Role of dimerized C16orf74 in aggressive pancreatic cancer: A novel therapeutic target


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Abstract

The therapeutic outcome for pancreatic ductal adenocarcinoma (PDAC), over the past 30 years remains stagnant due to the lack of effective treatments. We performed a genome-wide analysis to identify novel therapeutic targets for PDAC. Our analysis showed that Homo sapiens chromosome 16 open reading frame 74 (C16orf74) was upregulated in most of PDAC patients and associated with poor prognosis. Previously, we demonstrated that C16orf74 interacts with the catalytic subunit alpha of protein phosphatase 3 and plays an important role in PDAC invasion. However, the pathophysiological function of C16orf74 is still unclear. In this study, through the analysis of C16orf74 interaction, we demonstrate a new strategy to inhibit the growth and invasion of PDAC.

C16orf74 exists in the homodimer form under the cell membrane and binds integrin αVβ3 and is also involved in invasion by activating Rho family (Rac1) and MMP2. Considering that this dimeric form was found to be involved in the function of C16orf74, we designed an 11R-DB (dimer block) cell-permeable dominant negative peptide that inhibits the dimer form of C16orf74. 11R-DB suppressed invasion and proliferation of PDAC cell lines by inhibiting phosphorylation of Akt and mTOR and also by inactivation of MMP2. 11R-DB also showed anti-tumor effects in an orthotopic xenograft model and peritoneal metastasis model.

Thus, this study demonstrates that dimerized C16orf74, present in the cell membrane, is involved in pancreatic cancer invasion and proliferation.
Additionally, the C16orf74 dimer block cell-permeable peptide (11R-DB) has a potent therapeutic effect on PDAC in vitro and in vivo.

**Introduction**

The incidence of PDAC is increasing by about 1% per year, and an estimated 56,770 new cases and 45,750 deaths occurred in the US in 2019, even today, with progress in therapy, the 5-year combined survival rate of all stages is only 9% [American Cancer Society. Cancer facts & figures 2019. Atlanta, Georgia: American Cancer Society. 2019. http://www.cancer.org/research/cancerfactsstatistics/cancer-facts-and-figures-2019]. In recent years, FOLFIRINOX (a combination of oxaliplatin, irinotecan, fluorouracil, and leucovorin) and Gemcitabine (GEM) with nab-paclitaxel therapy have been developed and show higher antitumor effect than GEM for patients with metastatic PDAC. Conversely, it has also been reported that various adverse effects appear in the chemotherapy [1–3]. In addition, GEM with erlotinib was developed as a molecular target therapy, however, it did not have a significant effect compared with GEM single therapy (only 2 weeks of extension in median overall survival) [4]. Therefore, development of new molecular target therapy with strong therapeutic effect on PDAC is essential.

Through a genome-wide cDNA microarray analysis of PDACs, we have previously selected genes that are upregulated highly and commonly in PDAC compared with normal tissues [5]. Among the upregulated genes, we have reported a novel gene C16orf74 [Homo sapiens chromosome 16 open reading...
frame 74 (NM_206967)] as an ideal therapeutic target of PDAC. C16orf74 is frequently overexpressed in PDAC specimens associated with poor prognosis and was found to be essential to growth of PDAC [6]. Moreover, other reports have indicated that C16orf74 expression correlated with potential prognostic factor in other types of cancer [7–10]. With the functional analysis of C16orf74, previously, we have demonstrated that C16orf74 interacts with the catalytic subunit alpha of protein phosphatase 3 (PPP3CA) as well as the calcineurin (CN) isozyme. This interaction promotes proliferation and invasion of PDAC cells [6,11]. However, the pathophysiological functional analysis of C16orf74 is unclear.

In this study, we further analyzed the functional characteristics of C16orf74 and found a new strategy to inhibit the growth and invasion of PDAC.

Materials and Methods

Cell permeable peptide design

Three cell permeable peptides were synthesized by Sigma-Aldrich Japan (Tokyo, Japan) and were purified by preparative reversed-phase HPLC till they were >80% pure, with the expected amino acid composition and mass spectra. The peptides’ amino acid sequences are shown below.

11R-DB; RRRRRRRRRR-GGG-MGLKMSCLKGFQMCV
11R-C7A_C14A; RRRRRRRRRR-GGG-MGLKMSALKGFQMAV
11R-7_14AAA; RRRRRRRRRR-GGG-MGLKMAAAGKFQAAA
The cell lines: PANC-1, MIA PaCa-2, the embryonic kidney, HEK293, and COS7 were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). PK-1 and KLM-1 were purchased from RIKEN Bio Resource Center (Tokyo, Japan). PK-9 cells were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). PCI6 was previously obtained from PDAC patient in our laboratory [12]. Human Umbilical Vein Endothelial Cells (HUVEC) and Normal Human Dermal Fibroblasts (NHDF) were purchased from PromoCell (Heidelberg, German). All cells were cultured in appropriate media, and were incubated at 37 °C with 5 % CO₂. Dulbecco’s modified Eagle’s medium (nacalai tesque, Kyoto, Japan) was used for MIA PaCa-2, PANC-1, NHDF, and COS7. RPMI-1640 cell media (nacalai tesque, Kyoto, Japan) was used for PK-1, PK-9, KLM-1, and PCI6, while Endothelial Cell Media (PromoCell, Heidelberg, German) was used for HUVEC. Each medium was supplemented with 10% fetal bovine serum (Cell culture Bioscience, Tokyo, Japan) and 1% penicillin-streptomycin (Life Technologies, Tokyo, Japan).

Quantitative RT-PCR analysis

Total RNA from cells was extracted using an RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. cDNA was synthesized using Prime Script RT Master Mix (TAKARA BIO, Shiga, Japan). Using a StepOne Real-time PCR system (Applied Biosystems, Tokyo,
PCR reactions were performed. Data analysis was performed using the ΔΔCT method, and the housekeeping gene, *GAPDH*, was used as control. Primer specificity was confirmed by one peak melt curve. All experiments were performed in triplicates for each sample. The primers used in this study are described in Supplemental Table 1.

**Western blot analysis**

After washed twice with PBS, cells were harvested in RIPA buffer containing protease inhibitors (aprotinin and PMSF). The protein concentration was standardized by Bradford assay after the cells were homogenized. Proteins were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Darmstadt, Germany) and blocked with 5% skim milk or MaxBlot Solution 1 (MBL, Nagoya, Japan) for 1 h. Membranes were then incubated with the desired primary antibodies for what was being tested. Membranes were probed with the secondary antibodies (1:10,000 dilution) using goat anti-mouse or rabbit IgG. Immunoreactivity was detected with an enhanced chemiluminescence detection system (GE Healthcare). β-actin was used for equal loading confirmation. Primary antibodies and dilutions are shown in Supplemental Table 2. Secondary antibodies were purchased from Jackson ImmunoResearch.
Native PAGE

The cultured cells were harvested with RIPA buffer without SDS. Polyacrylamide gel electrophoresis was performed similar to as described above but at 4 °C and omitting SDS (in all solutions), 2-mercaptoethanol, and sample boiling.

Establishment of PANC-1 cells stably expressing C16orf74-FLAG

PANC-1 cells were plated at a density of $1 \times 10^5$ cells/well on 6-well plates and transfected with pCAGGS-C16orf74-3xFLAG using Lipofectamine® LTX Reagent & Plus™ Reagent (Invitrogen, CA, USA), according to manufacturer’s protocol. The medium was then replaced with selection medium containing G418 (800 μg/mL; Invitrogen). The resulting live cell colonies were plated at a density of 1 cell/well on 96-well plates, and single cell derived clones were obtained. Resulting clones were confirmed by immunofluorescence, qRT-PCR, and western blotting.

Establishment of PK-9 cells stably expressing tdTomato-Luc2

PK-9 cells were transfected with pCSII-CMV-tdTomato-Luc2 (provided by Vascular Biology and Molecular Pathology Graduate School of Dental Medicine, Hokkaido University, Sapporo, Japan), and the clones were selected for tdTomato-Luc expression sing 0.1 mg/mL bleomycin. The resulting colonies were plated at a density of 1 cell/well on 96-well plates, and single cell derived clones were obtained. The expression of luciferase in these cells was
confirmed via the *in vivo* optical imaging showing the quantitative correlation between the cell number and luminescent signal intensity (Promega, Madison, WI, USA).

**Immunofluorescence**

Cells were fixed with 3 % paraformaldehyde for 15 min at room temperature and permeabilized with 0.1 % Triton X-100 in PBS for 4 min. To block nonspecific binding of antibodies, cells were incubated in 1 % bovine serum albumin (BSA) and then further incubated with primary antibodies overnight at 4 °C, after which immune complexes were detected by incubation for 1 h at room temperature in the dark with secondary antibodies (1:250 dilution). FV-10i confocal microscope (Olympus, Tokyo, Japan) was used for imaging. The names and dilutions of the antibodies have been listed in Supplemental Table 3.

**Immunoprecipitation and western blotting (Pull down assay)**

After transfection 24 h, cells were harvested with RIPA buffer. Immunoprecipitation was performed with mouse anti-DDDDK (FLAG) tag (FLA-1, MBL, Nagoya, Japan) antibody or anti-HA tag (TANA2, MBL, Nagoya, Japan) antibody. By incubation with protein A agarose (sc-2001, Santa Cruz Biotechnology, Santa Cruz, CA, USA), the antibodies were removed, and the wash step was repeated 5 times. Proteins were extracted with SDS sample buffer and separated on a 4-20 % gradient SDS page (Bio-Rad, Tokyo, Japan).
To examine the interaction between flag-tagged proteins and HA-tagged proteins, immune complexes were analyzed by western blotting. To investigate the interactions between C16orf74 and integrin β3 as well as integrin αV and integrin αIIb, PANC-1 cells were harvested in RIPA buffer and mouse anti-DDDDK (FLAG) immunoprecipitation was performed using a tag antibody. Then, antibodies containing protein A agarose: sc-2001 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were collected and washed with PBS 5 times. Proteins were extracted with SDS sample buffer and separated by 4-20 % gradient SDS PAGE. Immunocomplexes were analyzed by western blotting using mouse antibodies (Supplementary Table 2).

Rac1 isolation

Rac1 isolation was performed using PAK-1 PBD agarose beads. PANC-1 cells stably expressing C16orf74 and MOCK (stable expressing empty vector) cells were lysed with MLB (magnesium-containing lysis buffer) (25 mM HEPES pH 7.5, 150 mM NaCl, 1 % lagepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 10 % glycerol, EMD Millipore, Temecula, CA, USA). PAK-1 PBD agarose (EMD Millipore) was added to each lysate and agitated for 1 h at 4 °C. The beads were collected by centrifugation and the supernatant was discarded. The beads were then washed 3 times with magnesium-containing lysis buffer.

Immunohistochemical staining
Paraffin-embedded sections were used for immunohistochemical staining. Same protocol was followed as our previous study [11]. Finally, the sections were counterstained with hematoxylin. Each antibody was shown in Supplemental Table 4.

**Cell proliferation assay**

The water-soluble tetrazolium salts (WST) cell proliferation assay was performed according to the manufacturer’s protocol provided with the cell counting kit-8 (Dojindo, Kumamoto, Japan). Briefly, cells were seeded on 96-well plates and the culture medium was replaced with various conditioned medium for 24 or 48 h. At the end of treatment, the WST-8 reagent was added and incubated for another 4 h. Finally, the plate was directly measured for absorbance at 450 nm.

**Matrigel invasion assay**

Same protocol was followed as our previous study [11]. At least 3 randomly selected fields were investigated and invaded cells were counted at 100× magnification.

**Hematogenic invasion assay**

A total of 2.5 × 10⁴ HUVEC were seeded on the surface of a BD Matrigel Matrix (BD BioCoat™ 24-Multiwell Tumor Cell Invasion System) in Endothelial Cell media (PromoCell, Heidelberg, German) to cover the upper
side of the trans-well inserts (8 μm pore size), and incubated at 37 °C, 5 % CO₂ for 1 day. After the confluence of HUVEC and monolayer formation, the rehydration solution was removed. 2.5 × 10⁴ stable GFP-expressing C16orf74 stable (C16orf74 stable/GFP) and MOCK (MOCK/GFP) cells were resuspended in 0.5 mL of medium (DMDM: Endothelial cell media=1:1) without FBS and loaded on the upper side of the trans-well inserts, and 0.75 mL medium (DMDM: Endothelial cell media=1:1) containing 10 % FBS was loaded on the lower side of the trans-well inserts to induce chemotaxis. The remaining cells on the upper side of the trans-well inserts were removed by cell scraping after incubation for 24 h at 37 °C and 5 % CO₂. The cells permeated through the HUVEC and trans-well inserts were counted by a fluorescence microscope (BZ-9000, KEYENCE, Tokyo, Japan). Cells were counted in full visual field (×100) per filter. The results were reported as means of triplicate assays.

Wound healing assay

Confluent cells were wounded by scraping with a 200 μL pipette tip to analyze cell motility. By phase-contrast microscopy, cell movements were observed at 0, 3, 6, 9, 12, 15, and 18 h.

Gelatin zymography

Cells were cultured in serum-free medium for 24 h and then washed twice with PBS. The conditioned medium was clarified by centrifugation at 2,000 x g for 15 min. Twenty microliters of 2X non-denaturing loading buffer
was mixed with an equal volume of conditioned medium, and separated by 10% SDS-PAGE containing 25 mg/mL gelatin. To remove SDS, gels were washed twice in 2.5% TritonX-100 PBS for 15 min at room temperature, and then twice in water, and incubated at 37 °C for 24 h in an incubation buffer (50 mM Tris, 5 mM CaCl₂・H₂O, 1 mM ZnCl₂). The resulting gel was stained, fixed with a 50 % methanol and 10 % acetic acid containing 1.25 mg / mL Coomassie Brilliant Blue for 30 min, and destained with 10 % methanol and 5 % acetic acid. The zymogram was imaged with ChemiDocTMXRS (Bio-Rad, Tokyo, Japan) and the intensity of the band corresponding to gelatinase activity was quantified using Image Lab™ software (Bio-Rad).

**Construction of the expression vector**

The entire coding sequence of each construct was amplified by RT-PCR and the PCR product was inserted into the EcoRI and XhoI sites of pCAGGS to express Flag or HA tagged protein [13]. RT-PCR primers were shown in Supplemental Table 5.

**Pancreatic cancer model mice and in vivo imaging**

Female Balb/cA Jcl nu/nu mice were purchased from CLEA Japan. At 6 weeks of age, each of the following models was constructed in the mice under anesthesia induced with ketamine (100 mg/kg) and xylazine (10 mg/kg).

- Subcutaneous tumor
PK-9 cells were adjusted to $5 \times 10^6$ cells/100 μL HBSS (Gibco, Carlsbad, CA, USA) containing Matrigel (Corning, NY, USA) and injected subcutaneously in the back using a 27 G needle and 100 μL Hamilton syringe. From 2 weeks after injection of the cancer cells, 11R-DB and a111R-7_14AAA (10 mg/kg, 5 times per week) or PBS only were administered in the subcutaneous tissue near the tumor for 3 weeks.

- Orthotopic xenograft model mice

PK-9 cells expressing luciferase (PK-9-tdTomato-luc2) were adjusted to $1 \times 10^6$ cells/50 μL HBSS containing Matrigel (Corning) and injected below the serosa of the pancreas tail using a 27 G needle and 100 μL Hamilton syringe [14]. One week post injection; 11R-DB and 11R-7_14AAA (10 mg/kg, 5 times per week) or PBS were administered intraperitoneally for 4 weeks. Using a post-intraperitoneal injection of VivoGlo Luciferin in vivo Grade (Promega), bioluminescent imaging was performed with the IVIS Spectrum imaging system (Caliper Life-Sciences, Hopkinton, MA). Mice were then sacrificed to assess tumor weight and pathological analysis of tumor and major organs such as, brain, lung, heart, liver, kidney, and spleen.

- Peritoneal dissemination model mice

PK-9-tdTomato-luc2 were adjusted to $3 \times 10^6$ cells/200 μL HBSS and injected intraperitoneally using a 27 G needle and 1000 μL syringe. From the day of transplantation of tumor cells until day 5, 11R-DB and 11R-7_14AAA (1 mg/kg, 10 μM) or PBS only were intraperitoneally administered. The mice were
analyzed by *in vivo* imaging on days 3, 7, 14, 21, and 28 after tumor cell implantation and engraftment of the tumor was confirmed.

All animal experiments were conducted according to the guidelines of the Hokkaido University Animal Experiment Implementation Manual and were approved by the Institutional Animal Care and Use Committee of Hokkaido University Graduate School of Medicine (No:17-0011). Based on their completion of required animal use and care training, all researchers who performed procedures using live animal were pre-approved by the Animal Welfare Committee of Hokkaido University.

**Statistical analysis**

The results are shown as the mean ± SEM or SD. Student's *t*-test or Mann-Whitney *U*-test was performed to analyze significant differences using JMP® version 13 (SAS Institute, Inc., Cary, NC). *P*-value < 0.05 was considered statistically significant.

**Results**

**Functional analysis of C16orf74 overexpressed stable transfectant cell line**

The morphological observation of C16orf74 stable transfectant (Red: f-actin, Green: paxillin), compared with MOCK stable transfectant demonstrated a strong polarity with cell membrane development in the C16orf74 stable cells (Figure 1A). From the above observation, we speculated that the Rho family
such as lamellipodium and stress fiber would be activated by C16orf74 overexpression. Active Rac1 (Rac1-GTP) was pulled down with GST-PAK-PBD-conjugated agarose beads from extracts of C16orf74 stable and MOCK cells and detected by Immunoblotting. Activated Rac1 was increased in C16orf74 stable cells as compared to MOCK cells (Figure 1B).

C16orf74 stable cells demonstrated increased cell proliferation, invasion, migration and vascular invasion compared to MOCK cells as observed from the results of WST assay (Figure 1C), matrigel invasion assay (Figure 1D), wound healing assay (Figure 1E), and hematogenic invasion assay (Figure 1F). Western blot analysis verified that C16orf74 increased phosphorylation of Akt and expression of MMP2 (Figure 1G). Therefore, it was suggested that activation of MMP2 and phosphorylation of Akt by C16orf74 may be involved in the increase of cell proliferation and invasiveness. C16orf74 stable cells showed an increase in activated MMP2 and activated MMP9 and a decrease in each of the inactivated form compared to MOCK cells as evidenced by gelatin zymography (Figure 1H). The expression of C16orf74 in C16orf74 stable and MOCK cell lines by quantitative RT-PCR, western blot and immunofluorescence were shown in Supplemental Figure 1.

**C16orf74 forms a homodimer and is localized under the cell membrane**

Western blot analysis of the C16orf74 protein revealed multiple bands, resulting in the deduction that C16orf74 form multimers or complexes. From the amino acid sequence of the C16orf74 protein, we predicted a dimer
formation at the S-S binding site C7 and C14 (Figure 2A). Native PAGE showed that two bands were recognized at 60 kDa and 120 kDa (2 fold) suggesting the possibility of dimer formation (Figure 2B). Co-transfection and pull-down assay showed that C16orf74/FLAG protein and C16orf74/HA protein were directly bound (Figure 2C). Hence, it was confirmed that C16orf74 exhibits a homodimeric form.

We also speculated that the amino acid sequence of G2 may have an important role in C16orf74 localization to the cell membrane as an N-myristoylation site. Administration of 2-hydroxymyristic acid (myristoylation inhibitor) showed that localization of C16orf74 was changed to the cytoplasm (Figure 2D). Using mutation analysis, the intracellular localization of C16orf74/G2A (mutant of the myristoylation site), C16orf74/C7A_C14A (mutant of the dimer formation site), C16orf74/Δ2-15 (deletion mutant of both the myristoylation and dimer formation site), C16orf74/T44A (mutant of the phosphorylation site that is important to PPP3CA interaction [6]) constructs were examined. C16orf74/WT and C16orf74/T44A localized directly under the cell membrane, oppositely, C16orf74/G2A, C16orf74/C7A_C14A, and C16orf74/Δ2-15 could not localize under the cell membrane (Figure 2E). To verify dimer formation, pull down assay was performed on each construct (with FLAG tag) and C16orf74/WT (with HA tag). It was found that C16orf74/WT and C16orf74/T44A formed dimers, but other constructs did not form dimers (Figure 2F).
These results indicate that the first 15 amino acid sequences are considered to be a “structural site” that regulates C16orf7 dimer formation and the localization to cell membrane. Additionally, the “functional sites” having a PDIIIT motif which is a PPP3CA binding site and T44 which is a phosphorylation site [6], are bound to the “structural site” via the -SSSSSS- structure (Figure 2G).

Suppression of cell proliferation, invasion, and migration by inhibiting homodimers of C16orf74 by dimer blocking peptides

We hypothesized that inhibition of C16orf74 homodimers would suppress PDAC cell proliferation and invasiveness, and so we designed a homodimer blocking cell permeable peptide. 11R-DB (dimer blocking) peptide was designed by a peptide homologous to the first 15 amino acid sequence of C16orf74 linking 11R (cell permeable peptide signal) with a hydrophilic spacer (-GGG-) (Figure 3A). The negative control peptides for 11R-DB, 11R-7_14AAA peptide (both sides of C7 and C14 were substituted with alanine) and the 11R-C7A_C14A peptide (C7 and C14 were substituted with alanine) were designed (Figure 3A). The dimer inhibition of C16orf74 using 11R-DB was evaluated by a pull down assay. 11R-DB was found to inhibit the dimerization of C16orf74 in a dose-dependent manner (Figure 3B). 11R-7_14 AAA (control peptide) did not inhibit the dimerization of C16orf74, and 11R-C7A_C14A inhibited the dimerization of C16orf74 partially (Figure 3C).
11R-DB showed inhibition of cell growth in C16orf74 over expressing PDAC cell lines (PK-1 and PK-9) but had a lower effect on C16orf74 low expressing PDAC cell line and normal cell line (PANC-1, MIA Paca2, and NHDF). 11R-C7A_C14A was weaker than 11R-DB but showed cell growth inhibition correlated with the expression level of C16orf74. 11R-7_14AAA (control peptide) showed no effect on any cell lines (Figure 3D). The same results were found in the Matrigel invasion assay and wound healing assay (Figure 3E, 3F). The expression of C16orf74 in PDAC or other cell lines by quantitative RT-PCR or western blot were shown in Supplemental Figure 2.

The morphological changes in pancreatic cancer cells under 11R-DB treatment were examined by immunofluorescence with Paxillin (Green: Alexa Fluor 488) and F actin (Red: Alexa Fluor 594). By 11R-DB treatment, the localization of the focal adhesion became scattered or pericytes, and the polarity was lost, and the forms of F actin changed randomly (Figure 3G). Similarly, the development of lamellipodium, thought to have developed by Rac1 activated by C16orf74 expression, was attenuated. The cell morphology of C16orf74 stable was changed to same as Mock (in Figure 1A) under the treatment of 11R-DB. C16orf74 (Green: Alexa Fluor 488) was localized in the cell membrane and was more strongly expressed in the cell to cell contact part (Figure 3H-a), however, after 11R-DB administration, the C16orf74 localization at the cell membrane was lost, and cell-cell contact was attenuated (Figure 3H-b). The actin fiber was also attenuated as seen when using Rho Inhibitor [15] (Figure 3H-c). The invasiveness of the C16orf74 stable transfectants were also
significantly suppressed by 11R-DB (Supplemental Figure 3). The above findings suggested that 11R-DB inhibits the cytoskeletal change for cancer cell invasion.

C16orf74 binds directly to Integrin αVβ3, and controls the PI3K/ Akt/ mTOR cascade

From C16orf74 localization under the cell membrane and stronger expression in cell to cell contact, we speculated that the integrin family and other molecules such as Integrin linked kinase (ILK), PDK1, and Paxillin would be involved in C16orf74 signals. The binding between C16orf74 and these molecules was examined by pull down assays. It was found that Integrin β3, ILK and PPP3CA (shown in previous study [6] as a control) directly bind to C16orf74 (Figure 4A). C16orf74 also showed co-localization with Integrin β3 by immunofluorescence analysis, in addition to the development of lamellipodium which was found at co-localized site (Figure 4B). In general, Integrin was present as a heterodimer of α-subunit and β-subunit, therefore the combination of integrin α-subunit and β-subunit was determined by Pull down assay. C16orf74 and Integrin αVβ3 were bound together (Figure 4C). The binding of C16orf74 and Integrin β3 was also inhibited by administration of 11R-DB (Figure 4D), indicating that dimer formation of C16orf74 is important for binding.

We examined intracellular signaling in PDAC cell lines by administration of 11R-DB (Figure 4E). It was found that 11R-DB reduces
phosphorylation of Akt and mTOR in C16orf74 high expression cell lines PK-1 and PK-9 (quantitative data in Supplemental Figure 4A, B) but does not affect phosphorylation of p42/44 MAPK. Additionally, 11R-DB did not affect the signaling of C16orf74 in low expression cell lines PANC-1 and MIA PaCa-2. These results suggest that C16orf74 might be involved in the PI3K/Akt/mTOR cascade but may not affect the MAPK pathway. In addition, 11R-DB decreased the activation of MMP2 but did not significantly affect MMP9 by zymography (Figure 4F).

Anti-tumor effect of 11R-DB peptide on human pancreatic cancer cells grown in nude mice

In order to assess the anti-tumor effect of 11R-DB in vivo, treatment of human pancreatic cancer cells, growing subcutaneously and in the pancreas (orthotopic animal model) and in peritoneal metastasis models of nude mice, was done with 11R-DB. In all experiments, PK-9 (C16orf74 overexpressed PDAC cell line) was used. Treatment schedules of each model are shown in Supplemental Figure 5A, Figure 5A, and Figure 6A. In subcutaneous model, tumor growth was suppressed with significant difference in the 11R-DB administration group (Supplemental Figure 5B, C). In orthotopic model, anti-tumor effect was examined by in vivo imaging (Figure 5B) and tumor growth was suppressed with significant difference in the 11R-DB administration group (11R-DB vs 11R-7.14AAA/PBS: *p<0.05, 11R-7.14AAA vs PBS: n.s.) (Figure 5C, D). No reduction in weight was observed in the peptide-treated mice after
treatment by 11R-DB, and no apparent negative side effects were found to the major vital organs confirmed by hematoxylin and eosin staining (Supplemental Figure 5D, E). 11R-DB suppressed Akt phosphorylation and mTOR phosphorylation in tumor specimen after treatment (Figure 5E) same as previously shown in vitro assays (Figure 4E). The Ki-67 Index of the tumor specimen was decreased after treatment with 11R-DB (Figure 5F).

In peritoneal metastasis model, tumor cell engraftment was significantly suppressed by 11R-DB administration (Figure 6B, C), and the number of disseminated nodules was suppressed with significant difference in the 11R-DB administration group (11R-DB vs 11R-7.14AAA/PBS: *p<0.05, 11R-7.14AAA vs PBS: n.s) (Figure 6D, E).

Discussion

In this study, C16orf74 was found to be localized just beneath the cell membrane and was seen to bind to integrin. From the morphological observation of C16orf74 stable cell lines, we acknowledged findings which strongly indicated the activation of the Rho family such as lamellipodium and stress fiber [16,17]. An increase in active Rac1 was thought to contribute to cell morphology and invasiveness [17-19]. C16orf74 can localize in the cell membrane only when present in a dimeric form and shows direct binding to Integrin αVβ3. By administration of 11R-DB, localization and binding of C16orf74 was inhibited. Administration of 11R-DB also caused a decrease in phosphorylation of Akt and mTOR and a decrease in MMP2 activity. Thus, we
suggest that 11R-DB inhibited the ILK/PI3K/Akt pathway by blocking the integrin signal. The results of our study suggest that dimerization of C16orf74 is important for binding to integrin αVβ3, and downstream phosphorylation of Akt contribute to activation of MMP2, and eventually invasiveness of pancreatic cancer.

Previous studies showed that C16orf74 interacted to PPP3CA (Calcineurin: CN) and was important for proliferation and invasion of PDAC [6,11]. This study demonstrated that C16orf74 might be the molecule that connected the integrin signal and CN signal. C16orf74 transfers signals from the integrin on the cell membrane to the cytoplasmic CN and activates cell proliferation and invasion through the Akt/mTOR pathway. The C16orf74 protein was found to consist of a localization domain for targeting the molecule inside the cell membrane and a functional domain for binding to CN. The localization domain is also important for dimer formation. Therefore, two blocking points were revealed in the development of therapy targeting C16orf74. One is dimer inhibition of C16orf74, that results in signal inhibition from integrin. The other is inhibition of binding between C16orf74 and PPP3CA, that is, signal inhibition to PPP3CA [11]. It is also possible to block both, however, the binding site between C16orf74 and PPP3CA has the consensus binding sequence of nuclear factor of activated T cells, therefore inhibition of binding has a risk of immunodeficiency by T cell inhibition [11]. The newly discovered dimer inhibition is C16orf74-selective and appears to be
extremely useful from the viewpoint of side effects in the development of therapeutic drugs.

The polyarginine signal achieve highly efficient nonspecific uptake of peptides into cells [20]. Thus, when DN-C16orf74 is used in a clinic, systemic administration of the peptide may not be able to achieve sufficient concentrations in tumor due to uptake by normal vascular endothelial cells. In this regard, cancer cell-specific cell-permeable peptide signals, which can penetrate only the target cells in vitro and in vivo [21], might be applicable in developing DN-C16orf74 for clinical use. Another important issue is the stability of peptides in the blood. In clinical, peptide drugs such as carperitide (α-human A-type natriuretic peptide) are used in continuous injections, because the half-life of the peptide is very short [22]. The clinical application of DN-C16orf74 requires technology that modifies the stabilization of peptides in blood. Another clinical approach for DN-C16orf74 is intraperitoneal administration (IP) for patients with PDAC with peritoneal metastases. A phase II clinical trial of IP paclitaxel has recently been reported for patients with PDAC with peritoneal metastases [23]. In our peritoneal metastasis model, IP DN-C16orf74 peptide treatment was extremely effective, therefore combination therapy can be considered for the peritoneal metastasis of PDAC for clinical trial in the future.

In conclusion, through the significant reduction of Akt and mTOR phosphorylation, DN-C16orf74 inhibited C16orf74 dimerization and suppressed PDAC proliferation in vitro and in vivo. These results suggest that DN-C16orf74 is a potential therapeutic option for patients with PDAC. However, the
limitations of our study are (1) the off-target effect of DN-C16orf74 peptides on
the binding inhibition of nonspecific proteins is not examined, (2) intravenous
peptide administration is not achieved, (3) the drug dynamics after the peptide
administration is not revealed. Therefore, further studies will be necessary to
overcome the above limitations.

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**Figure Legends**

Figure 1. Functional analysis of C16orf74 overexpressed stable transfectant cell line

(A) Observation of cell morphology of C16orf74 stable (A-1) and MOCK cells (A-2) by Immunofluorescence (Red: F actin, Green: paxillin). i: The polarity of the cell was strong, and the direction of cell movement (arrow) was clear. The development of lamellipodium was strong. ii: Filopodia was developing in the
direction of cell progression. The development of actin was strong. iii: The array of focal adhesions was irregular, and the development of Actin fiber was also weak. iv: Focal adhesion existed in the circumference and no polarity was recognized. (B) Pull down assay for Rac1-GTP. C16orf74 stable showed that expression of Rac1-GTP was increased compared to MOCK. GTPyS: positive control, GDP: negative control (C) WST assay (C16orf74 stable vs MOCK cells, comparison of 3 cell lines). C16orf74 stable showed an increase in cell proliferation compared with MOCK. (D) Matrigel invasion assay (C16orf74 stable vs MOCK cells, comparison of 3 cell lines). C16orf74 stable showed a significant increase in invasiveness. (E) Wound healing assay (C16orf74 stable vs MOCK cells). Cell migration ability was higher in C16orf74 with significant difference (* p < 0.01). (F) Hematogenic invasion assay (C16orf74 stable vs MOCK cells). C16orf74 stable had significantly more cells infiltrated beyond HUVEC compared to MOCK (** p < 0.05). (G) Western blot analysis for MMP2, MMP9, Akt, p-Akt, ERK, p-ERK, mTOR, p-mTOR in C16orf74 stable and MOCK cells. C16orf 74 stable cells showed increased phosphorylation form of Akt. (H) Gelatin zymography for MMP2 and MMP9 in C16orf74 stable and MOCK cells. Left: image of gels, Right: Band quantitative graph (value of C16orf74 based on MOCK=1). C16orf74 stable cells showed an increase in the amount of activated MMP2 (62 kDa) and a decrease in nonactivated MMP 2 (pro-MMP 2: 72 kDa) compared to MOCK. In addition, MMP9 showed an increase in activity although it was lower than MMP2.
Figure 2. C16orf74 forms a homodimer and localizes under the cell membrane.

(A) Amino acid sequence of C16orf74. (B) Native PAGE of C16orf74 stable (3 cell lines) and MOCK. Since C16orf74 stable showed two bands in Native PAGE, C16orf74 was considered to exhibit a homodimer form. Upper row: Native PAGE, lower row: SDS-PAGE, four lanes on the left: SDS and 2-mercaptoethanol (-), Boil (-) at the time of cell lysate collection, four lanes on the right: Cell lysate was collected as described earlier. (C) Proof of binding of C16orf74/FLAG and C16orf74/HA by pull down assay. C16orf74/FLAG and C16orf74/HA bind directly (form homodimers). (D) Immunofluorescence analysis of the changes in the localization of C16orf74 by administration of 2-hydroxymyristic acid to C16orf74 stable cells. Compared with Control (DMSO 0.125 %), in the 2-hydroxymyristic acid administration group, the localization of C16orf74 was changed from cell membrane to cytoplasm diffuse. (E) Intracellular localization of C16ord74/WT, C16orf74/G2A, C16orf74/C7A_C14A, C16orf74/Δ2-15, and T44A. C16orf74/WT and C16orf74/T44A localized under the cell membrane of COS7 cells. Conversely, C16ord74/G2A, C16orf74/C7A_C14A, and C16orf74/Δ2-15 could not localize to the cell membrane. (F) Pull down assay of C16orf74/G2A, C16orf74/C7A_C14A, C16orf74/Δ2-15, C16orf74/T44A (with FLAG tag) and C16orf74/WT (with HA tag). C16orf74/WT and C16orf74/T44A formed dimers, but other constructs did not form dimers. (G) Schematic representation of the
structure of C16orf74. We hypothesized that the first 15 amino acids of the C16orf74 protein is involved in the structure of the protein, dimer formation at disulfide binding site C7 and C14, and fixation to the cell membrane by G2 which is the N-myristoylation site.

Figure 3. Suppression of cell proliferation, invasion, and migration by inhibiting homodimers of C16orf74 by dominant negative peptides.

(A) Peptide design of 11R-DB (Dimer Block), 11R-C7A_C14A and 11R-7_14AAA and the schematic images of the effect of each peptide on dimerized C16orf74. (B) Pull down assay of C16orf74/FLAG and C16orf74/HA with 11R-DB (0-30 µM). The binding of each construct was inhibited in a concentration-dependent manner by 11R-DB. (C) Pull down assay of C16orf74/FLAG and C16orf74/HA with PBS, 11R-7_14AAA (30 µM), 11R-C7A_C14A (30 µM), 11R-DB (30 µM). Control (PBS) and 11R-7_14 AAA did not affect dimerization of C16orf74. 11R-DB inhibited each binding, the effect was weaker than 11R-DB, but 11R-C7A_C14A also inhibited binding. (D) WST assay for PDAC cell lines and NHDF (normal fibroblast) for each peptide (0-30 µM, 24 h). (E) Matrigel invasion assay of PDAC in presence of the 11R-DB peptide (0, 10, and 20 µM). (F) Wound healing assay for PDAC with 11R-DB peptide (0 or 10 µM). (G) Changes in cell morphology after 11R-DB administration (immunofluorescence of C16orf74 stable, Green: paxillin, Red: f actin). The localization of the focal adhesion became random (i) or pericytes (ii), and the number of polarity-lost cells were increased. Additionally, the form of actin changed randomly. (H) Changes in the localization of C16orf74 by 11R-DB administration
immunofluorescence of C16orf74 stable cells, Green: C16orf74/FLAG, Red: f 
actin). (a): Control (PBS), (b): 11R-DB. By 11R-DB administration, the 
localization of C16orf74 moved from the plasma membrane into the cytoplasm. 
Expression of C16orf74 between cells was strong and remained even after 
administration of 11R-DB (Yellow circle). (c): Actin fiber was broken as seen 
when using Rho Inhibitor, and development of cell membrane also weakened. 
NS; No significance, *; p < 0.05, **; p < 0.01.

Figure 4. C16orf74 binds directly to Integrin αVβ3, and controls the 
PI3K/Akt/mTOR cascade

(A) Search for molecules that bind C16orf74. Pull down assay of C16orf74/WT 
(with FLAG tag) and Integrin β1-4 (ITG B1-4), ILK, PPP3CA, PDK1 and paxillin 
(PXN) (with HA tag) revealed that C16orf74 and ITGB3, ILK and PPP3CA 
strongly bound to each other. (B) C16orf74/FLAG and Integrin β3/HA were co-
localized under the cell membrane of COS7 cells. Development of cell 
membrane was confirmed at co-localized site. (C) The binding of C16orf74, 
Integrin αV and Integrin αIIb was verified by pull down assay 
(immunoprecipitated with FLAG anti body and IgG2a as an isotype control) in 
C16orf74 stable cells (3 types) and MOCK. The degree of binding varied for 
each cell line. (D) Pull down assay of C16orf74/FLAG and Integrin β3/HA with 
11R-DB (0-30 µM). The binding of each construct was inhibited in a 
concentration-dependent manner by 11R-DB. (E) Western blotting of Akt, p-
Akt, mTOR, p-mTOR, p42/44 MAPK(ERK), p-p42/44 MAPK (p-ERK), MMP2 
and MMP9, and β-actin in 30 µM 11R-DB, 11R-C7A_C14A, 11R-7_14AAA, and
PBS (control), treated PK-1, PK-9, PANC-1 and MIA PaCA-2 cells. (F) Gelatin zymography of PK-1 and PK-9 cells with 11R-DB (0-30 µM). left: image of gels, right: Band quantitative graph. 11R-DB caused a decrease in activated MMP2, but did not significantly affect active MMP9.

**Figure 5. 11R-DB showed antitumor effect on pancreatic tumor model mice**

(A) Treatment schedule of orthotopic model. (B) Representative examples of bioluminescence imaging after tumor cell injection at 5 weeks (4 weeks after initiation of therapy). Representative cases of each groups are shown. (C) The graph shows the signal intensities recorded from individual mice for each week and group. The signal intensity at 1 week was set at 1 as a control for each mouse. Each group had six mice. Data are presented as mean ± SEM. (11R-DB vs 11R-7₋14AAA/PBS *p<0.05, 11R-7₋14AAA vs PBS: n.s.) (D) Tumor weights at 5 weeks after tumor cell injection in each group (n=6). (11R-DB vs 11R-7₋14AAA/PBS: *p < 0.05, 11R-7₋14AAA vs PBS: n.s.) (E) Immunohistochemical staining of phosphorylated Akt, phosphorylated mTOR and Ki-67 (MIB-1) in PDAC tumors in vivo. A representative example is shown. (F) Ki-67 Index in tumors of each group (PBS, 11R-7₋14AAA and 11R-DB).

**Figure 6. 11R-DB inhibited tumor cell engraftment of pancreatic cancer peritoneal dissemination model mice**

(A) Treatment schedule of peritoneal dissemination model. (B) The graph shows the signal intensities recorded from individual mice for each day and group (n = 5). (11R-DB vs 11R-7₋14AAA/PBS: *p < 0.05, 11R-7₋14AAA vs...
PBS: n.s.) Data are presented as the mean ± SEM. (C) Representative examples of bioluminescence imaging of peritoneal dissemination model mice. (D) The number of dissemination nodules at 35 days after tumor cell injection in each group (n=5). (11R-DB vs PBS: *p < 0.05, 11R-DB vs 11R-7.14AAA **p < 0.05 11R-7.14AAA vs PBS: n.s.) (E) Representative examples of bioluminescence imaging of peritoneal dissemination nodules (after sacrifice) in the three groups of mice (PBS, 11R-7.14AAA and 11R-DB).
Figure 1

**A**

<table>
<thead>
<tr>
<th>1</th>
<th>C16orf74</th>
<th>MOCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
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<td><img src="image2.png" alt="Image" /></td>
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<td>ii</td>
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**B**

<table>
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<th>IB:RAC1</th>
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<td>Control</td>
<td>GTPiS</td>
</tr>
<tr>
<td>C16orf74</td>
<td>MOCK</td>
</tr>
</tbody>
</table>

**C**

Graph showing relative cell viability (% day1) for different conditions.

**D**

Bar graph showing cells per field (×100) for different conditions.

**E**

Graph showing % of wound closure against time of incubation (h).

**F**

Bar graph showing cells per field (×100) for C16orf74 and MOCK.

**G**

Western blot analysis of various proteins including C16orf74.

**H**

Western blot analysis showing proMMP2 and MMP2, proMMP9 and MMP9.
Figure 2

A: C16orf74 protein

MGLKMSCLKGFMVCVSSSSSHDEA
PVLDNKHDLPVDIITPPPTGMMLPR
DLGSTWVLDEMYSCPDDGEIDPEA

*: N-myristoylation site
#: S-S binding site
□: PXIXIT motif (PPP3CA interaction)

B: IB FLAG

Dimer 120kDa
Monomer 60kDa

Native PAGE

SDS PAGE

Sample buffer (10% SDS, 2-mercaptoethanol)

C: IP:FLAG

Input

(kDa)

12

IB:HA

IB:FLAG

(kDa)

12

IB:HA

IB:FLAG

D: Control: DMSO 0.125% OH-myr 2μM

Green:C16orf74/FLAG

Green:C16orf74/FLAG

E: C16orf74 WT

C16orf74/C7A_C14A

C16orf74/G2A

C16orf74/Δ2-15

C16orf74/T44A

F: IP:FLAG

(kDa)

12

IB:HA

IB:FLAG

12

IB:HA

IB:FLAG

12

IB:HA

IB:FLAG

Input

(kDa)

12

IB:HA

IB:FLAG

12

IB:HA

IB:FLAG

12

IB:HA

IB:FLAG

12

IB:HA

IB:FLAG

12

IB:HA

IB:FLAG

12

IB:HA

IB:FLAG

12

IB:HA

IB:FLAG

12
Figure 3

A

B

C

D

E

F

G

H

11R-DB
11R-C7A.14A
11R-7.14AAA

IP:FLAG
IP:HA
Input

11R-DB
0 10 20 30 µM

11R-C7A.C14A
11R-7.14AAA

IP:FLAG
IP:HA
Input

Matrigel invasion assay

Cells per field (×100) (Normalized 0µM)

11R-DB
0µM 10µM 20µM 30µM

11R-DB
0µM 10µM 20µM 30µM

Red: f actin
Green: Paxillin

11R-DB (-)
11R-DB (+)

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Figure 4

A

B

C

D

E

F

Gammas and betas.

Phase contrast

Merge

C16orf74 /FLAG

ITG B3 / HA

MMP9 (active)

MMP2 (active)

PK-1

PK-9

MMP2

MMP9

p-AKT

AKT

p-ERK

ERK

p-mTOR

mTOR

Actin

PK-1

PK-9

MMP9

MMP2

p-AKT

AKT

p-ERK

ERK

p-mTOR

mTOR

Actin

PK-1

PK-9

Relative value quantity

0 1 2 3

0 1 2 3

0 1 2 3

0 1 2 3

0 1 2 3

0 1 2 3

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Figure 5

A

Day 0

Day 7 → Treatment → Day 35

11R-DB 5 times/week ip 10mg/kg/mouse

day 7  day 14  day 21  day 28  day 35

Sacrifice and analyze

In vivo imaging

B

C

Relative increase of photons (Photons/sec/mouse) vs. days

11R-DB  11R-7_14AAA  PBS

D

Tumor weight (mg)

PBS  11R-7_14AAA  11R-DB

E

Ki-67  p-AKT  p-mTOR

11R-DB  11R-7_14AAA  PBS

F

Ki-67 Proliferation index (%)

PBS  11R-7_14AAA  11R-DB

*  **
Figure 6

A

Day 0

Treatment

PK-9/Luc2

3 x 10^9 IP

Day 35

Sacrifice and analyze

In vivo imaging

B

photons

(Photons/sec/mouse)

3 7 14 21 28 35

days

PBS 11R-7_14AAA 11R-DB

C

PBS 11R-7_14AAA 11R-DB

Day 3

Day 7

Day 14

Day 28

D

Dissemination nodules (counts)

PBS 11R-7_14AAA 11R-DB

E

Luminescence

Radiance

(photons/sec/cm²/sr)

Color Scale

Min = 5.00e-7

Max = 5.00e-7

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