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論文内容の要旨

論文題目 **Identification and characterization of cell division cycle associated 5, as a novel prognostic biomarker and a therapeutic target for lung cancer**

（肺癌における新規予後予測バイオマーカー及び治療標的分子である**CDCA5**の同定と機能解析）

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<u>Introduction</u>

Lung cancer is the leading cause of cancer-related death in the world as 1.3 million patients died annually.　In spite of 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%.　Newly developed cytotoxic agents have emerged to offer multiple therapeutic options for patients with advanced non-small cell lung cancers (NSCLCs), but each of the new regimens can provide only modest survival benefits compared with conventional platinum-based therapies.　Although many genetic alterations involved in the development and/or progression of lung cancer have been reported, the molecular mechanism is not fully elucidated.　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; some of up-regulated 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 micro dissection. To verify the biological and clinicopathological significance of the respective gene products, we have established a screening system by a combination of the tumor-tissue microarray analysis of clinical lung-cancer materials with RNA interference (RNAi) technique.　In the course of those systematic studies we found that CDCA5 (cell division cycle associated 5; alias Sororin) was overexpressed in the great majority of lung cancers.

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We here report that CDCA5 is indispensable for growth of cancer cells and is possibly involved in the 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 molecular 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 four small-cell lung cancer cell lines (SCLC; DMS114, DMS273, SBC-3, and SBC-5).

Semiquantitative RT-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.

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 ^{32}P-labeled PCR product of *CDCA5* cDNA.

Immunohistochemistry analysis.   To investigate the significance of CDCA5 expression in normal tissues, we stained tissue sections using ENVISION+ kit/horseradish peroxidase .

RNA interference assay.   Two independent *CDCA5* siRNA oligonucleotides were designed using the *CDCA5* sequences. siRNAs were transfected into NSCLC cell line A549 , using 30 μl of Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

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).   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 GST-tagged full-length CDCA5 protein (pGEX-6p-1/CDCA5).   Briefly, 1.0 μg each of recombinant GST-CDCA5, MBP, or GST was incubated in 20 μl of kinase buffer supplemented with 1 μCi of [γ-^{32}P]-ATP (GE Healthcare) and 50 ng of ERK2 kinase for 20 minutes at 30°C. MBP was used as a positive substrate control and GST was used as a negative control.

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 NH2-terminus to a membrane transducing 11 poly-arginine (11R) sequence.   Three cell-permeable peptides were synthesized.

Results

Expression of CDCA5 in lung cancer and normal tissues.

We previously screened 27,648 genes or ESTs 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 *CDCA5* transcript to be up-regulated and confirmed by semiquantitative RT-PCR experiments its increased expression in all of 9 clinical lung cancer tissues, however its expression was hardly detectable in their adjacent normal lung tissues. High levels of *CDCA5* expression were also observed in all of 23 lung-cancer cell lines examined, but the transcript was hardly detectable in SAEC cells derived from normal airway epithelial cells.   In addition, we confirmed high levels of endogenous CDCA5 protein by western-blot analysis in lung cancer cell lines using anti-CDCA5 antibody.   Northern blot analysis using a *CDCA5* cDNA

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fragment as a probe identified a 2.8-kb transcript to be highly expressed in the testis, but its transcript was hardly detectable in any other normal tissues examined. Furthermore, we examined CDCA5 protein in 5 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 lung cancer cells, while its expression was hardly detectable in the remaining four normal tissues.

Growth promoting effect of CDCA5.   Using siRNA oligonucreotide for CDCA5, we attempted to knock down expression of endogenous CDCA5 in lung cancer cell line A549 which showed high levels of CDCA5 expression.   Two CDCA5-specific siRNAs (si-*CDCA5*-#1 and si-*CDCA5*-#2) significantly suppressed expression of CDCA5 in both transcript and protein levels compared with control siRNAs.   Colony formation and MTT assays revealed that reduction of CDCA5 expression by the two siRNAs significantly suppressed the growth of A549 and LC319 cells.   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.

Phosphorylation of CDCA5 by ERK kinase.

To analyze the function of CDCA5 in carcinogenesis, we focused on the possible phosphorylation of CDCA5 protein, because *in silico* approach suggested several consensus phosphorylation sites on CDCA5 by ERK kinase [x-x-S/T-P] that is one of important downstream components in the oncogenic 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 .

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 µM 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 the EGF stimulation, but the amount of the upper-shifted band was decreased at 60 minutes .

To examine the ERK-dependent *in vivo* phosphorylation sites on CDCA5 in cultured cells, we performed immunoprecipitation assay using anti-CDCA5 antibody and the lysates of EGF-treated or non-treated HeLa cells that were transfcted with both non-tagged CDCA5-expressing vectors and Myc/His-tagged ERK2-expressing vectors, and determined the phosphorylation sites by subsequent mass-spectrometric analysis. We found that serine-79 and serine-209 were ERK-dependently phosphorylated.   Phosphorylation of serine-21 was found in all three cells, indicating that it is unlikely to be an ERK-dependent phosphorylation site *in vivo*. 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). Using specific phospho-S209-CDCA5 antibody, phosphorylation of Ser 209 was observed in CDCA5 wild type or CDCA5-S79A, but not in CDCA5-S209A or CDCA5-S79A/S209A cells. In addition, the size of CDCA5-S79A and CDCA5-S209A appeared to be smaller compared to wild-type CDCA5.   These results indicated that both serine-79 and serine-209 are likely to be phosphorylated by ERK kinese in cells.

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Growth promoting effect of CDCA5 phosphorylation at Seine 209.

To examine whether ERK-dependent phosphorylation of CDCA5 could play important roles in lung carcinogenesis, we transfected into LC319 and A549 cells plasmids expressing non-tagged phospho-mimicking CDCA5 protein whose serine-79 or seine-209 residue was replaced with a glutamine acid (CDCA5-S79E and CDCA5-S209E).   We found by MTT assay that the growth of cells expressing phospho-mimicing CDCA5 at serine-209, but not that expressing serine-79 phospho-mimicing CDCA5, was significantly faster than those expressing wild-type CDCA5 (**Fig. 3**). The results could strongly suggest that phosphorylation of serine-209 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 the 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 NH2-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. The phosphorylation level of Ser209-CDCA5 was significantly suppressed by the treatment with p209-C peptide compared with two other peptides. Treatment of A549 and LC319 cells which strongly expressed CDCA5 with the p209-C peptide resulted in significant decreases in cell viability as measured by MTT assay.   Peptide p209-C revealed no significant effect on cell viability of human lung fibroblast-derived CCD19-Lu in which CDCA5 expression was hardly detectable.   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 minimum toxic effect on normal human cells in which CDCA5 expression was hardly detectable.

Conclusion

We here demonstrated that exogenous expression of phospho-mimicking CDCA5 protein whose serine-209 residue was replaced with glutamine acid further enhanced the growth of the cancer cells.   Since the phosphorylation of CDCA5 at the site was likely to be indispensable for the growth/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.

In summary, CDCA5 is likely to play a significant role in lung carcinogenesis through its phosphorylation at serine-209 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 type of anti-cancer drugs.

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