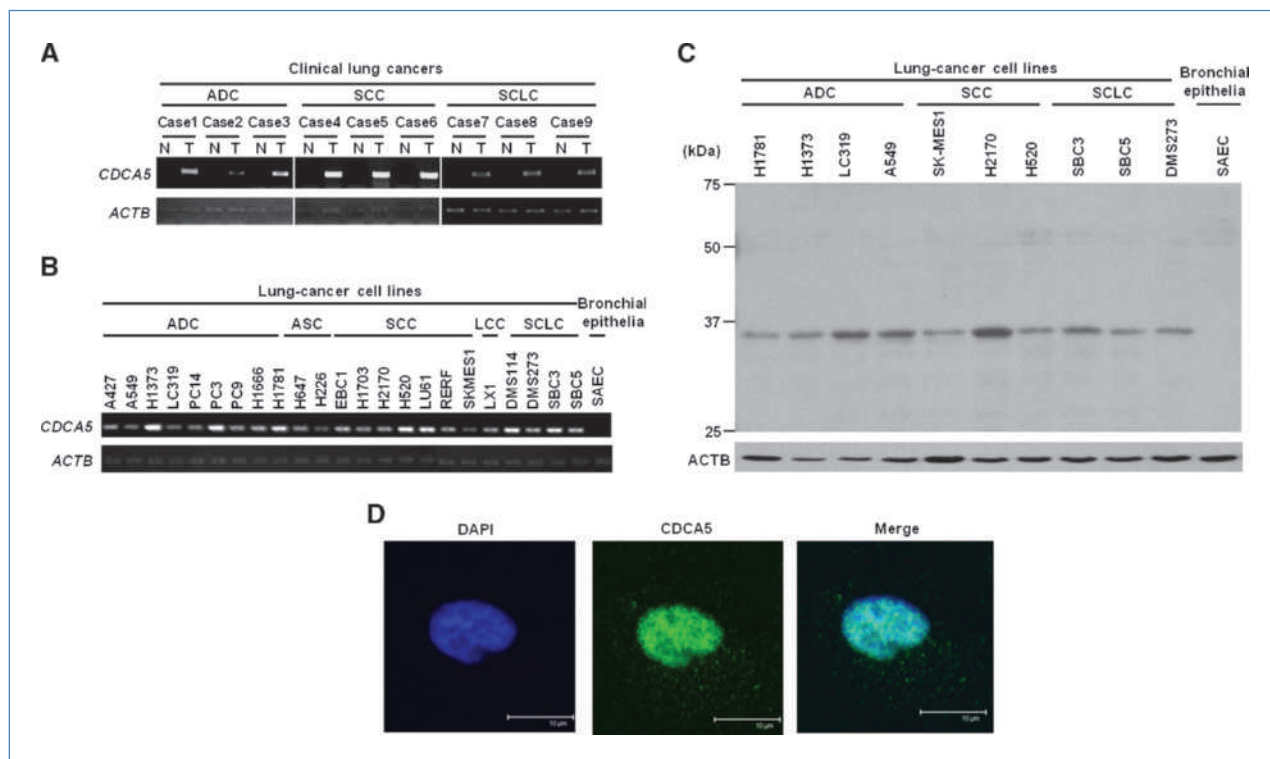
Two independent CDCA5 siRNA oligonucleotides were designed using CDCA5 sequences (Genbank accession no: NM080668). siRNAs (600 pmol/L) were transfected into two NSCLC cell lines, A549 and LC319, using 30  $\mu$ L of Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Transfected cells were cultured for 7 days. Cell numbers and viability were measured by Giemsa staining and triplicate MTT assays (cell counting kit-8 solution; Dojindo Laboratories). The siRNA sequences used were as follows: control-1 (si-LUC: luciferase gene from *Photinus pyralis*), 5'-CGUACGCGAAUACUUCGA-3'; control-2 (CNT: ON-TARGETplus siCONTROL Nontargeting siRNAs a pool of four oligos: 5'-UGGUUUACAUGUCGACUAA-3', 5'-UGGUUUACAUGUUUCUGA-3', 5'-UGGUUUACAUGUUUCUUA-3', and 5'-UGGUUUACAUGUUGUGUGA-3'); siRNA-CDCA5-#1 (si-CDCA5-#1: 5'-GCAGUUUGAUCUCCUGUUU-3'); siRNA-CDCA5-#2 (si-CDCA5-#2: 5'-GCCAGAGACUUG-GAAAUGUUU-3'). Downregulation of endogenous CDCA5 expression in the cell lines by siRNAs for CDCA5, but not by controls, was confirmed by semiquantitative RT-PCR and Western blot analyses.

#### Cell growth assay

To establish COS-7 cells stably expressing CDCA5, non-tagged CDCA5 expression vector (pCAGGSn-CDCA5) or mock vector (pCAGGSn) was transfected into COS-7 cells that weakly expressed endogenous CDCA5 using FuGENE6 transfection reagent (Roche). Transfected cells were incubated in the culture medium containing 0.4 mg/mL neomycin (Geneticin, Invitrogen) for 14 days. Then, 50 colonies were trypsinized and screened for stable transfectants by a limiting dilution assay. Expression of CDCA5 was determined in each clone by Western blotting and immunocytochemical staining. Viability of cells was evaluated by MTT assay at days 1, 3, 5, and 7.

#### In vitro kinase assay

We performed *in vitro* ERK kinase assay using glutathione *S*-transferase (GST)-tagged full-length CDCA5 protein (pGEX-6p-1/CDCA5). Briefly, 1.0  $\mu$ g each of recombinant GST-CDCA5, MBP, or GST was incubated in 20  $\mu$ L of kinase buffer [50 mmol/L Tris-HCl, 10 mmol/L MgCl<sub>2</sub>, 1 mmol/L EGTA, 2 mmol/L DTT, 0.01% Brij-35, and 1 mmol/L ATP (pH 7.5) at 25°C] supplemented with 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (GE Healthcare) and 50 ng of ERK2 kinase (Upstate) for 20 minutes at 30°C. The reactions were terminated with Laemmli SDS sample buffer to a final volume of 30  $\mu$ L; half of the samples were subjected to 5% to 15% gradient gel (Bio-Rad Laboratories); and phosphorylation was visualized by autoradiography. MBP was used as a positive substrate control, and GST was used as a negative control.



**Figure 1.** Expression of CDCA5 in lung tumors. A, expression of *CDCA5* gene in lung cancer tissues (T) and adjacent normal (N) lung tissues, detected by semiquantitative RT-PCR. B, expression of *CDCA5* gene in lung cancer cell lines, detected by semiquantitative RT-PCR. C, expression of CDCA5 protein in lung cancer cell lines, detected by Western blotting. ADC, adenocarcinoma; SCC, squamous cell carcinoma; ASC, adenosquamous carcinoma; LCC, large cell carcinoma. D, subcellular localization of endogenous CDCA5 protein in lung cancer LC319 cells. Cells were stained with a rabbit polyclonal anti-CDCA5 antibody (green) and DAPI (blue).

To determine *in vivo* ERK-dependent phosphorylation sites on CDCA5 in cultured cells, we performed colloidal CBB staining after immunoprecipitation of exogenously expressed CDCA5 protein in epidermal growth factor (EGF)-treated HeLa cells (50 ng/mL for 20 minutes) that were transfected with both CDCA5-expressing plasmid and ERK2-expressing plasmid, using anti-CDCA5 antibody. Samples were separated on SDS-PAGE gel. After electrophoresis, the gels were stained by Coomassie brilliant blue (CBB, R-250; Bio-Rad). Specific bands corresponding to CDCA5 were digested with trypsin as described elsewhere (43) and analyzed by matrix-assisted laser desorption/ionization mass spectrometry analysis (Shimadzu Biotech). Mass spectral data were evaluated using the Mascot search engine (<http://www.matrixscience.com>) to identify proteins from primary sequence databases.

**EGF stimulation assay**

HeLa cells were cultured in FCS-free medium for 20 hours. Then, cells were stimulated by 50 ng/mL of EGF with or without 10 μmol/L MEK inhibitor U0126 (Promega).

**Synthesized cell-permeable peptide**

Amino acid peptide sequences corresponding to a part of CDCA5 protein that contained Ser209, a site phosphorylated by ERK, were covalently linked at its NH<sub>2</sub> terminus to a membrane transducing 11 poly-arginine (11R) sequence (44).

Three cell-permeable peptides were synthesized: p209-A, RRRRRRRRRRRR-GGG-PDMTLPGISPPPEKQKRK; p209-B, RRRRRRRRRRRR-GGG-TLPGISPPPEKQKRKKKKM; and p209-C, RRRRRRRRRRRR-GGG-RVCAKPWAPDMTLPGISPPP. Peptides were purified by preparative reverse-phase high-pressure liquid chromatography. A549 and LC319 cells as well as human lung-derived CCD19-Lu cells were incubated with the 11R peptides at concentrations of 2.5, 5, 7.5, and 10 μmol/L for 7 days. The medium was changed every 48 hours at the appropriate concentrations of each peptide, and viability of cells was evaluated by MTT assay after 7 days of treatment with the peptide.

**Results**

**Expression of CDCA5 in lung cancer and normal tissues**

We previously screened 27,648 genes or expressed sequence tags on a cDNA microarray to identify transcripts showing 3-fold or higher expression in cancer cells than in normal cells in the majority of clinical lung cancer samples. Among them, we identified the *CDCA5* transcript to be up-regulated; its increased expression in all of nine clinical lung cancer tissues was confirmed by semiquantitative RT-PCR experiments, although its expression was hardly detectable in adjacent normal lung tissues (Fig. 1A). High levels of *CDCA5* expression were also observed in all of the 23 lung

cancer cell lines examined, but the transcript was hardly detectable in SAEC cells derived from normal airway epithelial cells (Fig. 1B). In addition, we confirmed high levels of endogenous CDCA5 protein by Western blot analysis in lung cancer cell lines using anti-CDCA5 antibody (Fig. 1C). Subsequent immunofluorescence analysis of lung cancer LC319 cells identified the subcellular localization of endogenous CDCA5 mainly in the nucleus and weakly in the cytoplasm (Fig. 1D). Northern blot analysis using a *CDCA5* cDNA fragment as a probe identified a 2.8-kb transcript to be highly expressed in the testis; however, this transcript was hardly detectable in other normal tissues examined (Fig. 2A). Furthermore, we examined CDCA5 protein in five normal tissues (heart, lung, liver, kidney, and testis) and lung cancer tissues by immunohistochemistry using anti-CDCA5 polyclonal antibodies, and detected its abundant expression in the testis as well as in lung cancer cells, whereas its expression was hardly detectable in the remaining four normal tissues (Fig. 2B).

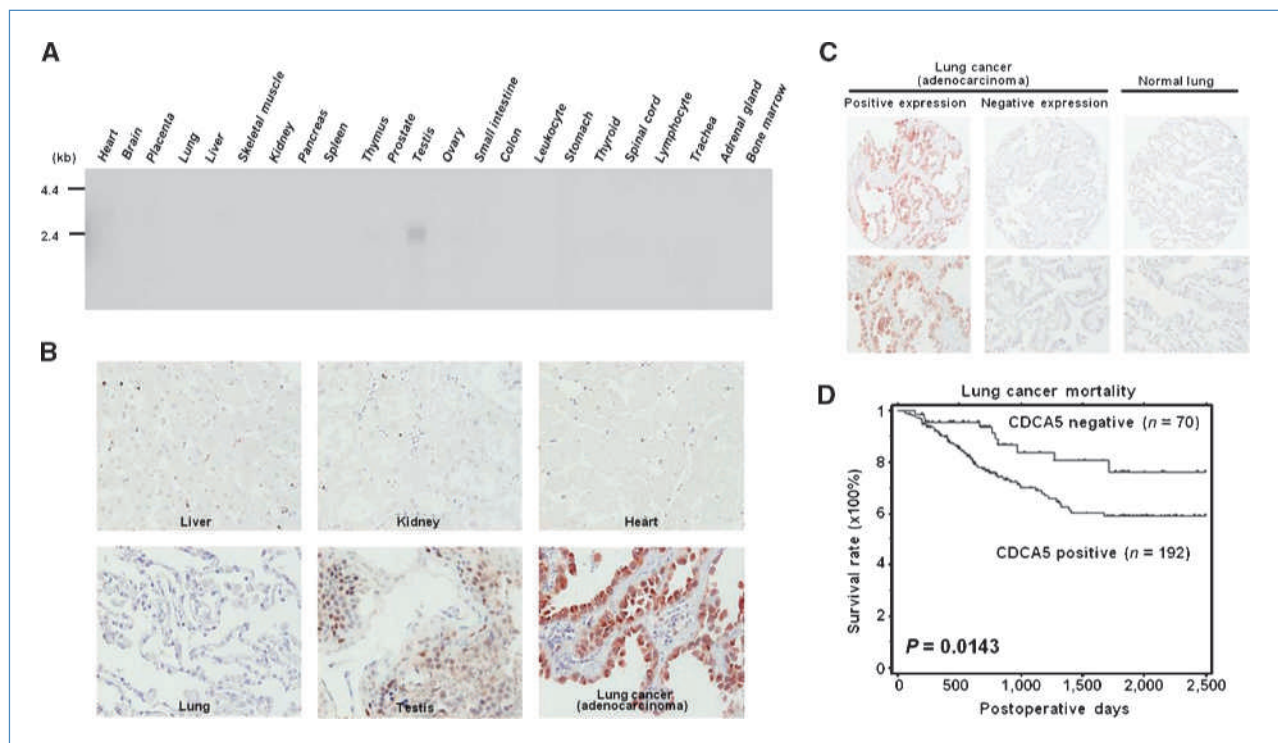
#### Association of CDCA5 expression with poor prognosis for NSCLC patients

Using tissue microarrays prepared from archived NSCLC samples from patients who underwent curative surgery, we performed immunohistochemical analysis with anti-CDCA5 polyclonal antibodies. We classified staining patterns of CDCA5 expression to be negative or positive as shown in

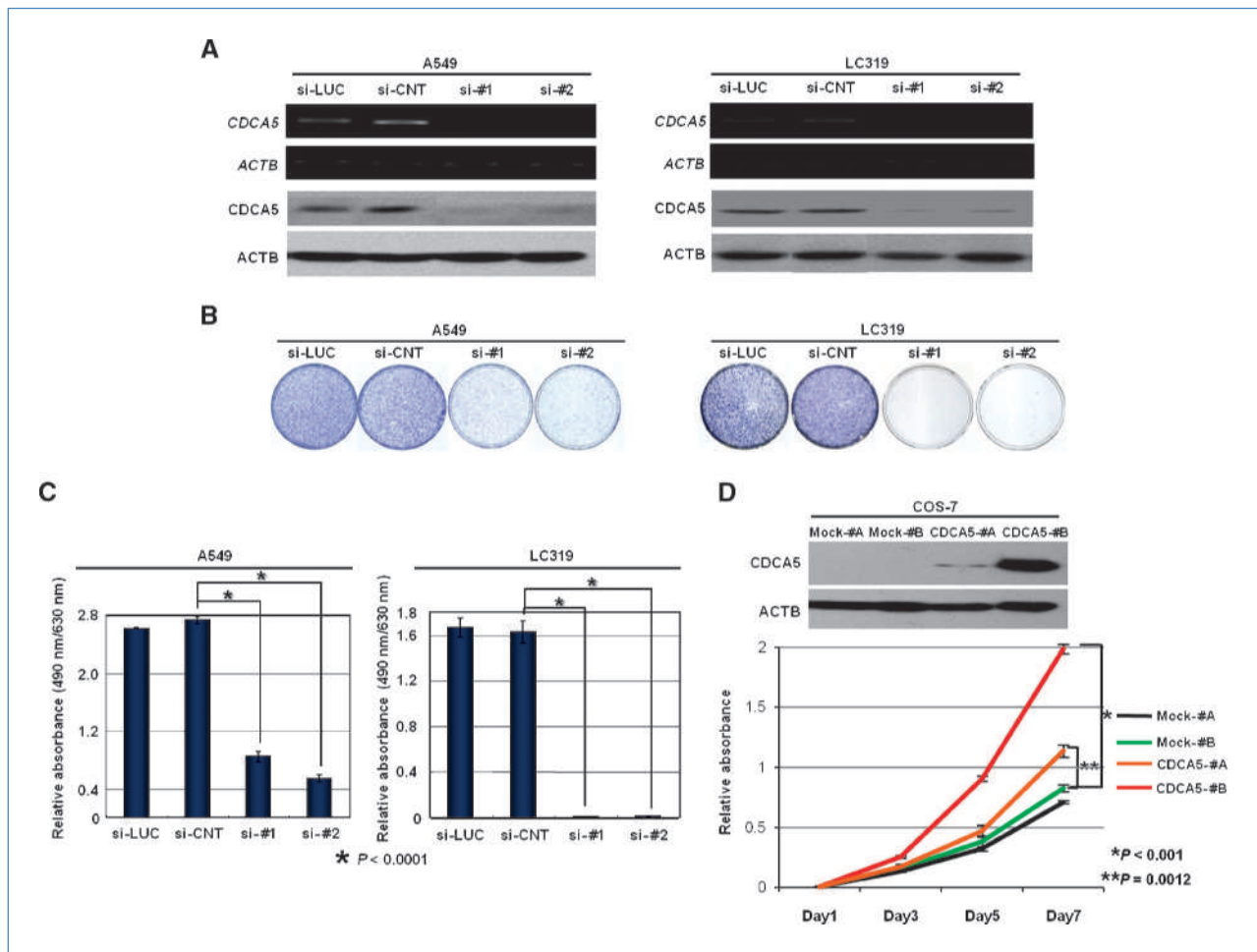
Fig. 2C. Of the 262 NSCLC cases examined, we judged 192 cases (73.3%) as positive and 70 cases (26.7%) as negative; no staining was observed in any of their adjacent noncancerous cells (Supplementary Table S3A). We then examined the association of CDCA5 expression with various clinicopathologic parameters of NSCLC patients, and found a significant association between CDCA5 positivity in NSCLCs and a shorter tumor-specific survival period ( $P = 0.0143$  by log-rank test; Fig. 2D and Supplementary Table S3B). We also applied univariate analysis to evaluate associations between patient prognosis and several factors including age, gender, smoking history, histologic type (adenocarcinoma versus nonadenocarcinoma), pT stage (tumor size; T<sub>1</sub> versus T<sub>2</sub>+T<sub>3</sub>), pN stage (node status; N<sub>0</sub> versus N<sub>1</sub>+N<sub>2</sub>), and CDCA5 status (negative versus positive expression). All of these parameters were significantly associated with poor prognosis (Supplementary Table S3C). Multivariate analysis indicates CDCA5 status to be an independent prognostic factor for surgically treated lung cancer patients enrolled in this study ( $P = 0.0244$ ), similar to pT and pN stages and age.

#### Growth-promoting effect of CDCA5

Using siRNA oligonucleotides for CDCA5, we attempted to knock down the expression of endogenous CDCA5 in lung cancer cell lines A549 and LC319, which showed high levels of CDCA5 expression. Two CDCA5-specific siRNAs



**Figure 2.** Expression of CDCA5 in normal tissues and its association with poor prognosis for NSCLC patients. A, Northern blot analysis of the *CDCA5* transcript in various normal human tissues. B, expression of CDCA5 protein in five normal tissues (liver, kidney, heart, lung, and testis) and a lung adenocarcinoma. C, immunohistochemical staining pattern of CDCA5 protein in representative lung adenocarcinomas using anti-CDCA5 polyclonal antibodies on tissue microarrays (top,  $\times 100$ ; bottom,  $\times 200$ ). D, Kaplan-Meier analysis of tumor-specific survival periods according to CDCA5 expression on tissue microarrays.



**Figure 3.** Growth-promoting effect of CDCA5. A, knock down of CDCA5 expression in A549 and LC319 lung cancer cells by specific oligonucleotide siRNAs for CDCA5 (si-#1 and si-#2) or control siRNAs (si-LUC and si-CNT), confirmed by semiquantitative RT-PCR analyses (top) and Western blotting (bottom). B, colony formation assays of A549 and LC319 cells transfected with the siRNAs. C, viability of A549 and LC319 cells evaluated by MTT assay in response to the siRNAs. All assays were performed in triplicate wells three independent times. D, enhancement of growth of COS-7 cells by exogenous introduction of CDCA5. Cell viability of two stable clones (COS-7-CDCA5-#A and -#B) and two control clones (COS-7-Mock-#A and -#B) was quantified with the MTT assay at days 1, 3, 5, and 7. All assays were performed in triplicate wells three independent times.

(si-CDCA5-#1 and si-CDCA5-#2) significantly suppressed expression of CDCA5 in both transcript and protein levels compared with control siRNAs (si-LUC and si-CNT; Fig. 3A). Colony formation and MTT assays revealed that reduction of CDCA5 expression by the two siRNAs significantly suppressed the growth of A549 and LC319 cells (Fig. 3B and C). To further examine the effect of CDCA5 on growth of mammalian cells, we transfected plasmids designed to express full-length CDCA5 (pcDNA3.1-CDCA5) or mock plasmids into COS-7 cells, and established two independent COS-7 cell lines overexpressing exogenous CDCA5 (COS-7-CDCA5-#A and -#B) and two control cells (COS-7-Mock-#A and -#B). We then carried out MTT assay of these COS-7-derived cells and compared the growth of COS-7-CDCA5 cells with control COS-7-Mock cells. Growth of the two COS-7-CDCA5 (COS-7-CDCA5-#A and -#B) cells was promoted at a significant degree in accordance with the amount of CDCA5 protein as detected by Western blot analysis (Fig. 3D).

### Phosphorylation of CDCA5 by ERK kinase

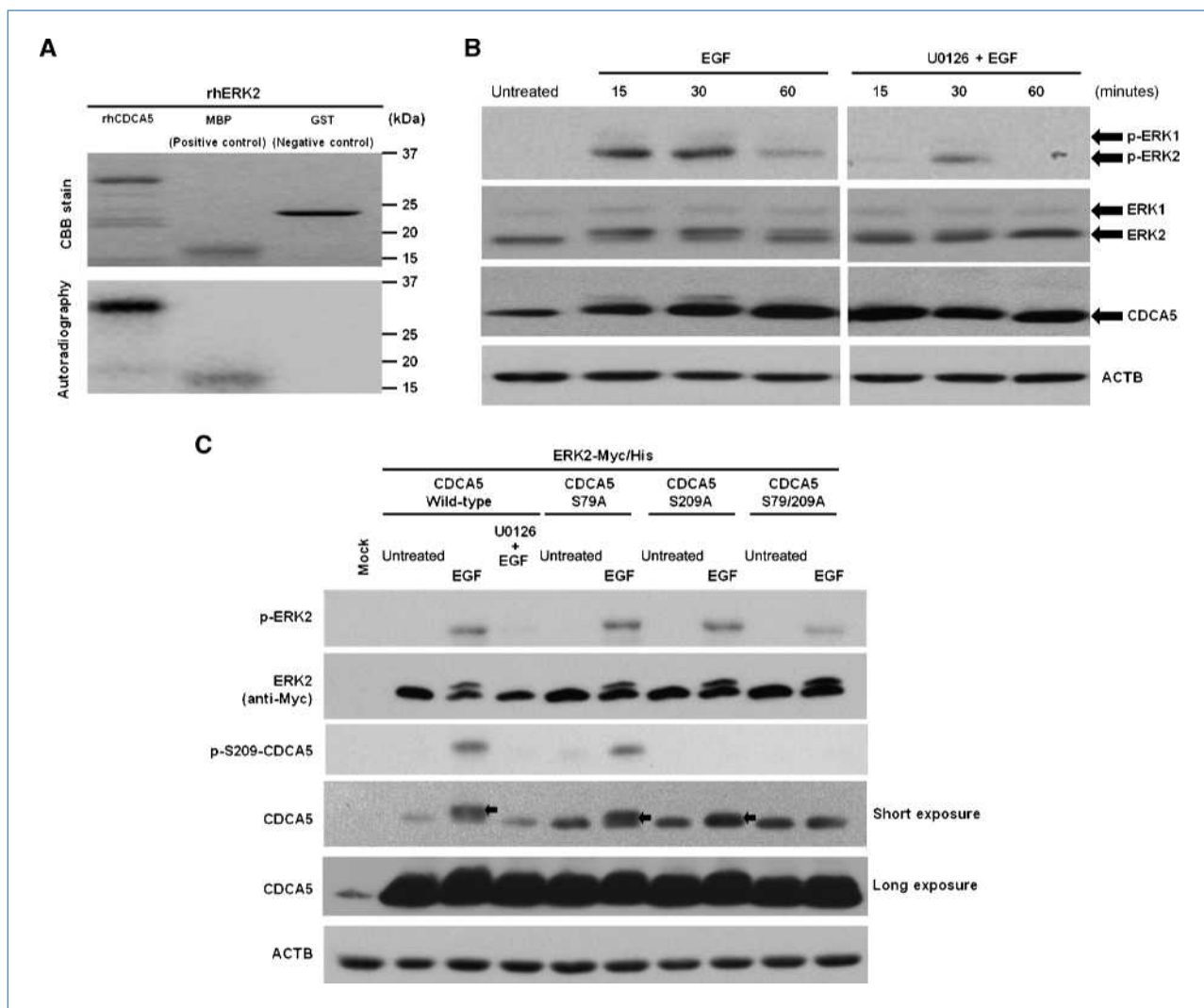
To analyze the function of CDCA5 in carcinogenesis, we focused on the possible phosphorylation of CDCA5 protein because the *in silico* approach suggested several consensus phosphorylation sites on CDCA5 by ERK kinase [x-x-S/T-P], which is one of the important downstream components in the oncogenic mitogen-activated protein kinase (MAPK) pathway. We first performed *in vitro* kinase assay by incubating rhERK2 with rhCDCA5 and found that CDCA5 was likely to be directly phosphorylated by ERK kinase (Fig. 4A).

To examine whether CDCA5 was phosphorylated by ERK in cells, serum-free cultured HeLa cells were stimulated with 50 ng/mL of EGF in the presence or absence of 10 μmol/L of MEK inhibitor U0126. Western blot analysis using anti-ERK antibody detected upper-shifted bands that corresponded to activated phospho-ERK1/2 at 15 and 30 minutes after EGF stimulation; however, the amount of the upper-shifted band

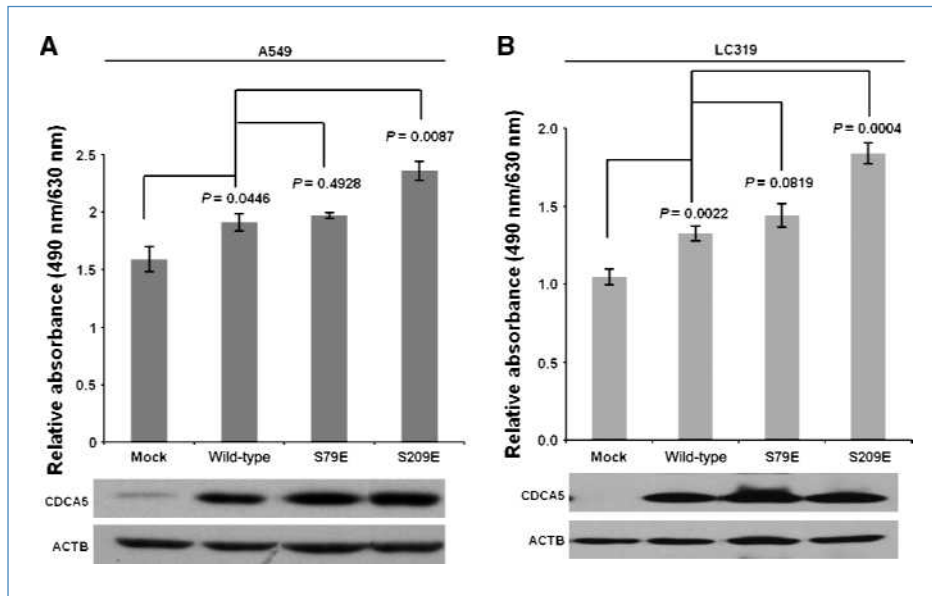
detected by anti-ERK antibody was decreased at 60 minutes (Fig. 4B, left panels). In the same condition, increase in phospho-ERK1/2 level at 15 and 30 minutes after EGF stimulation and its decrease at 60 minutes were also confirmed by directly detecting phosphorylated ERK using anti-phospho-ERK antibody. In accordance with the levels of endogenous ERK phosphorylation, an upper-shifted endogenous CDCA5 band was detected by anti-CDCA5 antibody at 15 and 30 minutes after the EGF stimulation, whereas the shifted CDCA5 band and activated phospho-ERK levels were decreased at 60 minutes. On the other hand, treatment of cells in the presence of U0126, an inhibitor for both EGF and MEK kinases, blocked ERK phosphorylation as well as

CDCA5 phosphorylation (Fig. 4B, right panels). These results suggest the possible phosphorylation of endogenous CDCA5 protein in the ERK-dependent pathway.

To examine the ERK-dependent *in vivo* phosphorylation sites on CDCA5 in cultured cells, we performed immunoprecipitation assay using anti-CDCA5 antibody and lysates of EGF-treated or untreated HeLa cells that were transfected with both nontagged CDCA5-expressing vectors and Myc/His-tagged ERK2-expressing vectors (Supplementary Fig. S1A), and determined the phosphorylation sites by subsequent mass spectrometric analysis. The three bands corresponding to immunoprecipitated CDCA5 in the cells that were treated with or without EGF, or treated with



**Figure 4.** *In vitro* and *in vivo* phosphorylation of CDCA5 by ERK protein kinases. **A**, *in vitro* phosphorylation of recombinant human CDCA5 (rhCDCA5) by recombinant human ERK2 (rhERK2). **B**, phosphorylation of endogenous CDCA5 in HeLa cells after EGF stimulation (50 ng/mL) at various time points (15, 30, or 60 minutes) in the presence or absence of 10  $\mu$ M of the MEK inhibitor U0126. **C**, exogenous expression of Myc/His-tagged ERK2 and nontagged wild-type and mutant CDCA5 proteins (CDCA5-S79A, CDCA5-S209A, and CDCA5-S79A/S209A) in HeLa cells, detected by Western blotting using anti-Myc antibody or antibodies to total CDCA5, phospho-CDCA5 (Ser209), or phospho-ERK after EGF stimulation (50 ng/mL) for 20 minutes in the presence or absence of 10  $\mu$ M of U0126.



**Figure 5.** Growth-promoting effect of CDCA5 phosphorylation at Ser209. A and B, enhanced growth-promoting activity of phospho-mimicking CDCA5 protein in which serine at 209 was replaced with glutamine acid. Cell viability of A549 or LC319 cells transfected with mock vectors or vectors expressing wild-type or phospho-mimicking CDCA5 proteins (CDCA5-S79E and CDCA5-S209E) was quantified by the MTT assay at 6 days after transfection.

both EGF and U0126, were excised and subjected to mass spectrometric analysis (Supplementary Fig. S1B); 70%, 77%, or 66% of the CDCA5 protein were individually analyzed. We found that Ser79 and Ser209 were ERK-dependently phosphorylated. Phosphorylation of Ser21 was found in all three cells, indicating that it is unlikely to be an ERK-dependent phosphorylation site *in vivo* (data not shown). To confirm the results of this mass spectrometric analysis, we performed Western blot analysis using HeLa cells transfected with vectors expressing wild-type or mutant CDCA5 protein whose Ser79 and/or Ser209 residues were replaced with alanine (CDCA5-S79A, CDCA5-S209A, and CDCA5-S79A/S209A). Myc/His-tagged ERK2-expressing vector was cotransfected with either wild-type or mutant CDCA5-expressing vector under the same conditions with/without EGF treatment as described above. Upper-shifted bands were detected in lanes corresponding to wild-type and two mutant CDCA5 proteins (CDCA5-S79A and CDCA5-S209A) after EGF stimulation, whereas no shifted band was detected in those corresponding to the mutant CDCA5 (CDCA5-S79A/S209A) containing double substitutions (Fig. 4C). Using anti-phospho-Ser209-CDCA5 antibody, phosphorylation of both ERK2 and Ser209 of CDCA5 after EGF stimulation was detected in lanes corresponding to wild type CDCA5 and CDCA5-S79A, but not in those corresponding to CDCA5-S209A or CDCA5-S79A/S209A. In addition, CDCA5-S79A and CDCA5-S209A seemed to be smaller compared with wild-type CDCA5. These results indicate that both Ser79 and Ser209 are likely to be phosphorylated by ERK kinase in cells.

**Growth-promoting effect of CDCA5 phosphorylation at Ser209**

To examine whether ERK-dependent phosphorylation of CDCA5 could play important roles in lung carcinogenesis,

we transfected into LC319 and A549 cells plasmids expressing nontagged phospho-mimicking CDCA5 protein whose Ser79 or Ser209 residue was replaced with a glutamine acid (CDCA5-S79E and CDCA5-S209E). We found by MTT assay that growth of cells expressing phospho-mimicking CDCA5 at Ser209, but not those expressing Ser79 phospho-mimicking CDCA5, was significantly faster than those expressing wild-type CDCA5 (Fig. 5A and B). The results could strongly suggest that phosphorylation of Ser209 of CDCA5 plays a pivotal role in cancer cell growth.

**Growth inhibition of lung cancer cells by cell-permeable peptides**

To investigate the functional significance of Ser209 phosphorylation on CDCA5 protein by ERK for growth or survival of lung cancer cells, we developed bioactive cell-permeable peptides that were expected to inhibit the *in vivo* phosphorylation of CDCA5 by ERK. We synthesized three different peptides that included the Ser209 phosphorylation site on CDCA5 protein. These peptides were covalently linked at its NH<sub>2</sub> terminus to a membrane transducing 11 arginine residues (11R). We first investigated the inhibition of these three p209-CDCA5 peptides on the phosphorylation level of Ser209-CDCA5 by ERK in cells, using anti-phospho-Ser209-CDCA5 antibody. We incubated HeLa cells, which were transfected with vectors designed to express nontagged CDCA5 and ERK2-Myc/His, with each peptide for 48 hours. Then, we stimulated the cells with EGF for 20 minutes. The phosphorylation level of Ser209-CDCA5 was significantly suppressed by the treatment with p209-C peptide compared with two other peptides (Fig. 6A). We also performed immunoprecipitation using Myc-agarose to precipitate ERK2 protein and found that the amount of CDCA5 protein binding to ERK was significantly decreased in cells treated with p209-C peptide (data not shown). Treatment of A549 and LC319 cells



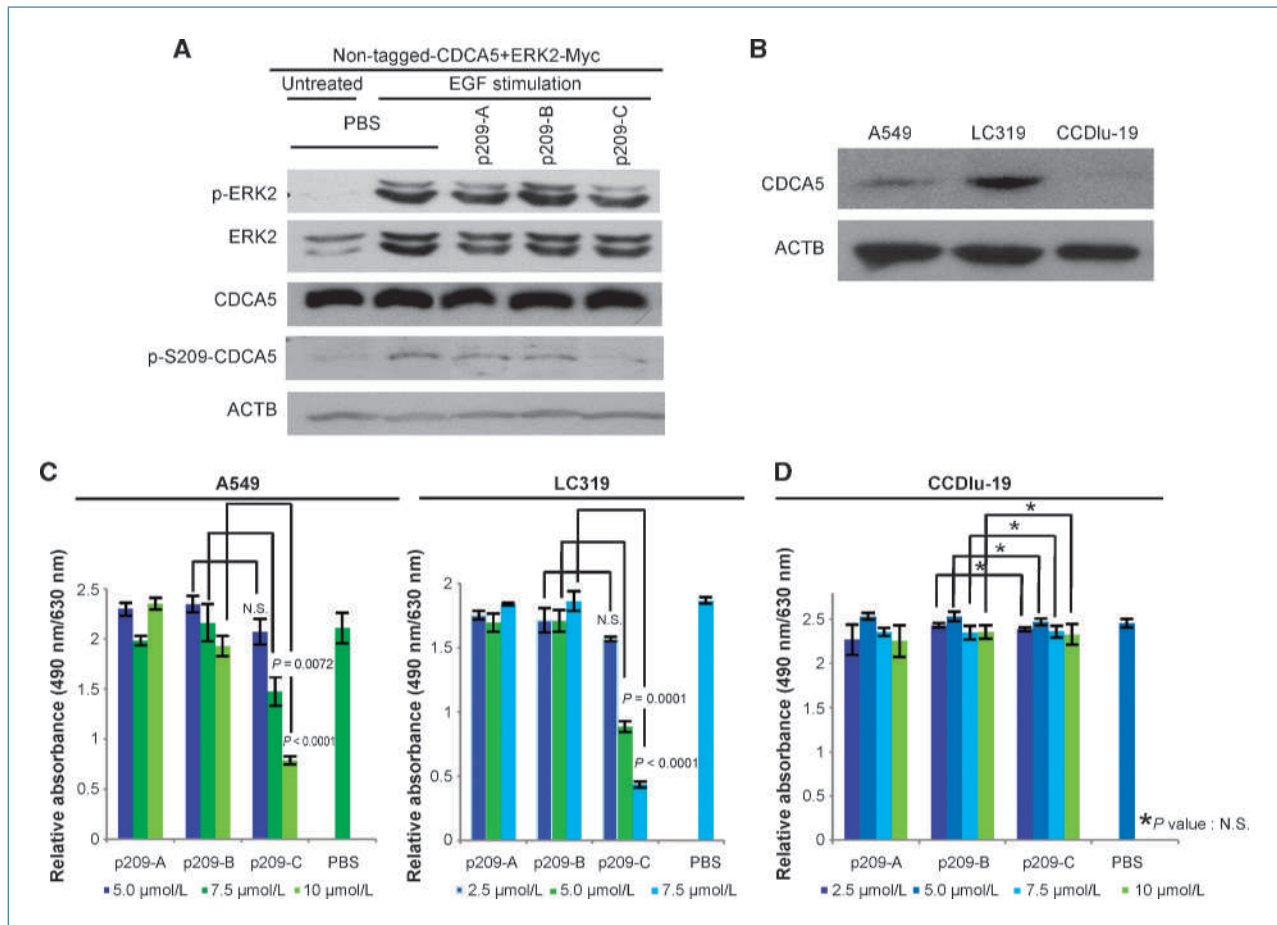
that strongly expressed CDCA5 with the p209-C peptide resulted in significant decreases in cell viability, as measured by the MTT assay (Fig. 6B and C). Peptide p209-C revealed no significant effect on cell viability of human lung fibroblast-derived CCD19-Lu in which CDCA5 expression was hardly detectable (Fig. 6D). The data indicate that the p209-C peptide could specifically inhibit the ERK kinase activity for CDCA5 phosphorylation through the interference of their interaction, and have no or minimal toxic effect in normal human cells in which CDCA5 expression was hardly detectable.

## Discussion

Despite the improvement of surgical techniques and adjuvant chemoradiotherapy, lung cancer still has very poor prognosis among malignant tumors. Therefore, it is urgently required to develop novel diagnostic biomarkers for early detection of cancer and for a better choice of adjuvant treatment modalities to individual patients, as well as for developing new types of anticancer drugs and/or cancer

vaccines. To identify appropriate diagnostic and therapeutic target molecules, we combined genome-wide gene expression analysis (5–9) for selecting genes that were overexpressed in lung cancer cells with high-throughput screening of loss-of-function effects by means of the RNAi technique and tumor tissue microarray analysis (10–36). Using this systematic approach, we found *CDCA5* to be frequently upregulated in clinical lung cancer samples and showed that this gene product plays an indispensable role in growth of lung cancer cells.

Although CDCA5 was suggested to play some roles in cell cycle progression through its interaction with cohesion on chromatin in some immortalized cell lines (38), no study has shown that CDCA5 could be involved in carcinogenesis. Our study has indicated that CDCA5 is a putative oncogene that is highly and frequently expressed in lung cancers. We found by tissue microarray analysis that patients with NSCLC having positive CDCA5 expression represented a shorter tumor-specific survival period, indicating that CDCA5 is likely to contribute to a malignant potential of lung cancers. In addition,



**Figure 6.** Inhibition of growth of lung cancer cells by cell-permeable CDCA5 peptides. A, reduction of the ERK-dependent Ser209 phosphorylation on CDCA5 by cell-permeable CDCA5 peptides (p209-C), detected using anti-phospho-Ser209-CDCA5 antibody. B, expression of CDCA5 protein in normal human lung fibroblast-derived CCD19-Lu cells, compared with lung cancer cell line A549 and LC319, examined by Western blot analysis. C, MTT assay of A549 and LC319 cells, detecting a growth-suppressive effect of p209-C peptide. Columns, relative absorbance of triplicate assays; bars, SD. D, MTT assay, detecting no significant off-target effect of the p209-C peptide on CCD-Lu-19 cells that scarcely expressed CDCA5 protein.

we showed that CDCA5 was phosphorylated by ERK at two phosphorylation sites, Ser79 and Ser209, whose sequences matched to a consensus ERK phosphorylation site and are highly conserved in many species (data not shown). Furthermore, phosphorylation of Ser209 by ERK seemed to be critically important for cancer cell growth.

ERK is a member of the MAPK family that functions at an integration point for multiple biochemical signals and is involved in a wide variety of cellular processes, such as proliferation, differentiation, transcription regulation, and development (45–47). Dysfunction of MAPK cascades is well known to play a pivotal role in carcinogenesis (48). The Raf-MEK-ERK pathway is a key downstream effector in epidermal growth factor receptor (EGFR) signaling (45–47). Thus, the EGFR-Ras-Raf-MEK-ERK signaling network has been the subject of intense research scrutiny, leading to the discovery of new anticancer drugs. Currently, inhibitors of the Raf-MEK-ERK cascade, such as the geldanamycin analogue 17-allylamino-17-demethoxygeldanamycin, are under evaluation in clinical trials (49). In addition, multiple small-molecule inhibitors for MEK and Ras have been developed (50). Although these drugs are indicated to be effective in preclinical studies and/or in certain proportions of cancer patients, clinical response was not likely to be precisely correlated with the activation levels of the target protein(s). More importantly, severe adverse reactions due to their non-specific cytotoxicity have been reported in some cases. Therefore, development of drugs that more selectively target this pathway based on the precise information for downstream oncogenic signals may be one of the alternative promising approaches.

We here showed that exogenous expression of phosphomimicking CDCA5 protein whose Ser209 residue was replaced with glutamine acid further enhanced the growth of cancer cells, and that functional inhibition of interaction between

CDCA5 and ERK kinase by a cell-permeable peptide corresponding to a 20-amino-acid sequence part of CDCA5, which included the Ser209 phosphorylation site by ERK, significantly reduced phosphorylation of CDCA5 and resulted in growth suppression of lung cancer cells. Because phosphorylation of CDCA5 at the site was likely to be indispensable for growth and survival of lung cancer cells, selective targeting of CDCA5-ERK enzymatic activity as well as interaction between CDCA5 and its unknown oncogenic binding partners could be a promising therapeutic strategy that is expected to have a strong biological activity against cancer, with a minimal risk of adverse reactions. Further analyses of the mechanism of growth suppression by specific inhibition of CDCA5 phosphorylation by ERK and the detailed function of downstream effectors of CDCA5 may have the great benefit toward the development of new types of anticancer agents.

In summary, CDCA5 is likely to play a significant role in lung carcinogenesis through its phosphorylation at Ser209 through the activation of the MAPK pathway. Inhibition of CDCA5 itself as well as its functional interaction with ERK kinase could be a promising therapeutic strategy for the development of new types of anticancer drugs.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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