Activation of CDCA1-KNTC2, Members of Centromere Protein Complex, Involved in Pulmonary Carcinogenesis

Satoshi Hayama,1,2 Yataro Daigo,1 Tatsuya Kato,1 Nobuhisa Ishikawa,1 Takumi Yamabuki,1 Masaki Miyamoto,2 Tomoo Ito,1 Eiju Tsuchiya,1 Satoshi Kondo,1 and Yusuke Nakamura1

1Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan; Departments of Surgical Oncology and Surgical Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; and Kanagawa Cancer Center Research Institute, Kanagawa, Japan

Abstract

We found cotransactivation of cell division associated 1 (CDCA1) and kinetochore associated 2 (KNTC2), members of the evolutionarily conserved centromere protein complex, in non–small cell lung carcinomas (NSCLC). Immunohistochemical analysis using lung cancer tissue microarray confirmed high levels of CDCA1 and KNTC2 proteins in the great majority of lung cancers of various histologic types. Their elevated expressions were associated with poorer prognosis of NSCLC patients. Knockdown of either CDCA1 or KNTC2 expression with small interfering RNA significantly suppressed growth of NSCLC cells. Furthermore, inhibition of their binding by a cell-permeable peptide carrying the CDCA1-derived 19-amino-acid peptide (11R-CDCA1398-416) that corresponds to the binding domain to KNTC2 effectively suppressed growth of NSCLC cells. As our data imply that human CDCA1 and KNTC2 seem to fall in the category of cancer-growth of NSCLC cells. Furthermore, inhibition of their binding by a cell-permeable peptide carrying the CDCA1-derived 19-amino-acid peptide (11R-CDCA1398-416) that corresponds to the binding domain to KNTC2 effectively suppressed growth of NSCLC cells. As our data imply that human CDCA1 and KNTC2 seem to fall in the category of cancer-growth/survival of lung cancer, selective suppression of CDCA1 or KNTC2 activity and/or inhibition of the CDCA1-KNTC2 complex formation could be a promising therapeutic target for treatment of lung cancers. (Cancer Res 2006; 66(21): 10339–48)

Introduction

Lung cancer is one of the most common cancers in the world, and non–small cell lung cancer (NSCLC) accounts for 80% of those cases (1). Many genetic alterations involved in development and progression of lung cancer have been reported, but the precise molecular mechanisms still remain unclear (2). In the last few decades, newly developed cytotoxic agents, including paclitaxel, docetaxel, gemcitabine, and vinorelbine, have emerged to offer multiple therapeutic choices for patients with advanced NSCLC. However, because those regimens provide a limited survival benefit compared with cisplatin-based therapies (3, 4), new therapeutic strategies are eagerly awaited.

Systematic analysis of expression levels of thousands of genes using cDNA microarrays is an effective approach for identifying a set of molecules that are involved in carcinogenic pathways and can also be applicable as candidate targets for development of novel therapeutics and diagnostics. Toward this goal, we have done genome-wide expression profile analysis of lung cancers with the cDNA microarray containing 27,648 genes (5–8) and have been examining the biological and clinicopathologic significance of the respective gene products. We did high-throughput screening of loss-of-function effects by means of the RNA interference (RNAi) technique as well as tumor tissue microarray analysis of clinical lung cancer materials (9–14). This systematic approach revealed that cell division associated 1 (CDCA1; alias Nuf2) and kinetochore associated 2 (KNTC2; alias Ndc80) were frequently overexpressed in various histologic types of lung cancer and were essential to growth or survival of lung cancer cells. The highly conserved CDCA1-KNTC2 complex in prokaryotic and eukaryotic cells was reported to play an important role in the kinetochore function and the spindle checkpoint (15–18).

CDCA1 and KNTC2 are members of the Ndc80 complex that is composed of the two subcomplexes of Ndc80 (KNTC2)-Nuf2 (CDCA1) and Spec24-Spec25 (15). The Ndc80 complex was first isolated in budding yeast, and its homologues have been identified in worm, frog, chicken, and human (15–18). The attachment sites of the CDCA1-KNTC2 complex within the kinetochore outer plate generate microtubule dependent forces for chromosomal movement and regulate spindle checkpoint protein assembly at the kinetochore. Yeast cells that lost members of the complex or had mutated members were known to exhibit loss of kinetochore-microtubule attachment without global loss of kinetochore structure (19). Yeast Nuf2 also disappears from the centromere during meiotic prophase when centromeres lose their connection to the spindle pole body and plays a regulatory role in segregation of chromosomes (20). Human CDCA1 was identified as a member of genes that were coexpressed with known cell cycle genes, including CDC2, cyclin, topoisomerase II, and others (21), and was reported to be associated with centromeres of mitotic HeLa cells, which suggests a possibility that this protein is a functional homologue of yeast Nuf2 (19). On the other hand, human KNTC2 was identified as an interacting protein with the COOH terminus of the retinoblastoma protein (RB1) using a yeast two-hybrid screening and was suggested to be one of several proteins involved in spindle checkpoint signaling (22, 23). This surveillance mechanism involving KNTC2 recruits the MPS1 kinase and MAD1/MAD2 complexes to kinetochores and assures correct segregation of chromosomes during cell division by detecting unaligned chromosomes and causing prometaphase arrest until the proper bipolar attachment of chromosomes is achieved (24). In spite of these biological studies, there has been no report describing the significance of coactivation of the CDCA1-KNTC2 complex in human cancer progression and its potential as therapeutic and prognostic targets.

We here show that coactivation of CDCA1 and KNTC2 and their cognate interactions play significant roles in pulmonary carcinogenesis, and that inhibition of the complex would lead to novel therapeutic strategy for treatment of lung cancer.

Requests for reprints: Yusuke Nakamura, Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, Japan. E-mail: yusuke@ims.u-tokyo.ac.jp.

© 2006 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-06-2137

www.aacrjournals.org 10339 Cancer Res 2006; 66: (21). November 1, 2006

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2006 American Association for Cancer Research.
Cancer Research

Materials and Methods

Lung cancer cell lines and tissue samples. The human lung cancer cell lines used in this study were as follows: lung adenocarcinomas A427, A549, LC319, PC9, PC9, and NCI-H1373; bronchioloalveolar cell carcinomas NCI-H1666 and NCI-H1781; lung squamous cell carcinomas (SQC) RERF-LC-AL, SK-MES-1, EBC-1, L616, NCI-H226, NCI-H520, NCI-H647, NCI-H1703, and NCI-H217b; a lung large cell carcinoma LX1; and SCLC DMS114, DMS275, SBC-3, and SBC-5. All cells were grown in monolayers in appropriate medium supplemented with 10% FCS and were maintained at 37°C in atmospheres of humidified air including 5% CO2. Human small airway epithelial cells (SAEC) were grown in optimized medium (SAGM) purchased from Cambrex Bio Science, Inc. (East Rutherford, NJ). Sixteen primary NSCLCs, including eight adenocarcinomas and eight SCCs, were obtained with written informed consent along with adjacent normal lung tissue samples from patients undergoing surgery at Hokkaido University and its affiliated hospitals (Sapporo, Japan). A total of 282 NSCL and adjacent normal lung tissue samples for immunostaining on tissue microarray were also obtained from patients who underwent surgery at Hokkaido University and its affiliated hospitals (Sapporo, Japan). This study and the use of all clinical materials were approved by individual institutional ethical committees.

Semiquantitative reverse transcription-PCR. Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s protocol. Extracted RNAs were treated with DNase I (Nippon Gene, Tokyo, Japan) and reversely transcribed using oligo (dT) primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following synthesized CDCA1-specific primers, KNTC2-specific primers, or with β-actin (ACTB)–specific primers as an internal control: CDCA1, 5’-GAGAAGCTAAGGATCCGAGAAT-3’ and 5’-CTGATACCTCATTGCTTCAC-3’; KNTC2, 5’-AAAAAGAAGCTATGCTAGATGTC-3’ and 5’-CCGGAGATCTTCCAGATCT-3’; ACTB, 5’-GAGGCTGAGATGAGCTTGTC-3’ and 5’-CAATGCTATGGTACAGGATGCC-3’. PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

Northern blot analysis. Human multiple-tissue blots (BD Biosciences Clontech, Palo Alto, CA) were hybridized with 32P-labeled PCR product of CDCA1 or KNTC2. The cDNA probes of CDCA1 and KNTC2 were prepared by RT-PCR using the primers described above. Prehybridization, hybridization, and washing were done according to the supplier’s recommendations. The blots were autoradiographed at room temperature for 30 hours with intensifying BAS screens (Bio-Rad, Hercules, CA).

Antibodies. We purified rabbit polyclonal anti-CDCA1 antibody (originally generated to the peptides, DSPEKLKNYKEKMKDV and DSPEKLKNYKEKMKD) using standard protocols. This antibody was used for immunodetection of CDCA1 or KNTC2 protein. The immune complexes were stained with a horseradish peroxidase-linked antibody reaction. Then the cells were incubated with antibody to human CDCA1 or KNTC2 protein. The immune complexes were stained with a donkey anti-rabbit secondary antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR) and donkey anti-goat secondary antibody conjugated to Alexa 594 (Molecular Probes) and viewed with a laser confocal microscope (TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany). The number of cells was counted in five independent fields and normalized for 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 paraaffin blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue was punched from each case. Five-micrometer sections of the resulting microarray block were stained with hematoxylin. Tumor tissue microarrays were constructed as published previously, using formalin-fixed NSCLCs (25, 26). 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 paraaffin blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue was punched from each case. Five-micrometer sections of the resulting microarray block were stained with hematoxylin.

Immunohistochemistry and tissue microarray analysis. To investigate the significance of CDCA1/KNTC2 overexpression in clinical NSCLCs, we stained tissue sections using ENVISION+™ kit/horseradish peroxidase (HRP; DakoCytomation, Glostrup, Denmark). Affinity-purified anti-CDCA1 antibody or anti-KNTC2 antibody (Abcam) was added after blocking of endogenous peroxidase and proteins, and each section was incubated with HRP-labeled anti-rabbit or anti-goat 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 (25, 26). 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 paraaffin blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue was punched from each case. Five-micrometer sections of the resulting microarray block were stained with hematoxylin.

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 pathologic tumor-node-metastasis (TNM) stage, with the expression levels of CDCA1 or

Cancer Res 2006; 66: (21). November 1, 2006 10340 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2006 American Association for Cancer Research.
KNTC2 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 CDCA1 or KNTC2 expression; differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate analysis was done with the Cox proportional-hazard regression model to determine associations between clinicopathologic variables and cancer-related mortality.

RNAi assay. We had previously established a vector-based RNAi system, psh1IBX3.0, that was designed to synthesize small interfering RNAs (siRNAs) in mammalian cells (9, 11, 12, 14); 10 µg each of siRNA expression vector was transfected using 30 µL of LipofectAMINE 2000 (Invitrogen) into NSCLC cell lines, A549 and LC319. The transfected cells were cultured for 7 days in the presence of appropriate concentrations of genetin (G418); the number of colonies was counted by Giemsa staining; and viability of cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 7 days after the treatment. Briefly, cells with known-8 solution (DOITND, Kumamoto, Japan) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37°C for additional 4 hours. Absorbance was then measured at 490 nm, and at 630 nm as a reference, with a Microplate Reader 580 (Bio-Rad). To confirm suppression of CDCA1 or KNTC2 mRNA expression, semiquantitative RT-PCR experiments were carried out with the following synthesized CDCA1-specific primers and KNTC2-specific primers according to the standard protocol. The target sequences of the synthetic oligonucleotides for RNAi were as follows: control 1 (enhanced green fluorescent protein (EGFP) gene, a mutant of Aequorea victoria GFP), 5'-GAAAGACGACGACTCTTTCA-5'; control 2 (Scramble/SCR: chloroplast Euglena gracilis gene coding for 55 and 16s rRNAs), 5'-CGCGGCGTTGTTAGGATTG-3'; siRNA-CDCA1-1, 5'-AATGTCGACAGAAGTGGT-3'; siRNA-CDCA1-2, 5'-AAGATGCGTGGAAAGGAGA-5'; siRNA-KNTC2-1, 5'-GGTTGGAAGAGGTTCCA-3'; siRNA-KNTC2-2, 5'-GCTGGATGATCTTTACCAA-3'.

Synthesized dominant-negative peptide. Twenty- or 19-amino-acid sequence derived from minimized KNTC2-binding domain in CDCA1 (codons 368-416; see Fig. 3A) was covalently linked at its NH2 terminus to a membrane transducing 11 poly-arginine sequence (11R; ref. 27). Four dominant-negative peptides were synthesized covering the codons 368–416 region: 11R-CDCA1_368-387, RRRRRRRRRRR-GGG-QYKRT-VIEDCNKVQERGAV; 11R-CDCA1_387-397, RRRRRRRRRRR-GGG-NVKQER-GAVYERTVITQEG; 11R-CDCA1_388-407, RRRRRRRRRRR-GGG-YERTVITQIEIQKIKGLIQQ; 11R-CDCA1_398-416, RRRRRRRRRRR-GGG-IQKIRGILQIQKDAER. Scramble peptides derived from the most effective 11R-CDCA1_398-416, peptides were synthesized as a control: Scramble, RRRRRRRRRRR-GGG-IQKIREKDAAEQICLKGLP. Peptides were purified by preparative reverse-phase high-performance liquid chromatography and were >95% purity.

LC319, A549, and normal human lung fibroblasts-derived MRC5 cells line were incubated with the 11R peptides at different concentrations and the viability of cells was evaluated by MTT assay at 7 days after the treatment. Flow cytometric analysis was done as described previously (14).

Results

Coactivation of the CDCA1 and KNTC2. Using a cDNA microarray representing 27,648 genes, we identified CDCA1 to be highly transactivated in the major group of lung cancers. We subsequently confirmed its transactivation by semiquantitative RT-PCR experiments in 10 of 16 additional NSCLC cases (four of eight adenocarcinomas and six of eight SCCs; Fig. 1A, top). High level of CDCA1 expression was also observed in all of the 23 NSCLC and SCLC cell lines examined, whereas the transcript was hardly detectable in SAEC cells derived from normal bronchial epithelium (Fig. 1B, top). We further confirmed overexpression of CDCA1 protein on Western blots using anti-CDCA1 antibody in all of 11 lung cancer cell lines (Fig. 1C, top). Northern blot analysis using CDCA1 cDNA as a probe identified a 2.4-kb transcript exclusively and abundantly in testis among the 23 normal human tissues (Fig. 1D, top).

CDCA1 is known to be a member of a highly conserved Ndc80 complex that plays a significant role in spindle checkpoint signaling. To elucidate the function of CDCA1 activation in lung cancer cells, we first confirmed the expression status of other components of the Ndc80 complex using our gene expression database covering 101 lung cancers and 31 normal human organ tissues (27 adult and 4 fetal organs; refs. 5–8, 28, 29). This procedure suggested that the expression of KNTC2 was very likely to correlate with that of CDCA1 because KNTC2 was frequently overexpressed in lung cancers but not expressed in normal tissues except the testis. We subsequently reexamined primary NSCLC tissues and lung cancer cell lines by semiquantitative RT-PCR experiments and Western blot analysis and found increased KNTC2 expression in 11 of 16 NSCLC clinical samples as well as in all of lung cancer cell lines examined (Fig. 1A-C, middle). The expression pattern of the CDCA1 gene showed good concordance with that of KNTC2 gene. These two genes and their products were also coactivated in all lung cancer cell lines examined. Northern blotting using KNTC2 cDNA as a probe identified a 2.5-kb transcript, exclusively and abundantly in testis, indicating that both human CDCA1 and KNTC2 could be categorized to the cancer-testis antigen (Fig. 1D, bottom).

Cognate interaction between CDCA1 and KNTC2 and their localization in NSCLC cells. We examined by immunoprecipitation experiment the cognate interaction between endogenous human CDCA1 and KNTC2 using LC319 lung cancer cells, in which these two genes were expressed abundantly (Fig. 2A). We then investigated their subcellular localization in LC319 lung cancer cells synchronized with aphidicolin by immunocytochemical analysis using a rabbit polyclonal anti-CDCA1 and goat polyclonal anti-KNTC2 antibodies. Colocalization of the proteins was detected mainly in the centrosome in the cells at G1 and S phases and predominantly in the centromere at G2 and M phases (representative images are shown in Fig. 2B).

Association of overexpression of CDCA1 and KNTC2 with poor prognosis. Using tissue microarrays prepared from paraffin-embedded NSCLCs, we did immunohistochemical analysis with affinity-purified anti-CDCA1 and anti-KNTC2 polyclonal antibodies. We classified patterns of CDCA1/KNTC2 expression as absent/weak (scored as 0–1+) or strong (scored as 2+). Of the 282 NSCLC cases examined, 95 (33.7%) revealed strong CDCA1 staining (score 2+); 113 (40.1%) were stained weakly (score 1+); and no staining (score 0) was observed in 74 cases (26.2%). For KNTC2, strong staining (score 2+) was observed in 112 cases (39.7%), weak staining (score 1+) in 122 cases (43.3%), and no staining (score 0) in 48 cases (17%). All of these tumors were surgically resected NSCLC cases, and no staining was observed in any of their adjacent normal lung tissues (Fig. 2C). 189 of the 282 tumors were positive (scored as 1+–2+) for both CDCA1 and KNTC2, and 29 were negative for the both proteins. Nineteen of the 282 cases were positive for only CDCA1, and 45 were positive for only KNTC2. The fact that the expression pattern of CDCA1 protein was significantly concordant with KNTC2 protein expression in these tumors (P < 0.0001, χ2 test) further confirmed the results by RT-PCR and Western blotting, suggesting that there might be common transcriptional regulator(s) for CDCA1 and KNTC2. We found that strong expression of CDCA1 in NSCLCs
was significantly associated with tumor size (classified according to the postsurgical pathologic TNM stage: pT1 versus pT2-T4; \( \chi^2 = 5.473, P = 0.019 \)) and with tumor-specific 5-year survival after the curative resection of primary tumors ( \( P = 0.0233 \), log-rank test; Fig. 2D, left). Strong expression of KNTC2 in NSCLCs was significantly associated with tumor size (pT1 versus pT2-T4; \( \chi^2 = 11.664, P = 0.0006 \)) and 5-year survival ( \( P = 0.0384 \), log-rank test; Fig. 2D, right). NSCLC patients whose tumors expressed neither CDCA1 nor KNTC2 could reveal the longest survival period, whereas those with strong positive values for both markers suffered the shortest tumor-specific survival ( \( P = 0.0250 \), log-rank test; data not shown). Using univariate analysis, we found that node status (pN0 versus pN1,pN2: \( P < 0.0001 \), score test), tumor size (pT1 versus pT2-T4: \( P < 0.0001 \), score test), and high CDCA1/KNTC2 expression ( \( P = 0.0233 \) and 0.0384, respectively, score test) were important correlative features for shorter tumor-specific survival times of patients with NSCLC.

Inhibition of growth of NSCLC cells by specific siRNA against CDCA1 and KNTC2. To assess whether CDCA1 and KNTC2 are essential for growth or survival of lung cancer cells, we constructed plasmids to express siRNA against CDCA1 (si-CDCA1-1 and si-CDCA1-2) or KNTC2 (si-KNTC2-1 and si-KNTC2-2), using siRNAs for EGFP and SCR as controls. Transfection of either of the plasmids (si-CDCA1-2 or si-KNTC2-2) into A549 or LC319 cells significantly suppressed expression of endogenous CDCA1 or KNTC2 compared with the control siRNAs (EGFP and SCR), si-CDCA1-1, or si-KNTC2-1 and resulted in significant decreases in cell viability and colony numbers measured by MTT and colony formation assays ( \( P = 0.0008 \) and 0.0019, respectively, in MTT assays by unpaired t test; representative data of A549 cells was shown in Fig. 3A and B).

Identification of the KNTC2-binding region in CDCA1. We subsequently investigated the biological importance of the association of these two proteins and its potential as therapeutic targets for lung cancer. First, to determine the domain in CDCA1...
that is required for interaction with KNTC2, we transfected into LC319 cells one of five partial constructs of CDCA1 with FLAG sequence at its COOH-terminal (CDCA1149-464, CDCA1200-464, CDCA11-148, CDCA1149-306, and CDCA1306-464; Fig. 4A). Immunoprecipitation with monoclonal anti-Flag antibody indicated that CDCA1149-464, CDCA1200-464, and CDCA1306-464 were able to interact with KNTC2, but CDCA11-148 and CDCA1149-306 were unable (Fig. 4B, top). To further define the minimal and high-affinity KNTC2-binding domain in CDCA1, we transfected into LC319 cells one of additional four partial constructs of CDCA1 (CDCA1277-416, CDCA1277-367, CDCA1319-416, and CDCA1319-367; Fig. 4A) and found that CDCA1277-367 and CDCA1319-367 were unable to interact with KNTC2 (Fig. 4B, bottom). These experiments suggested that the COOH-terminal 49-amino-acid polypeptide (codons 368-416) in CDCA1 should play an important role in the interaction with KNTC2.

Growth inhibition of NSCLC cells by dominant-negative peptides of CDCA1. To investigate the functional significance of CDCA1-KNTC2 interaction for growth or survival of lung cancer cells, a partial segment of CDCA1 that lacked the NH2-terminal portion of CDCA1 but had shown strong binding-affinity to endogenous KNTC2 (CDCA1149-464; see Fig. 4B, top) was examined for a dominant-negative function of inhibiting the interaction between CDCA1 and KNTC2 in lung cancer cells. We transfected into LC319 cells the CDCA1149-464 or CDCA1149-306 construct, the latter of which was unable to interact with endogenous KNTC2 as control, and confirmed that transduction of CDCA1149-464 construct to LC319 cells reduced complex formation between endogenous CDCA1 and KNTC2 by an immunoprecipitation experiment using an antibody to the NH2-terminal portion of CDCA1 (Fig. 4C). Expectedly, transfection of the dominant-negative fragment of CDCA1149-464 resulted in significant decreases in cell viability as measured by MTT assay (P = 0.0026, unpaired t test; CDCA1149-464 versus control CDCA1149-306; Fig. 4D).

As shown in Fig. 4A, 49-amino-acid peptides of CDCA1368-416 were supposed to contain the region to interact with KNTC2. To develop the bioactive cell-permeable peptides that can inhibit the functional association of CDCA1 with KNTC2, we synthesized four different kinds of 19-amino-acid polypeptides covering the KNTC2-binding domain in CDCA1368-416 that was added a membrane-permeable 11 residues of arginine (11R) at its NH2 terminus. To test the effect of these polyarginine-linked peptides on lung cancer cell growth/survival, we treated LC319 and A549 cells with each of the four peptides. Addition of the 11R-CDCA1398-416 into the culture media inhibited the complex formation between endogenous CDCA1 and KNTC2 (Fig. 5A), and resulted in significant decreases in cell viability, as measured by MTT assay (representative data of LC319 cells was shown in Fig. 5B; P = 0.001 for 10 and 0.0004 for 20 μmol/L peptide treatment by unpaired t test). Seventy-two hours after the 11R-CDCA1398-416 treatment, several cells had progressed through the cell cycle and blocked at mitosis, exhibiting a rounded cellular morphology that was very similar to the effect of either CDCA1 or KNTC2 suppression with siRNAs (data not shown). In contrast, there

---

**Figure 2.** Interaction of CDCA1 with KNTC2 in NSCLC cells and association of CDCA1 and KNTC2 overexpression with poor prognosis of NSCLC patients. A, immunoprecipitation of endogenous CDCA1 and KNTC2 from extracts of lung cancer cell line LC319. IP, immunoprecipition; IB, immunoblot. B, colocalization of endogenous CDCA1 (green) and endogenous KNTC2 (red) in LC319 cells. White arrow indicates centrosome (left). C, immunohistochemical evaluation of representative samples from surgically resected SCC tissues, using anti-CDCA1 (top) and anti-KNTC2 (bottom) polyclonal antibodies on tissue microarrays (×100). D, Kaplan-Meier analysis of tumor-specific survival times according to CDCA1 expression (left) and KNTC2 expression (right) on tissue microarrays.
was no difference in cellular morphology between the nontreated cells and those treated with noneffective peptides, both of which exhibited normal distribution of interphase cells in spread shape and mitotic cells in rounded shape. To clarify the mechanism of tumor suppression by 11R-CDCA1398-416 peptides, we did flow cytometric analysis of the tumor cells treated with these peptides and found that the cells caused the G2-M arrest and sub-G1 fraction at 72 hours after the treatment was significantly increased (Fig. 5C). 11R-CDCA1 revealed no effect on cell viability of normal human lung fibroblast derived MRC5 cells that expressed a hardly detectable level of CDCA1 and KNTC2 (Fig. 5D). These data suggest that transducible 11R-CDCA1398-416 peptides could inhibit a functional complex formation of CDCA1 and KNTC2 and have no toxic effect on normal human cells that do not express these proteins.

Discussion

A number of molecular targets for lung cancer therapy have been reported. However, suppression of some of such molecules also caused serious adverse reactions in vivo. Therefore, we have established an effective screening system to identify therapeutic targets and their functionally relevant partners toward the goal of developing small molecular compounds that have more specific and efficient anticancer effect with minimum risk of adverse effects than current therapies. The strategy was as follows: (a) to identify up-regulated genes in lung cancer by genome-wide screening using the cDNA microarray system (5–8); (b) to verify the candidate genes for its no or very low level of expression in normal tissues by Northern blot analysis (28, 29); (c) to validate clinicopathologic significance of its overexpression by means of tissue microarray containing hundreds of archived lung cancer samples (10–14); and (d) to verify whether the target gene is essential for cell growth or survival of cancer cells by RNAi assay (9, 11, 12, 14). Through this systematic approach, we identified that CDCA1 and KNTC2 are co-overexpressed in the great majority of clinical lung cancer samples as well as cancer cell lines, and that a complex of the two proteins is indispensable for growth and progression of lung cancer cells. CDCA1 and KNTC2 were indicated to be involved in the regulation of mitosis. A large proportion of the proteins that

![Figure 3. Growth suppression of NSCLC cells by siRNAs against CDCA1 and KNTC2. A and B, top left, knock-down effect in response to si-CDCA1-1, si-CDCA1-2, si-KNTC2-1, si-KNTC2-2, or control siRNAs (EGFP or SCR) in A549 cells, analyzed by semiquantitative RT-PCR. Bottom left and right, results of colony formation and MTT assays of A549 cells transfected with specific siRNAs or control plasmids. Bars, SD of triplicate assays.](image-url)
regulate mitosis are aberrantly expressed in human tumor cells when compared with their normal counterparts, and some of them are known to function as oncogenes (30). A subset of them has also been expected to represent a possible source of target molecules for development of novel anticancer agents. For example, highly conserved aurora kinases represent one of such families that are critical as mitotic regulators (31). Indeed, several Aurora kinase inhibitors, including ZM447439, Hesperadin, and VX-680, have recently been described as anticancer drugs (31, 32). In this study, we found by tissue microarray analysis that NSCLC patients

Figure 4. Identification of the KNTC2-binding region in CDCA1 and inhibition of growth of NSCLC cells by dominant-negative fragment of CDCA1. A, schematic drawing of nine COOH-terminal FLAG-tagged CDCA1 deletion mutants lacking either or both of the terminal regions. Molecular weights of each deletion mutants are described in the parenthesis. Top, relative polypeptide size (1-200) of the constructs (amino acids). B, identification of the region in CDCA1 that binds to KNTC2 by immunoprecipitation experiments. The CDCA11-148 and CDCA1149-464 constructs, which lacked COOH-terminal 158-amino-acid polypeptides in CDCA1, did not retain any ability to interact with endogenous KNTC2 in LC319 cells (top). The CDCA1277-367 and CDCA1319-367 constructs, which lost 49-amino-acid polypeptides corresponding to CDCA1368-416, were unable to interact with endogenous KNTC2, suggesting that the 49-amino-acid segment (codons 368-416) was supposed to be important to interact with endogenous KNTC2 (bottom). C, reduction of the complex formation detected by immunoprecipitation between endogenous CDCA1 and KNTC2 proteins in LC319 cells that were transfected with the CDCA1149-464 construct (top; black arrow). Input fractions (bottom). D, MTT assay of LC319 cells indicates a dominant-negative effect of CDCA1149-464. CDCA1149-306 was served as a control. Bars, SD of triplicate assays.
showing abundant expression of CDCA1/KNTC2 revealed a shorter tumor-specific survival period, thus suggesting that CDCA1, along with KNTC2, plays important roles in progression of lung cancers.

Novel therapeutic targets are expected to have a powerful biological activity against cancer with minimal adverse events. We found that a human homologue of a highly conserved cell cycle regulator complex, CDCA1-KNTC2, could belong to cancer-testis antigens that are coactivated in the great majority of lung cancers. Furthermore, we showed for the first time that growth of lung cancer cells overexpressing CDCA1-KNTC2 complex was suppressed effectively by blocking the interaction by the dominant-negative form of the CDCA1 segment as well as a cell-permeable peptide.

Figure 5. Inhibition of growth of NSCLC cells by dominant-negative peptides of CDCA1. A, reduction of the complex formation detected by immunoprecipitation between endogenous CDCA1 and KNTC2 proteins in LC319 cells that were treated with the 11R-CDCA1398-416 peptides (top; black arrow). Input fractions (bottom).

B, MTT assay shows growth suppressive effect of 11R-CDCA1398-416 peptides that were introduced into CDCA1-KNTC2 overexpressing LC319 cells. Bars, SD of triplicate assays.

C, cell cycle analysis of LC319 cells after treatment with 11R-CDCA1398-416 peptides or Scramble peptides.

D, expressions of CDCA1 and KNTC2 proteins in normal human lung fibroblast-derived MRC5 cells compared with three lung cancer cell lines, examined by Western blot analysis (left). MTT assay shows no off-target effect of the 11R-CDCA1398-416 peptides on MRC5 cells that scarcely expressed CDCA1 and KNTC2 protein (right).
polypeptide that corresponded to a part of the specific binding region to KNTC2. We detected a distinct G2-M arrest at 24 hours and subsequent increase in sub-G1 fraction at 120 hours after the treatment (representative data at 72 hours are shown in Fig. 5C), suggesting that the cell-permeable polypeptide induced mitotic arrest and subsequent apoptosis of the cancer cells. To date, several apoptotic pathways through the inhibition of proper mitosis were partly reported. Apoptosis induced by kinesin inhibitor or taxanes is mediated by caspase-dependent pathway (33), whereas sulfono-namides induce apoptosis through the Bcl-2-dependent pathway (34). However, the detailed mechanism of apoptosis induced by the spindle checkpoint system remains unclear (35). Further analyses of the mechanism of growth suppression by inhibiting CDCA1 and KNTC2 binding may be of the great benefit towards the development of novel type of anticancer drugs.

Recently, several groups reported that inhibition of the protein-protein interaction could effectively block the function of the complex in vivo and in vitro. For example, cell-permeable peptide derived from the AMP1 specifically blocked AMP1/cortactin binding and effectively inhibited breast cancer invasion and metastasis (36). The covalent linkage of a short cell-permeable peptide to a 20-amino-acid sequence derived from the c-Jun NH2-terminal kinase (JNK)–binding domain of JNK-interacting protein-1 has lead to improvement of insulin resistance and ameliorated glucose tolerance in diabetic mice (37). Blocking nuclear factor-κB protects β cells from interleukin-1β–induced apoptosis (38). Similar approaches have also been taken successfully to block activating protein 1, a nuclear factor of activated T cells, and signal transducer and activator of transcription 1 nuclear import (39). These preclinal evidences suggest a possible approach for the cancer therapy by the use of small peptides or compounds to interfere the functional association of some protein complexes. Selective killing of tumor cells with no or minimum toxic effect to normal cells is the most desirable treatment to cancer patients. Chen et al. reported that cell membrane–permeable peptides containing the motif inhibiting the phosphorylation of substrates by cyclin A (CCNA)/cyclin-dependent kinase 2 (CDK2) or cyclin E (CCNE)/CDK2–inhibited cancer cells to undergo apoptosis at a relative higher level than nontransformed cells (40). The anti-MDM2 peptide blocking p53-MDM2 interaction was also reported to induce rapid accumulation of p53, activation of apoptosis-inducing genes, preferential killing of retinoblastoma cells, and minimal retinal damage after intravitreal injection (41). Our results using cell permeable peptides specifically targeting cancer cells suggest that inhibition of CDCA1-KNTC2 complex provide a rationale for development of novel antagonists as antineoplastic agents.

In summary, we have found that human CDCA1-KNTC2 cancer-testis antigen complex has an important functional role in growth and/or survival of lung cancer cells. The data strongly imply the possibility for designing new anticancer peptides as well as small compounds to specifically target the activity of CDCA1-KNTC2 as a promising therapeutic strategy for treatment of lung cancer patients.

Acknowledgments

Received 6/12/2006; revised 8/14/2006; accepted 8/17/2006.

Grant support: Japan Society for the Promotion of Science Research for the Future Program grant #00L01402 (Y. Nakamura).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

33. Weaver BA, Cleveland DW. Decoding the links between mitosis, cancer, and chemotherapy: the mitotic checkpoint, adaptation, and cell death. Cancer Cell 2005;8:7–12.
Activation of CDCA1-KNTC2, Members of Centromere Protein Complex, Involved in Pulmonary Carcinogenesis

Satoshi Hayama, Yataro Daigo, Tatsuya Kato, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/21/10339

Cited articles
This article cites 39 articles, 20 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/21/10339.full.html#ref-list-1

Citing articles
This article has been cited by 28 HighWire-hosted articles. Access the articles at:
/content/66/21/10339.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.