Oncogenic Role of KIAA0101 Interacting with Proliferating Cell Nuclear Antigen in Pancreatic Cancer

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Abstract

To isolate novel diagnostic markers and therapeutic targets for pancreatic cancer, we earlier did expression profile analysis of pancreatic cancer cells using a genome-wide cDNA microarray combined with microdissection. Among dozens of trans-activated genes in pancreatic cancer cells, this study focused on KIAA0101 whose overexpression in pancreatic cancer cells was validated by immunohistochemical analysis. KIAA0101 was previously identified as p15PAF [proliferating cell nuclear antigen (PCNA)–associated factor] to bind with PCNA; however, its function remains unknown. To investigate for the biological significance of KIAA0101 overexpression in cancer cells, we knocked down KIAA0101 by small interfering RNA (siRNA) in pancreatic cancer cells and found that the reduced expression by siRNA caused drastic attenuation of their proliferation as well as significant decrease in DNA replication rate. Concordantly, exogenous overexpression of KIAA0101 enhanced cancer cell growth, and NIH3T3 derivative cells expressing KIAA0101 revealed in vivo tumor formation, implying its growth-promoting and oncogenic property. We also showed that the expression of KIAA0101 was regulated tightly by the p53-p21 pathway. To consider the KIAA0101/PCNA interaction as a therapeutic target, we designed the cell-permeable 20-amino-acid dominant-negative peptide and found that it could effectively inhibit the KIAA0101/PCNA interaction and resulted in the significant growth suppression of cancer cells. Our results clearly implicated that suppression of the KIAA0101 and PCNA oncogenic activity, or the inhibition of KIAA0101/PCNA interaction, is likely to be a promising strategy to develop novel cancer therapeutic drugs. [Cancer Res 2007;67(6):2568–76]

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the western world and shows one of the worst mortality rates among the common malignancies, with a 5-year survival rate of only 5% (1, 2). Approximately 33,730 new cases are expected to be diagnosed with pancreatic cancer in 2006, and nearly 32,300 die of the disease in the United States (3). The great majority of pancreatic cancer patients are diagnosed at an advanced stage where no effective therapy is available. Surgical resection is the only way to offer a possibility for cure at present, but 80% to 90% of patients who undergo curative surgery suffer from relapse and die due to the metastatic or disseminated disease (1, 2). Some approaches in a combination of surgery with chemotherapy based on gemcitabine or 5-fluorouracil, with or without radiation therapy, can improve the quality of life of patients (1, 2). However, such treatments have a very limited effect on the improvement of their long-term survival because pancreatic cancers are biologically extremely aggressive and usually chemoresistant and radiation resistant. Hence, development of novel molecular therapies for pancreatic cancer through identification of molecular targets is urgently required now.

Earlier, we did detailed and accurate expression profile analysis of pancreatic cancer cells using a genome-wide cDNA microarray consisting of ~30,000 genes, in combination with laser microbeam microdissection to purify pancreatic cancer cell population (4). Among the genes we identified as being trans-activated in pancreatic cancer cells, we here focused on KIAA0101 as a novel molecular target for cancer therapy. KIAA0101 was previously identified as p15PAF [proliferating cell nuclear antigen (PCNA)–associated factor] to bind with PCNA by yeast two-hybrid screening (5). PCNA is an essential scaffold molecule for DNA replication/repair through interacting with several DNA replication proteins (6–8). The crystal structure showed that PCNA is a ring-shaped homotrimeric protein and functions as a clamping platform necessary to recruit to DNA proteins involved in DNA synthesis or metabolism, such as DNA polymerases, endonuclease, and DNA ligase (9, 10). It has been established that high expression of PCNA is a hallmark of cell proliferation, and in the clinic, PCNA serves as a general proliferative marker, especially in the prognosis of tumor development as well as Ki67/MIB-1 (11). KIAA0101/p15PAF protein is shown to interact with PCNA; however, its function in cell proliferation or cancer progression is still largely unknown (5, 12).

In this study, we showed that overexpression of KIAA0101 revealed oncogenic activity, probably through its interaction with PCNA. Suppressing KIAA0101 expression in pancreatic cancer cells by small interfering RNA (siRNA) and inhibiting its interaction with PCNA by cell-permeable dominant-negative peptides resulted in growth suppression of cancer cells, implicating that KIAA0101 could be a promising target for development of novel anticancer therapies.

Materials and Methods

Cell lines. Pancreatic cancer cell lines MIA-PaCa2 and Panc-1 and mouse fibroblast cell-line NIH3T3 were purchased from the American Type Culture Collection (Rockville, MD). Other pancreatic cancer cell lines, such as PK-59,
KLM-1, PK-1, and PK-1, were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Colon cancer cell lines HCT116 p53 wild-type (p53+/−) and its derivative (p53−/−) and HCT116 p21 wild-type (p21+/−) and its derivative (p21−/−) were gifts from Dr. B. Vogelstein (Johns Hopkins University; ref. 13). All cells were cultured under conditions recommended by their respective depositors.

**Semiquantitative reverse transcription-PCR.** Microdissection of pancreatic cancer cells and normal pancreatic ductal cells were described previously (4). RNA from the pancreatic cancer cells and normal pancreatic ductal cells were subject to two-round of T7-based RNA amplification (Epizence Technologies, Madison, WI) and subsequent synthesis of single-strand cDNA. Total RNAs from human pancreatic cancer cell lines were extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendation. Extracted RNAs were treated with DNase I (Roche, Mannheim, Germany) and reversely transcribed to single-stranded cDNAs using oligo (dT)12-18 primer with Superscript II reverse transcriptase (Invitrogen). The primer sequences were 5′-AGCTTTGTGAAAGCTTATT-3′ and 5′-GGCCAGCAATCAACAATAGC-3′ for KIAA0101 (NM_017436), 5′-ACCACCCATGAAAAGAGA-3′ and 5′-TACCTGGGACAAGATGTCG-3′ for β2MG, and 5′-AAGGATTATGAGGAGGTTGGTGT-3′ and 5′-CTTGGCTG-TGAACAAAAGGATC-3′ for x-tubulin (TUBA). Each PCR regimen involved a 55°C, 5 min initial denaturation step followed by 23 cycles (for TUBA and β2MG) and 28 cycles (for KIAA0101) at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, on a Gene Amp PCR system 9600 (PE Applied Biosys, Foster City, CA).

**Northern blot analysis.** We extracted total RNAs from several pancreatic cancer cell lines using TRizol reagent (Invitrogen) and did Northern blot analysis. After treatment with DNase I (Nippon Gene, Osaka, Japan), mRNA was purified with Micro-FastTrack (Invitrogen), according to the manufacturer’s protocols. A 1-µg aliquot of each mRNA from pancreatic cancer cell lines, as well as those isolated from normal human adult heart, lung, liver, kidney, brain, and pancreas (BD Biosciences, Palo Alto, CA), were separated on 1% denaturing agarose gels and transferred onto nylon membranes. The 702-bp probe specific to KIAA0101 was prepared by PCR using the following primer set: forward, 5′-TCCTGCTCTCCTCTCTC-CTTCT-3′ and reverse, 5′-GGCCAGCATCACAACATACATG-3′. Hybridization with a random-primer, α32P-dCTP–labeled probe was carried out according to the instructions for Megaprime DNA labeling system (Amersham Biosciences, Buchinghamshire, United Kingdom). Prehybridization, hybridization, and washing were done according to the supplier’s recommendations. The blots were autoradiographed with intensifying screens at −80°C for 3 days.

**Recombinant protein and antibody.** The cDNA fragment encoding full-length KIAA0101 (111 amino acids, NP_055551) was generated using KOD-Plus polymerase (Toyobo, Osaka, Japan) and cloned into pET28 vector (Novagen, Darmstadt, Germany). The recombinant KIAA0101 protein fused with polyhistidine tag at COOH terminus was expressed in Escherichia coli, BL21 codon plus (Stratagene, La Jolla, CA), and purified with TALON Superflow Metal Affinity Resin (BD Biosciences) under native condition according to the supplier’s protocol. Further purification was done by use of high-performance liquid chromatography AKTA explorer (Amersham Biosciences) equipped with MonoS HR 5/5 (Amersham Biosciences). The purified recombinant KIAA0101 protein was immunized into rabbits, and the immune sera were purified on affinity-columns packed with Affi-Gel 10 activated affinity media (Bio-Rad Laboratories, Hercules, CA) conjugating recombinant KIAA0101 protein with accordance of basic methodology. The affinity-purified anti-KIAA0101 polyclonal antibody was used for detection of KIAA0101 protein.

**Immunohistochemical staining.** Conventional sections from pancreatic cancer tissues were obtained from surgical specimens that were resected in Osaka Medical Center for Cancer and Cardiovascular Diseases under the appropriate informed consent. Sections from normal pancreas were purchased from Biochain (Hayward, CA). The sections were deparaffinized and autoclaved at 108°C in DakoCytomation Target Retrieval Solution High pH (Dako, Carpinteria, CA) for 15 min. After blocking of endogenous peroxidase and proteins, the sections were incubated with anti-KIAA0101 antibody (diluted by 1:200) at room temperature for 30 min. After washing with PBS, immunodetection was done with peroxidase-labeled anti-rabbit immunoglobulin (Envision kit, DAKO). Finally, the reagents were developed with 3,3′-diaminobenzidine (DAKO). Counterstaining was done using hematoxylin.

**siRNA-expressing constructs and growth assay.** To knock down endogenous KIAA0101 expression in pancreatic cancer cells, we used psiU6B3X0 vector for expression of short hairpin RNA against a target gene as described previously (14). The target sequences of the synthetic oligonucleotides for siRNA against KIAA0101 were as follows: #38si, 5′-CGTCTCTCTTTTCTCTCTGG-3′; #759si, 5′-GCCATATTGTCCTCCTCTA-3′; and EGFPsi, 5′-GAAGCAGACACGCTCTCTC-3′ as a negative control. Pancreatic cancer cell lines KLM-1 and MIA-PaCa2, which highly expressed KIAA0101, were plated onto 10-cm plates and transfected with 8 µg plasmid designed to express siRNA to KIAA0101 using FuGENE6 (Roche) according to manufacturer’s instruction. Cells were selected by 0.5 mg/mL (for KLM-1) or 0.8 mg/mL (for MIA-PaCa2) of Geneticin (Sigma-Aldrich, St. Louis, MO) for 5 days and then harvested to analyze knockdown effect on KIAA0101 expression by reverse transcription-PCR (RT-PCR) using the primers described above. For colony formation assay, transfected expressing siRNAs were grown for 14 days in media containing Geneticin. After fixation with 4% paraformaldehyde, transfected cells were stained with Giemsa solution to assess colony formation. Cell viability was quantified using Cell counting kit-8 (DOJINDO, Kumamoto, Japan). After 14 days of culture in the Geneticin-containing medium, the solution was added at a final concentration of 10%. Following incubation at 37°C for 2 h, absorbance at 450 nm was measured with a Microplate Reader 550 (Bio-Rad).

**Establishment of stably KIAA0101-expressing cells and their growth in vitro and in vivo.** KIAA0101 cDNA was prepared by PCR amplification using the forward primer that included the Kozak sequence and NorI linker and the reverse primer including a NorI linker. The PCR product was inserted into the NorI site of the mammalian expression vector pcAGGS/HA for expressing a hemagglutinin (HA)-tagged protein. The pcAGGS-KIAA0101/HA or empty pcAGGS/HA mock vector was transfected into PK45P, which showed low expression of KIAA0101, and NIH3T3 cells, which exhibited hardly detectable expression of mouse KIAA0101 homologue (NP_080791), by FuGENE6 (Roche), according to the manufacturer’s protocol. Then, the Geneticin-resistant clones were selected in the culture medium containing 0.5 mg/mL Geneticin for PK45P and 0.9 mg/mL Geneticin for NIH3T3. The exogenous KIAA0101 expression in each clone was confirmed by Western blot analysis using anti-HA tag antibody (Sigma-Aldrich), and anti-β-actin antibody (Sigma-Aldrich) as a loading control. For growth assays, 7,500 cells of each of KIAA0101-HA or empty pcAGGS/HA mock vector was transfected into PK45P, which showed low expression of KIAA0101, and NIH3T3 cells, which overexpressed KIAA0101, were lysed in hypolysis buffer [10 mmol/L Tris-HCl (pH 7.6), 2.5 mmol/L MgCl2, 0.5% NP-40, 1 mmol/L DTT, 0.2 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA)]. Equal amounts of total proteins were incubated at 4°C for 1 h with 2 µg of anti-KIAA0101 polyclonal antibody or a rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were incubated with protein G Sepharose (Zymed Laboratories, South San Francisco, CA) for 1 h and washed with lysis buffer. Coprecipitated proteins were separated in 24-well culture dish and incubated in the medium containing 10% fetal bovine serum. Cell viability was quanified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The experiment was repeated at least thrice. For in vivo transformation, 5 × 103 cells of three stable clones NIH3T3-KIAA0101 and three NIH3T3-Mock clones were inoculated in the right and left flank of 8-week-old nude mice, respectively, and the tumors were harvested after 4 weeks. Each of the tumors was weighed and immunostained by anti-KIAA0101 antibody.

**Immunoprecipitation and mass spectrometric analysis for KIAA0101-associated complexes.** To isolate proteins that associated with KIAA0101 protein, we did immunoprecipitation experiments using the anti-KIAA0101 antibody. Pancreatic cancer cell lines KLM-1 and PK-9, which overexpressed KIAA0101, were lysed in hypolysis buffer [10 mmol/L Tris-HCl (pH 7.6), 2.5 mmol/L MgCl2, 0.5% NP-40, 1 mmol/L DTT, 0.2 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA)]. Equal amounts of total proteins were incubated at 4°C for 1 h with 2 µg of anti-KIAA0101 polyclonal antibody or a rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were incubated with protein G Sepharose (Zymed Laboratories, South San Francisco, CA) for 1 h and washed with lysis buffer. Coprecipitated proteins were separated in 5% to 20% gradient SDS-PAGE and stained by silver-staining kits (Invitrogen). Bands that differentiated proteins precipitated with anti-KIAA0101 polyclonal antibody from those
precipitated with control rabbit IgG were excised, digested in-gel with trypsin, and analyzed for peptide-mass fingerprints using an AXIMA-CFR MALDI-TOF mass spectrometer (Shimadzu Corp., Tsukuba, Japan). Peptide masses were searched with 10-ppm mass accuracy, and protein database searches were done using the database-fitting program IntelliMarque (Shimadzu). The protein binding identified by this strategy was validated by immunoprecipitation using anti-KIAA0101, anti-PCNA antibody (PC-10, Santa Cruz Biotechnology), anti-POLD1 antibody (A-9, Santa Cruz Biotechnology), and anti-FEN1 antibody (B-4, Santa Cruz Biotechnology).

**Immunocytochemistry.** KLM-1 cells grown on cover glasses were washed with PBS and treated for 10 min on ice with cold 0.1% Triton X-100 buffer containing 0.1% Triton X-100, 20 mmol/L HEPES-KOH (pH 7.4), 50 mmol/L NaCl, 3 mmol/L MgCl2, and 0.3 mol/L sucrose. Treated cells were washed PBS and fixed in 4% paraformaldehyde for 20 min on ice followed by quenching at room temperature for 30 min with 0.1 mol/L glycine in PBS. Cells were permeabilized with 0.5% Triton X-100 buffer containing 0.5% Triton X-100, 20 mmol/L HEPES-KOH (pH 7.4), 50 mmol/L NaCl, 3 mmol/L MgCl2, and 0.3 mol/L sucrose; covered with blocking solution (3% bovine serum albumin/PBS) for 60 min at room temperature; and finally incubated for 60 min at room temperature with rabbit anti-KIAA0101 polyclonal antibody and anti-PCNA mouse antibody (PC-10, Santa Cruz Biotechnology) in blocking solution. After washing with PBS, the cells were stained by Alexa 594–conjugated anti-rabbit IgG and Alexa 488–conjugated anti-mouse IgG secondary antibodies (Molecular Probes, Eugene, OR). Stained preparations were mounted with VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole and visualized with Spectral Confocal Scanning Systems (Leica, Bensheim, Germany).

**Bromodeoxyuridine incorporation assay using KIAA0101-siRNA inducible expression vector system.** To examine the effect of downregulation of endogenous KIAA0101 expression in pancreatic cancer cells, we applied a doxycycline-inducible siRNA expression system (14, 15). A pTER vector was kindly provided by Dr. van de Wetering (Hubrecht Laboratory, Center for Biomedical Genetics, Utrecht, Netherlands; ref. 15). This vector is designed to contain a doxycycline-regulated form of the RNA polymerase III H1 promoter and allow inducible knockdown of gene expression by short hairpin RNAs. To construct the pTER-siKIAA0101 vector, we phosphorylated the oligonucleotide sequences of KIAA0101-si#759 described above and the negative control (EGFPs), by incubating the oligonucleotides with T4-polynucleotide kinase (Toyobo) before ligation into the pTER vector. A tetracycline-repressor expression construct, pCDNA6/TR (Invitrogen) was used to generate clones expressing the tetracycline repressor (15). The pCDNA6/TR vector was transfected into KLM-1 cells, using Bacterialin (Sigma-Aldrich) to select stable transfectants. Clones that stably expressed the tetracycline repressor were subsequently transfected with pTER-KIAA0101-si#759 or pTER-siEGFP and selected using Zeocin (Invitrogen). In this way, we obtained KLM-1 derivative cells containing pTER-KIAA0101-si#759 (si#759-1 and si#759-2) and pTER-siEGFP (si#EGFP). After inducing the KIAA0101-specific siRNA by treatment with doxycycline (1.0 μg/mL; Sigma-Aldrich) for 48 h, we analyzed selected clones by Western blotting for their knockdown effect on endogenous KIAA0101, using anti-KIAA0101 antibody. We seeded cells in 96-well plates and, 24 h later, changed media with or without 1.0 μg/mL doxycycline. After a 48-h incubation, cell number was counted by MTT assay, and DNA replication rate was evaluated by bromodeoxyuridine (BrdUrd) incorporation assay using Cell Proliferation ELISA Biotrak System, version 2 (Amersham Biosciences) according to the manufacturer’s recommended procedures. To evaluate ability of DNA replication, relative ratio of BrdUrd absorbance/MTT absorbance in each clone with doxycycline treatment was calculated by comparing with those without doxycycline treatment as a control.

**Induction of p53/p21 and KIAA0101 expression.** To examine the expression of KIAA0101 in response to p53/p21 induction, HCT116 p53+/+ and p53−/− cells, or HCT116 p21+/+ and p21−/− cells, were seeded 1 × 10⁶ per 10-cm dish, treated with 0.5 μg/mL Adriamycin for 2 h, or γ-irradiated at 14 Gy using a 60Co source. After 0 to 72 h, cells were harvested. The expression of endogenous KIAA0101, p53, and p21 in each cell lysates were confirmed by RT-PCR and Western blot analysis using anti-KIAA0101 antibody, anti-p53 antibody (DO-1, Santa Cruz Biotechnology), and anti-p21 antibody. We used cells in 96-well plates and, 24 h later, changed media with or without 1.0 μg/mL doxycycline. After a 48-h incubation, cell number was counted by MTT assay, and DNA replication rate was evaluated by bromodeoxyuridine (BrdUrd) incorporation assay using Cell Proliferation ELISA Biotrak System, version 2 (Amersham Biosciences) according to the manufacturer’s recommended procedures. To evaluate ability of DNA replication, relative ratio of BrdUrd absorbance/MTT absorbance in each clone with doxycycline treatment was calculated by comparing with those without doxycycline treatment as a control.
Cell-permeable peptide treatment and KIAA0101-PCNA interaction. Several PCNA-associated proteins can bind to PCNA through their conserved PCNA-binding motif (PIP box, QxxL/Mxx(F/Y)[F/Y]; refs. 8–10), and KIAA0101 also has this PIP motif in its middle portion (IQKGGEFF). To inhibit the interaction between KIAA0101 and PCNA in the dominant-negative manner, we designed the PIP box motif peptide of KIAA0101 conjugating with cell-permeable arginine (R)-repeat peptide (16). PIP29 was RRRRRRRRRRRGGG-VRTPPKWQKGGEFFRLSPK (PIP box motif is underlined, and the conserved residues are shown in bold). PIP29mt replaced the conserved residues in the PIP box motif with alanine (bold): RRRRRRRRRRRGGG-IFQWPRGETPKWRPLPSKG. They were synthesized by Sigma-Aldrich and purified by high-performance liquid chromatography to >95% grade. Cancer cell line KLM-1, in which high expression of KIAA0101 was detected, and mouse cell line NIH3T3, in which no expression of KIAA0101 homologue (Kiaa0101, NP_080791) was observed, were treated with serial concentration (5, 10, and 20 μmol/L) of each of these cell-permeable peptides. At days 1 and 3, the cells were exposed with each peptide, and at day 5, viable cell numbers were evaluated by MTT assay as described above.

Results

Overexpression of KIAA0101 in pancreatic cancer cells. Our microarray data had shown KIAA0101 overexpression (>10-fold than normal ductal cells) in all of the 14 informative pancreatic cancers, and subsequent semiquantitative RT-PCR confirmed KIAA0101 overexpression in eight of the nine pancreatic cancer cases examined (Fig. 1A). Northern blot analysis using a KIAA0101 cDNA fragment as a probe identified a transcript of about 1.2 kb that was also highly expressed in all pancreatic cancer cell lines we examined; no expression was observed in any vital organs, including lung, heart, liver, and kidney (Fig. 1B). Immunohistochemical analysis using a polyclonal antibody specific to KIAA0101 also showed strong signals in the nuclei of pancreatic cancer cells (Fig. 1C). Among the 17 pancreatic cancers examined by the immunohistochemical analysis, 12 showed positive staining in their nuclei. On the other hand, no staining was observed in normal pancreatic ductal or acinar cells (Fig. 1C), or in any of the vital normal organs, including lung, heart, liver, and kidney (data not shown).

Effect of KIAA0101 knock down by siRNAs on growth of pancreatic cancer cells. To investigate the biological significance of KIAA0101 overexpression in cancer cells, we constructed several siRNA expression vectors specific to KIAA0101 and transfected each of them into KLM-1 and MIA-PaCa2, in which KIAA0101 mRNA was endogenously expressed abundantly. A significant knockdown effect was observed by semiquantitative RT-PCR when #759si construct was transfected into KLM-1 (Fig. 2A). Colony formation assays and MTT assays (Fig. 2B and C) using KLM-1 revealed a drastic reduction in the number of cells transfected with #759si, compared those with #38si or EGFPsi, which revealed no knockdown effect. Similar effects were observed in MIA-PaCa2 cell line (data not shown), indicating that KIAA0101 is likely to play important roles of cancer cell viability.

Exogenous overexpression of KIAA0101 promoted cancer cell growth and transformed NIH3T3. To further explore the potential oncopgenic property of KIAA0101, we established six PK-45P derivative clones (PK45P-KIAA0101-1-6), in which exogenous KIAA0101 was expressed constitutively. We also established control PK-45P cells transfected with the mock vector (PK45P-Mock) and compared their growth rates. Western blot analysis in Fig. 3A showed expression levels of exogenous KIAA0101 in these six clones. The growth curve measured by MTT assay revealed that all of the six PK45P-KIAA0101 clones (Fig. 3B, red lines) grew significantly more rapidly than the four PK45P-Mock clones (Fig. 3B, blue lines). Because mouse fibroblast NIH3T3 cells did not express a homologue of human KIAA0101, we introduced KIAA0101 expression vector in NIH3T3 cells and investigated whether KIAA0101 could transform normal cells to tumor cells in vivo. As shown in Fig. 3C, three NIH3T3-KIAA0101 derivative clones formed tumors at the right flank of nude mice, whereas NIH3T3-Mock cells that were...
inoculated at the left flank of the same mice did not form tumors. The subsequent immunohistochemical staining of the tumors in these mice validated a high level of exogenous KIAA0101 expression in the nuclei of tumor cells (Fig. 3D). These results implicated that KIAA0101 had an oncogenic property that was able to transform normal cells to tumor cells.

**Involvement of KIAA0101 with DNA replication.** KIAA0101 can interact with PCNA (5), which is an essential molecule for DNA replication/repair through interacting with several DNA replication proteins. Our mass spectrometric analysis following immunoprecipitation by anti-KIAA0101 antibody using cell lysates from pancreatic cancer cell lines KLM-1 and PK59 identified polymerase δ p125 subunit (POLD1) and flap endonuclease-1 (FEN1) to be interacting partners with KIAA0101 as well as PCNA. These interactions were confirmed by immunoprecipitation experiment using specific antibody to each protein (KIAA0101, PCNA, POLD, DNA replication/repair through interacting with several DNA replication proteins. Our mass spectrometric analysis following immunoprecipitation by anti-KIAA0101 antibody using cell lysates from pancreatic cancer cell lines KLM-1 and PK59 identified polymerase δ p125 subunit (POLD1) and flap endonuclease-1 (FEN1) to be interacting partners with KIAA0101 as well as PCNA. These interactions were confirmed by immunoprecipitation experiment using specific antibody to each protein (KIAA0101, PCNA, POLD,
Immunocytochemical analysis in pancreatic cancer cell KLM-1 showed that KIAA0101 was colocalized with PCNA in the nucleus of cancer cells within replication foci, preferentially in S phase of the cell cycle (Fig. 4B). These findings implicated that KIAA0101 is very likely to be a member of a DNA-replication complex with POLD1, FEN1, and PCNA in replication foci.

To further examine the involvement of KIAA0101 on DNA replication, we established the stable clones in which endogenous KIAA0101 expression was regulated by an absence or a presence of doxycycline that could induce expression of siRNA specific to KIAA0101. We evaluated DNA replication by BrdUrd incorporation assay in the condition of the absence or the presence of endogenous siRNA to KIAA0101. Western blot analysis (Fig. 4C) showed that doxycycline treatment clearly suppressed endogenous KIAA0101 expression in two clones of KLM1-si#KIAA0101 (si#759-1 and si#759-2), whereas no suppression of KIAA0101 expression was observed in the control clone KLM1-si#EGFP. In this condition,

Figure 4. Involvement of KIAA0101 with DNA replication. A, the KIAA0101-interacting proteins were identified by a matrix-assisted laser desorption/ionization time-of-flight system after in-gel trypsin digestion following immunoprecipitation by anti-KIAA0101 antibody; they were identified as PCNA, POLD1, and FEN1. These interactions were confirmed by immunoprecipitation experiments. All of these proteins are involved with DNA replication, and POLD1 and FEN1 also bind to PCNA as well as KIAA0101. B, in early-mid and late S phase, endogenous KIAA0101 was colocalized with PCNA in the nucleus of KLM-1 cells within replication foci. The overlap between KIAA0101 staining (red) and PCNA (green) is in yellow (merge, right). C, for a doxycycline (Dox)–inducible siRNA-expression system, Western blot analysis validated knockdown of KIAA0101 by doxycycline treatment in si#759-1 and si#759-2 clones but not in a control clone (pTER-si#EGFP). Western blotting of anti-β-actin antibody served as a quantitative control. D, BrdUrd incorporation assay evaluated the ability of DNA replication of si#759-1 and si#759-2 clones and pTER-si#EGFP with or without doxycycline treatment. Relative ratio of BrdUrd absorbance/MTT absorbance in each clone with doxycycline treatment was calculated (Y-axis) comparing with those without doxycycline treatment as a control. DNA synthesis was significantly decreased when KIAA0101 expression in KLM1-si#759-1 and KLM1-si#759-2 clones was suppressed by doxycycline treatment (P < 0.05, Student’s t test), although no effect on DNA synthesis was observed in the control KLM1-si#EGFP clone by doxycycline treatment.
BrdUrd incorporation was significantly ($P < 0.05$, Student’s $t$ test) decreased in the cells in which KIAA0101 expression was suppressed with KLM1-si#759-1 and KLM1-si#759-2 (Fig. 4D). On the other hand, no suppression of KIAA0101 expression was observed in HCT116 p53+/+ cells. These findings suggest that KIAA0101 could play important roles in cancer cell growth through the control of DNA replication.

Regulation of KIAA0101 expression by the p53-p21 pathway.

The expressions of several DNA replication factors, such as PCNA (17), POLD1 (18), and FEN1 (19), were regulated by p53 pathway. Our extensive expression analysis for p53-regulated genes using cDNA microarray (20) also found a possibility that KIAA0101 expression was down-regulated by adenovirus-mediated introduction of p53 (data not shown). To further investigate the relation between the KIAA0101 expression and p53, wild-type HCT116 cells (HCT116 p53+/+) or HCT116-derivative cells lacking p53 (HCT116 p53−/−) were treated with Adriamycin or exposed to irradiation and then examined the expression levels of KIAA0101, p53, and p21 by RT-PCR and Western blot analysis. As shown in Fig. 5A, endogenous KIAA0101 expression was significantly decreased in the HCT116 p53+/+ cells at the protein level (top column) and mRNA level (bottom column) in accordance with induction of endogenous p53 by Adriamycin treatment (left) or irradiation exposure (right). On the other hand, no suppression of KIAA0101 expression was observed in HCT116 p53−/− cells. β-Actin protein and β2MG RNA served as quantitative controls.

B, RT-PCR and Western blot analysis of KIAA0101 expression in HCT116 p21+/+ and p21−/− cells at various time points after treatment with 0.5 ng/mL Adriamycin or after exposure to 14 Gy γ-ray. KIAA0101 expression was not suppressed by Adriamycin treatment (left) or irradiation exposure (right), whereas KIAA0101 expression was drastically decreased in the wild-type HCT116 p21+/+ cells in its RNA and protein level.

Figure 5. Regulation of KIAA0101 expression by the p53-p21 pathway. A, RT-PCR and Western blot analysis for KIAA0101 expression in HCT116 p53+/+ and p53−/− cells at various time points after treatment with 0.5 ng/mL Adriamycin or after exposure to 14 Gy γ-ray. KIAA0101 expression was decreased in the HCT116 p53+/+ cells in protein level (top column) and mRNA level (bottom column) in accordance with induction of endogenous p53 by Adriamycin treatment (left) or irradiation exposure (right). On the other hand, no suppression of KIAA0101 expression was observed in HCT116 p53−/− cells. β-Actin protein and β2MG RNA served as quantitative controls. B, RT-PCR and Western blot analysis of KIAA0101 expression in HCT116 p21+/+ and p21−/− cells at various time points after treatment with 0.5 ng/mL Adriamycin or after exposure to 14 Gy γ-ray. KIAA0101 expression was not suppressed by Adriamycin treatment (left) or irradiation exposure (right), whereas KIAA0101 expression was drastically decreased in the wild-type HCT116 p21+/+ cells in its RNA and protein level.

Inhibition of the interaction between KIAA0101 and PCNA by cell-permeable dominant-negative peptide. To investigate the biological significance of an interaction between KIAA0101 and PCNA in pancreatic carcinogenesis, we attempted to inhibit the interaction between KIAA0101 and PCNA using a cell-permeable dominant negative peptide.
interaction by the use of the dominant-negative peptide. Several PCNA-associated proteins can bind to PCNA through their conserved PCNA-binding motif (PIP box, Qxx[L/I/M][x][F/Y][F/Y]; refs. 8–10). KIAA0101 has this PIP motif in its middle portion (QKGIGEFF), and we validated that KIAA0101 could bind to PCNA through its PIP box domain (data not shown). Then we designed the 20-amino-acid PIP box peptide that was conjugated with arginine (R)-repeat to facilitate cell permeability (Fig. 6A). In vitro immunoprecipitation experiment clearly showed effective inhibition of the KIAA0101/PCNA interaction by cell-permeable PIP20 peptide, whereas PIP20mt, in which the conserved residues were replaced with alanine or the scramble peptide, showed no influence to coimmunoprecipitation of the two proteins (Fig. 6B). Then we further evaluated whether these peptides could inhibit cancer cell growth or not, by treating cancer cells and normal fibroblast NIH3T3 cell with these peptides. We examined KIAA0101 expression of a number of established human cell lines, but all of them were found to be expressed KIAA0101 more or less. However, because mouse fibroblast NIH3T3 did not express its homologue (NP_080791) that has high similarity to human KIAA0101 (85% identical in amino acid), and because the amino acid sequences corresponding to this dominant-negative peptide were 100% identical, we used NIH3T3 cells as a negative control. PIP20 peptide treatment clearly suppressed the growth of cancer cells in a dose-dependent manner but showed no effect on the growth of NIH3T3 cells, excluding a possibility of the off-target effect of this peptide. PIP20mt and the scramble peptide did not affect the growth of cancer cells.

**Discussions**

In this study, we focused on KIAA0101 that is one of the genes overexpressed in pancreatic cancer cells. In our extensive microarray analysis for a variety of human cancers (21, 22), KIAA0101 was indicated to be overexpressed in cancer cells arisen from multiple organs in addition to the pancreas. Our immunohistochemical analysis using anti-KIAA0101 antibody validated a high level of KIAA0101 expression in cancer cell, and a low level of the expression in the crypt cells of normal intestinal mucosa as well as the germinal center of normal lymph node where active proliferation was usually observed. Knockdown of KIAA0101 by means of siRNA introduction suppressed cell proliferation.

**Figure 6.** Inhibition of the interaction between KIAA0101 and PCNA by cell-permeable dominant-negative peptides. A, a dominant-negative peptide (PIP20) containing PIP box (QKGIGEFF indicated by box, the conserved residues: gray letters) and its mutant peptide (PIP20mt) with the conserved PIP box residues replaced with alanines (AKGAGEAA, underlined) were designed and conjugated them with arginine (R)-repeat to facilitate cell permeability. B, in in vitro study, immunoprecipitation validated the inhibition of the interaction between PCNA and KIAA0101 by PIP20 treatment, but PIP20mt and scramble peptide did not affect the interaction between PCNA and KIAA0101. C, PIP20 treatment significantly suppressed cell growth of KIAA0101-expressing KLM-1 dose-dependently ($P = 0.000163$, Student’s $t$ test), whereas PIP20mt and scramble peptide (sc) did not. On the other hand, PIP20 did not affect the growth of mouse normal cell line (NIH3T3 cells) that did not express KIAA0101. The numbers of viable cells were measured by MTT assay. Y-axis, absorbance at 490 nm (MTT assay), and at 630 nm as reference, measured with a microplate reader.
Furthermore, exogenous overexpression of KIAA0101 promoted cancer cell proliferation and transformed NIH3T3 cells in vivo, indicating its critical involvement in carcinogenesis.

PCNA is an essential and central auxiliary protein for the processes of DNA replication/repair, interacting with many enzymes involved in DNA synthesis or metabolism (6, 7). Our mass spectrometric analysis following immunoprecipitation by anti-KIAA0101 antibody identified POLD1 and FEN1 to be interacting partners with KIAA0101, as well as PCNA, and it is presumed that KIAA0101 is very likely to be a member of a DNA-replication complex with POLD1, FEN1, and PCNA in replication foci. Furthermore, we here showed that KIAA0101 expression was regulated by the p53-p21 pathway, like PCNA, POLD1, and FEN1 (17–19). KIAA0101 interaction with PCNA could be inhibited by p21 competitively (5), and both expression and function of KIAA0101 could be strictly regulated by the p53-p21 pathway as one of the critical factors of DNA replication.

We designed the dominant-negative peptide that was conjugated with cell permeable arginine-repeat and successfully showed that the 20-amino-acid peptide corresponding to the PIP domain (PIP20) inhibited the interaction between PCNA and KIAA0101 and resulted in suppression of the cancer cell growth in a dose-dependent manner. In addition, because PIP20 showed no effect on the growth of NIH3T3 cells in which KIAA0101 expression was hardly detectable, we considered the effect of PIP20 was highly specific to the cells in which KIAA0101 was overexpressed.

Intracellular protein-protein interactions are very important in regulation of many signaling pathways, but it has been indicated that the inhibition of these interactions is a difficult target for small-molecule chemistry because a protein interface is extensive, shallow, and hydrophobic (23). Although dominant-negative peptides are attractive candidates for stabilizing or disrupting protein-protein interactions, their efficacy as in vivo reagents is severely compromised by their loss of secondary structure, susceptibility to proteolytic degradation, and difficulty in penetrating into cells. However, a recent report indicated a promise that the modified peptides may be applicable to inhibit the protein interaction (24). Further structural analysis of targeted protein-protein interaction (23) or DDS improvement can open the novel road to a big success for inhibiting the protein-protein interaction as novel therapeutic approach, such as PIP20, we reported here.

In conclusion, our expressional and functional analysis for KIAA0101 suggested that the inhibition of KIAA0101 function or the specific interaction between KIAA0101 and PCNA is likely to be a promising strategy to develop novel cancer therapeutic drugs.

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