CKAP4, a DKK1 receptor, is a biomarker in exosomes derived from pancreatic cancer and a molecular target for therapy

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Running title: CKAP4 may be a biomarker and a therapeutic target for PDAC

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**Translational Relevance**

Pancreatic cancer is one of the most frequent causes of cancer death in the world, thus, novel molecularly targeted therapy and companion diagnosis are required. Here, we asked whether cytoskeleton-associated protein 4 (CKAP4), a novel Dickkopf1 (DKK1) receptor, is a candidate for pancreatic cancer diagnosis and therapy. We found that CKAP4 is released with exosomes from pancreatic cancer cells, in which DKK1 was overexpressed and CKAP4 was localized to the plasma membrane, and that the secretion of CKAP4-containing exosomes is mediated by DKK1-dependent endocytosis routes. We developed enzyme-linked immuno-sorbent assay to detect CKAP4 in pancreatic cancer patient sera using anti-CKAP4 monoclonal antibodies (mAbs), which also exhibited anti-tumor effect. CKAP4 secreted with exosomes in serum may represent a biomarker for pancreatic cancer, and anti-CKAP4 mAbs could be useful for the development of a molecularly targeted therapy and a companion diagnostic agent for pancreatic cancer.
Abstract

**Purpose:** The survival rate of pancreatic ductal adenocarcinoma (PDAC) is poor, thus novel molecularly targeted therapy and companion diagnostics are required. We asked whether cytoskeleton-associated protein 4 (CKAP4), a novel Dickkopf1 (DKK1) receptor, is a candidate for PDAC diagnosis and therapy.

**Experimental Design:** Whether CKAP4 can be secreted with small extracellular vesicles (SEVs) from PDAC cells was examined. It was also investigated whether CKAP4 can be detected in sera from PDAC patients by enzyme-linked immuno-sorbent assay (ELISA) using newly generated anti-CKAP4 monoclonal antibodies (mAbs) and whether anti-CKAP4 mAbs can show anti-tumor activity *in vivo*.

**Results:** CKAP4 was secreted with SEVs from PDAC cells, and the SEVs exhibited the characteristics of exosomes. The secretion of CKAP4-containing exosomes was mediated by DKK1-dependent endocytosis routes and required exosome biogenesis molecules. Two ELISAs capable of detecting tumor-secreted CKAP4 were developed. The serum CKAP4 levels were higher in PDAC patients than healthy control individuals. CKAP4 was highly detected in the sera of pancreatic tumor-bearing xenografted mice and PDAC patients, whereas CKAP4 was barely detectable in sera from normal mice and post-operative patients. Anti-CKAP4 mAbs with different epitopes demonstrated the inhibitory activities for the binding of DKK1 and CKAP4, AKT activity, and proliferation and migration of PDAC cells. Anti-CKAP4 mAbs also suppressed xenograft tumor formation in immunodeficient mice and extended the survival of mice receiving intraperitoneal or orthotopic injection of PDAC cells.

**Conclusions:** CKAP4 secreted in exosomes may represent a biomarker for PDAC. Anti-CKAP4 mAbs can contribute to the development of novel diagnostic methods and therapeutics.
Introduction

Pancreatic cancer is extremely aggressive and exhibits poor prognosis compared with other gastrointestinal cancers; the 5-year survival rate is only 5% and a median survival time is less than 6 months (1). Although surgical resection is the only curative treatment, more than 80% of patients have either locally advanced or metastatic disease and the tumor is unresectable at the time of diagnosis (1, 2). Chemotherapy with gemcitabine (GEM), a nucleoside analog, alone or in combination with nab-paclitaxel or FOLFIRINOX for pancreatic cancer prolonged overall survival (3, 4). However, satisfactory long-term prognoses were not achieved and a more effective drug is needed to further improve prognosis.

Cytoskeleton-associated protein 4 (CKAP4), a type II transmembrane protein, is an endoplasmic reticulum (ER) protein and organizes the overall structure of the ER by binding to microtubules (5, 6). CKAP4 has also been shown to be localized to the plasma membrane (PM) where it functions as a receptor of various ligands (7). In addition, it has been reported that CKAP4 is a receptor for Dickkopf (DKK) family proteins (8-10). DKK1 is a secretory protein that inhibits the β-catenin-dependent pathway (β-catenin pathway) in Wnt signaling by binding to a Wnt receptor, low-density lipoprotein receptor-related protein 6 (11, 12). DKK1 and DKK3 have oncogenic properties in various cancer types (11, 13-15). Furthermore, the DKKs-CKAP4 signaling axis is shown to be involved in tumorigenesis through the activation of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway (7, 8). Simultaneous expression of DKK1 and CKAP4 in pancreatic ductal adenocarcinoma (PDAC), lung adenocarcinoma, lung squamous cell carcinoma (SCC), and esophageal SCC is associated with poor prognosis in patients (8-10). Furthermore, an anti-CKAP4 polyclonal antibody (pAb) can inhibit xenograft tumor formation caused by these cancer cell lines, suggesting that CAKP4 represents a novel molecular target for diagnosis and therapy of cancers.

Cancer cells secrete small extracellular vesicles (SEVs, also known as exosomes) that function in an autocrine and paracrine manner to promote tumor-induced immune suppression, angiogenesis,
and premetastatic niche formation (16). SEVs are membrane vesicles that originate in large multivesicular bodies (MVBs) and are released into the extracellular milieu upon fusion of MVBs with the PM (17, 18). SEV proteins are promising candidates for cancer biomarkers (19). For instance, epithelial cell adhesion molecule is a SEV biomarker found in the ascites of ovarian cancer patients (20) and glypican-1 released with SEVs has been proposed as a biomarker for the early diagnosis of pancreatic cancer (21). Since CKAP4 is localized to the PM in certain cancer types, we hypothesized that CKAP4-containing SEVs might be released from cancer cells.

In this study, we found that CKAP4 is released with exosomes from PDAC cells. We subsequently developed methods to detect CKAP4 in PDAC patient sera using anti-CKAP4 monoclonal antibodies (mAbs). In addition, anti-CKAP4 mAb efficiently suppressed xenograft tumor formation and extended the overall survival of mice receiving intraperitoneal or orthotopic injection of PDAC cells. Thus, CKAP4 might represent a biomarker and molecular target for PDAC therapy.
Materials and Methods

Materials and chemicals

All cell lines, antibodies, and other chemicals used in this study are shown in Supplementary Table S1 and Table S2, respectively. Cell line authentication and mycoplasma testing were not performed. The target sequences for siRNA experiments are shown in Supplementary Table S3. Primers used in qPCR experiments are shown in Supplementary Table S4. GEM-resistant (GEM-R) cells were generated by culturing with GEM, of which concentrations were increased from 50 nM to 5 μM. Anti-CKAP4 pAb was generated previously (8).

Sandwich enzyme-linked immuno-sorbent assay (ELISA) for measuring CKAP4 concentration

ELISA were performed as previously described (22, 23). Ninety six-well plates were coated with 100 μl of anti-CKAP4 mAb (3F11-2B10) (2 μg/ml) and incubated at RT overnight. After washing away any unbound antibody using wash buffer, reagent diluent was added to the wells and incubated for one hour at RT. After washing, 50 μl of concentrated CM or blood sample were added to the wells and incubated for two hours at RT. After washing away any unbound substances, 100 μl of biotinylated anti-CKAP4 mAb (1G4-4A9) (1 μg/ml) were added to each well and incubated for one hour at RT. After washing, 50 μl of HRP-streptavidin (DY998, R&D Systems, Inc.) was added to the wells and incubated for 20 minutes at RT. Then, a substrate solution (DY999, R&D Systems, Inc.) was added to the wells and allowed to react for ten minutes. The reaction was stopped by adding 50 μl of STOP solution (Cell Signaling Technology, Beverly, MA). Color intensity was calculated by subtracting the reading at 540 nm from the reading at 450 nm on a multimode plate reader. As a reference, a standard curve was prepared for every assay by making serial dilutions of recombinant CKAP4 protein. When the CKAP4 concentration was less than 1 ng/ml (the lower limit of the standard curve), the value was defined as zero.
Exosome ELISA for measuring CKAP4 in SEVs

Fifty ml of CM from cultured cells were subjected to sequential centrifugation steps of 2,000 x g and 10,000 x g; the supernatant was concentrated to one ml using an ultrafiltration unit (Vivaspin 20). CKAP4 in SEVs was measured using a PS Capture™ Exosome ELISA Kit (Wako) according to the manufacturer’s recommendations. One hundred µl of biotin-labeled anti-CKAP4 mAb (5A6-17A11) (100 ng/ml) was used as the detection antibody.

Inter- and intra-variability of the ELISA

Serum CKAP4 levels of four cases were measured in triplicate (well) by exosome ELISA and sandwich ELISA in three independent experiments (plates). For log-transformed CKAP4 levels, the Gaussian linear mixed-effect model (24) with intercept and plate as random-effects was applied to evaluate intra- and inter-variabilities. The variances of intercept, plate, and residual were interpreted as those of inter-subject, intra-plate, and intra-well variances, respectively, and the proportions of the variances of the inter-subject, intra-plate, and intra-well relative to the total variance were examined.

Human serum and tissues samples

Serum samples were obtained with informed consent from 20 PDAC patients (median age of 71 with a range of 50 to 87 years) who underwent surgical resection at Osaka University hospital between August 2012 and July 2017 and 27 PDAC patients (median age of 68 with a range of 32 to 84 years) admitted to Osaka University Hospital from April 2014 to July 2015. In PDAC cases, tissue samples were obtained from the former 20 cases. In the latter 27 cases, 7 resectable cases and 20 unresectable cases were included. In total, 27 resectable cases and 20 unresectable cases were analyzed. A total 47 PDAC patients were newly diagnosed and previously untreated and their tumors were pathologically diagnosed. Sera from 20 colon polyp patients (median age of 62 with a range of 49 to 69) admitted to Osaka University Hospital from September 2015 to September 2016 were used to measure serum
CKAP4 levels as the control group. In colon polyp cases, there were no cancerous lesions pathologically. Serum was obtained at the time of diagnosis. The pathological diagnosis for surgical resection specimens was performed as previously described (8, 9). Eighteen healthy control (HC) individuals serum samples were purchased from Clinical Trials Laboratory Services (London, United Kingdom), Jackson ImmunoResearch Inc. (West Grove, PA, USA), and BioIVT (Westbury, NY, USA).

**Generation of anti-CKAP4 mAb**

A mouse mAb that specifically recognized CKAP4 was generated based on the mouse medial iliac lymph node method (25) or spleen method (26) at Cell Engineering Corporation (Osaka, Japan). In the medial iliac lymph node method, 10-week-old female mice were injected at the tail base with 100 μl of emulsions containing GST-human CKAP4 (aa 128-602) and Freund’s complete adjuvant. Seventeen days after the first immunization, an additional immunization of CKAP4 protein was performed without adjuvant into the tail base of the mice. Four days after the additional immunization, cells from the iliac lymph nodes of the immunized mouse were fused with mouse myeloma Sp2/0-Ag14 cells at a ratio of 5:1 in 50% polyethylene glycol.

In the spleen method, 8-week-old CKAP4 KO mice were injected intraperitoneally with 100 μl of emulsions containing GST-human CKAP4 (aa 468-602) and Freund’s complete adjuvant. An additional immunization of CKAP4 protein was performed intraperitoneally with Freund’s complete adjuvant in the mice a total of four times every 14 days from the first immunization. Spleen cells from immunized mice were harvested 14 days after the last immunization, then fused with mouse myeloma Sp2/0-Ag14 cells at a ratio of 5:1 in 50% polyethylene glycol. The resulting hybridoma cells were plated onto 96-well plates and cultured in HAT selection medium.

Anti-CKAP4 antibodies were screened using the following methods: ELISA, detection of endogenous CKAP4 by western blotting, immunostaining of CKAP4 on the PM, and blocking the
binding of DKK1 and CKAP4 using hybridoma supernatants. Finally, hybridoma clones producing mAb named 83-2C8, 73-1C12, and 52-2G9 were obtained from the medial iliac lymph node method and those producing 1G4-4A9, 3F11-2B10, and 5A6-17A11 were obtained from the spleen method. The mAbs 83-2C8, 73-1C12, 1G4-4A9, and 3F11-2B10 were found to be an IgG2b (k), while 52-2G9 and 5A6-17A11 were found to be an IgG2a (k) subtype using a mouse isotyping kit.

**Generation of CKAP4 KO mice**

CKAP4 KO mice were produced by pronuclear injection of the circular pX330 plasmid as described previously (27). Four single guide RNAs (sgRNAs) targeting mouse *Ckap4* exon 1 were designed with the help of CRISPR Genome Engineering Resources (http://crispr.mit.edu/). Genotyping was performed by direct sequencing following PCR. Forward and reverse primers were as follows; 5’-CCATGCCCTCGGCCAAACAAAG-3’ and 5’-AGGACGCGGAGGACGCGTTG-3’. For validation of the efficiency of sgRNAs in DNA double-strand break at the target DNA site, green fluorescence reconstitution by homology-directed repair of an EGFP expression cassette was employed.

The pCAG-EGxxFP plasmid containing the sgRNA target sequence between split EGFP fragments and the pX330 plasmid with sgRNA sequences targeting CKAP4 were co-transfected into HEK293T cells. EGFP fluorescence, which was induced by cutting and repair via HDR upon the overlapped EGFP sequences in the split EGFP cassette, was observed 48 hours after transfection. The sgRNA that exhibited a high activity in an *in vitro* assay was selected for zygote injection (C57 BL/6N). Homozygous mice that have a 67 bp deletion resulting in frameshift mutations yielding prematurely truncated proteins were obtained by breeding of F1 heterozygous mice. Mating of CKAP4 KO mice produced litters of normal size and progeny that appeared phenotypically normal, indicating that homozygous mutant mice are fertile.
Peritoneal dissemination and orthotopic transplantation assays

Six-week-old male BALB/cAnNCrj-nu immunodeficient mice were anesthetized and received an intraperitoneally or orthotopic injection of S2-CP8 cells (3 x 10^6 cells suspended in 200 µl of PBS for intraperitoneally injection and 1 x 10^6 cells suspended in 50 µl of PBS with 1% Matrigel for orthotopic transplantation). Orthotopic transplantation assay was performed as previously described (28) with modification. After 2 days, anti-CKAP4 mAb 3F or control IgG (50 µg/body in intraperitoneally injection assay or 200 µg/body in orthotopic transplantation assay) were injected into the intraperitoneal cavity twice per week for 3 weeks. In orthotopic transplantation assay, at least four immunodeficient mice in each group were then sacrificed at 20 days and pancreatic tumors were weighed.

Statistics

All experiments except experiments with human serum were repeated at least three times and the results are expressed as means ± s.d. Statistical analyses were performed using JMP version 11 and SAS version 9.4 (SAS Institute. Inc., Cary NC). Mean of continuous outcome variables were tested with the Student’s t test. The absorbance values in exosome ELISA and the concentrations of CKAP4 in sandwich ELISA were tested with Wilcoxon rank sum test. The areas under the curves (AUCs) were calculated on the receiver operating characteristics (ROC) curves. Cumulative probability of overall survival was computed with the Kaplan-Meier method and the log-rank test was used to assess statistical significance. P-values less than 0.05 were considered statistically significant. Western blotting data are representative of at least three independent experiments except Figs. 1F and 4B (they were done twice).

Study approval

The protocol for human specimens was approved by the ethical review board of the Graduate School
of Medicine, Osaka University, Japan (No. 13455 and 17160) under Declaration of Helsinki, and were performed in accordance with the Committee guidelines and regulations. The written informed consent was obtained from all patients. All protocols used for animal experiments in this study were approved by the Animal Research Committee of Osaka University, Japan (No. 21-048-1).
Results

**CKAP4 is secreted with exosomes from PDAC cells**

CKAP4 was expressed in various cells, including non-tumor and cancer cells, but its PM localization was variable (7). CKAP4 was present in the PM of PDAC S2-CP8, HPAF-II, and PANC-1 cells and non-tumor X293T, Eph4, and MDCK cells, yet it was barely detectable in the PM of PDAC BxPC-3 cells (Fig. 1A). CKAP4 was found in 100,000 x g precipitates, which is thought to include SEVs, of CM from S2-CP8, HPAF-II, and PANC-1 cells; CKAP4 was present at lower levels in SEVs from X293T cells and barely detectable in SEVs from BxPC-3, Eph4, or MDCK cells (Fig. 1A). CKAP4 in 100,000 x g precipitates was increased by overexpression of CKAP4 in S2-CP8 cells and was lost by knockout (KO) of CKAP4, while levels of SEV markers, clathrin, and tumor susceptibility gene 101 (TSG101), were unchanged (Supplementary Fig. S1A). CKAP4 was minimally found in 10,000 x g precipitates, also known as microvesicles (29), of CM from S2-CP8 cells (Supplementary Fig. S1B). Fetal bovine serum (FBS) and exosome-depleted FBS did not affect the amount of CKAP4 in 100,000 x g precipitates from CM of S2-CP8 cells and CKAP4 was not found in 100,000 x g precipitates from FBS itself (Supplementary Fig. S1C).

Localization of CKAP4 in SEVs was further confirmed using the following experiments. When 100,000 x g precipitates were subjected to a discontinuous sucrose gradient, CKAP4 sedimented with CD81, TSG101, and clathrin at a density of 1.136 - 1.172 (Fig. 1B). It was reported that a phosphatidylserine-binding protein, T-cell immunoglobulin- and mucin-domain-containing molecule (Tim4), was able to precipitate SEVs (30). In the assay using Tim4, CKAP4 was detected in SEVs from CM of S2-CP8 and S2-CP8/CKAP4-HA cells, but not S2-CP8/CKAP4 KO cells (Supplementary Fig. S1D).

DKK1 was expressed more abundantly in S2-CP8 cells than non-tumor cells (Fig. 1A), and CKAP4-containing SEVs were decreased after KO of DKK1 (Fig. 1C). Expression of wild-type
(WT) DKK1 rescued the phenotype, but DKK1Δcysteine-rich domain1 (CRD1), which failed to bind to CKAP4, did not (Fig. 1C). Similar results were also observed in MDCK cells (Supplementary Fig. S1E). In addition, knockdown of clathrin or treatment with monodansylcadavelin that impairs clathrin-mediated endocytosis (31) inhibited the secretion of CKAP4 with SEVs from S2-CP8 cells; further, the amounts of PM-localized CKAP4 increased in reverse proportion to the amount of CKAP4 found with SEVs (Fig. 1D and Supplementary Fig. S1F). Therefore, CKAP4 outside of the cells is likely to originate from the PM, and CKAP4 levels in the SEVs can reflect cell surface expression of CKAP4 in cancer cells.

Many proteins are involved in SEV biogenesis (17). They are the endosomal sorting complex required for transport with the associated proteins (TSG101, HRS, and ALIX), the tetraspanin proteins (CD9, CD63, and CD81), small G proteins (Rab27a and Rab27b), and vacuole sorting proteins (vacuolar protein sorting (VPS) 26a and VPS35). The protein composition of SEVs varies by vesicle (32). When SEV markers were knocked down in S2-CP8 cells, the amount of CKAP4 associated with SEVs was clearly decreased in ALIX-, Rab27a-, Rab27b-, VPS26a-, and VPS35-depleted cells (Fig. 1E and Supplementary Fig. S1G). Taken together, these results indicate that CKAP4-containing SEVs are exosomes.

To examine whether CKAP4 was released with exosomes in vivo, S2-CP8 or HPAF-II cells were inoculated into the dorsal subcutaneous tissue of immunodeficient mice. The mice were bled when tumors reached a volume of 1,000 mm³; exosomes in mouse serum were precipitated using Tim4 (MagCapture™ Exosome Isolation Kit PS). Using Western blotting analysis, CKAP4 was detected in exosomes from the serum of mice transplanted with cancer cells, although CKAP4 barely detectable in the normal serum of non-transplanted mice (Fig. 1F). In order to easily measure CKAP4 in exosomes from serum with high sensitivity, we generated anti-CKAP4 mAbs and develop ELISA.

**Anti-CKAP4 mAbs are generated and characterized**
CKAP4 KO mice were generated using the CRISPR/Cas9 system (Supplementary Fig. S2A-C) to analyze CKAP4 functions. CKAP4 mRNAs and proteins were expressed in various organs in WT mice (Supplementary Fig. S2D and E), while CKAP4 protein expression was completely lost in CKAP4 KO mice (Supplementary Fig. S2E). However, CKAP4 KO mice appeared phenotypically normal and were indistinguishable from their heterozygous and WT littermates from birth to 18 months. Detailed histological analysis of various organs in CKAP4 KO mice did not show any differences from WT mice (Supplementary Fig. S2F). Since Human and mouse CKAP4 have high similarity at the amino acid (aa) level, especially in the extracellular domain (ECD) in which they have 82% identity and 90% similarity, CKAP4 KO mice were used to obtain anti-CKAP4 mAbs efficiently.

WT and CKAP4 KO mice were immunized with the ECD, aa 128-602 and 468-602, respectively, from human CKAP4 and six different anti-CKAP4 mAbs were obtained (Fig. 2A). Three hybridomas established from WT mice produced CKAP4 mAbs, 83-2C8 (83, IgG2b), 52-2G9 (52, IgG2a), and 73-1C12 (73, IgG2b). These three mAbs recognized different deletion mutants of CKAP4 (Supplementary Fig. S3A and B), suggesting that they recognize different epitopes. To define the CKAP4 epitopes recognized by these mAbs, 38 different 17-aa peptides, in which 7-aa are overlapping adjacent peptides, were generated based upon the human CKAP4 aa sequence (Pepspot). The predicted epitopes recognized by mAbs 83, 73, and 52 were aa 302-310, aa 451-455, and aa 481-485, respectively (Fig. 2B and Supplementary Table S5).

Three additional hybridomas selected from CKAP4 KO mice produced CKAP4 mAbs: 1G4-4A9 (1G, IgG2b), 3F11-2B10 (3F, IgG2b), and 5A6-17A11 (5A, IgG2a). mAb 1G recognized CKAP4-(Δ525-602), but not CKAP4-(Δ503-602) (Supplementary Fig. S3A). Pepspot analysis revealed that mAb 1G recognizes aa 502-510 (Fig. 2B and Supplementary Table S5). mAb 5A bound to CKAP4-(Δ590-602) strongly, bound weakly to CKAP4-(Δ587-602), and did not bind to CKAP4-(Δ585-602) (Fig. 2C). Unlike mAb 5A, mAb 3F still bound to CKAP4-(Δ570-602) and
CKAP4-(Δ525-602) weakly but did not bind to CKAP4-(Δ503-602) (Fig. 2C). However, the epitopes recognized by mAbs 3F and 5A were difficult to determine using pepspot analysis (data not shown), suggesting that both antibodies recognize the three-dimensional structure of the C-terminal region of CKAP4. Thus, six anti-CKAP4 mAbs with different epitopes were obtained (Fig. 2A).

All of the anti-CKAP4 mAbs detected human CKAP4 in S2-CP8 cells. In addition, mAb 1G recognized dog CKAP4 in MDCK cells, while mAbs 3F and 5A cross reacted with mouse and dog CKAP4 in Eph4 and MDCK cells (Supplementary Fig. S3C). When S2-CP8 cells were stained with mAb 3F without permeabilization, cell surface CKAP4 was detected (Supplementary Fig. S3D). All CKAP4 mAbs bound to the ECD of CKAP4 in a dose-dependent manner as determined by ELISA (Fig. 2D). mAb 1G exhibited the highest binding ability among the six mAbs, while mAb 83 exhibited the lowest binding ability (Fig. 2D). The $K_{D}$ of the mAbs were calculated to be 0.05 ~ 1.45 nM. Monoclonal Abs 1G, 3F, and 5A all immunoprecipitated endogenous CKAP4 from lysates of S2-CP8 cells with high efficiency (Fig. 2E).

**CKAP4 is detected in PDAC patient sera**

Two types of ELISAs capable of detecting CKAP4-containing SEVs were developed to measure serum CKAP4: an exosome ELISA (PS Capture ™ Exosome ELISA Kit) and a sandwich ELISA (Supplementary Fig. S4A). Among the six anti-CKAP4 mAbs generated, mAb 5A detected CKAP4 in CM from S2-CP8/CKAP4-HA cells most efficiently in the exosome ELISA (Supplementary Fig. S4B). By concentrating CM from WT S2-CP8 cells 25- or 50-fold, endogenous CKAP4 was detected using the exosome ELISA (Fig. 3A).

The exosome ELISA detected CKAP4 in sera (100 μl) from mice xenografted by S2-CP8 cells, but CKAP4 was not detected in normal mouse sera (Fig. 3B). When an absorbance value higher than 0.1 was defined as positive, the exosome ELISA was able to detect CKAP4 as positive in 24 of 47
(51.1%) sera samples from PDAC patients without dilution (100 µl) and 14 of 47 cases (29.8%) in 10-fold diluted sera (equivalent to 10 µl) (Fig. 3C).

A sandwich ELISA for quantitative measurement of serum CKAP4 was also developed. The combination of mAbs 3F and 1G as the capture and detector mAbs, respectively, exhibited the strongest signal (highest absorbance) (Supplementary Fig. S4C) with a range of 1 to 25 ng/ml CKAP4 proteins (Supplementary Fig. S4D). In sera from 47 PDAC patients used in Fig. 3C, the concentrations of CKAP4 were calculated to be >200 ng/ml in 3 cases, 50~200 ng/ml in 11 cases, 1~50 ng/ml in 4 cases, and less than 1 ng/ml in 29 cases (Fig. 3D). For the exosome ELISA, the proportions of intra-plate, intra-well, and inter-subject variances were estimated as 6.3%, 8.2%, and 85.5%, respectively. For the sandwich ELISA, the corresponding proportions were 1.8%, 0.3%, and 97.9%, respectively. These results strongly support high reproducibility in both ELISAs. A positive correlation was found between the values measured by the exosome ELISA and sandwich ELISA (R=0.559) (Fig. 3E).

Among 47 PDAC cases, 20 cases who underwent surgical resection were stained with anti-CKAP4 Ab immunohistochemically, and they were classified into four categories (Negative; < 5%, 6 cases, Low; 5-20%, 3 cases, Medium; 20-50%, 3 cases, and High; 50-95%, 8 cases. The percentage refers to ratios of tumor areas positive for CKAP4). When the medium and high cases were defined as immunohistochemically CKAP4-positive, 11 cases were CKAP4-positive (Supplementary Fig. S5A). Serum CKAP4 levels of immunohistochemically CKAP4-positive cases in the exosome ELISA and sandwich ELISA were significantly higher than those of CKAP4-negative cases, HC individuals, and colon polyp cases (p=0.037, 0.0006, and 0.004, respectively in the exosome ELISA and p=0.003, 0.012, and 0.0001, respectively in the sandwich ELISA) (Fig. 3F and G). However, serum CKAP4 levels were higher in some HC individuals and colon polyp cases than PDAC cases immunohistochemically negative for CKAP4 (Fig. 3F). It was reported that exosome protein concentrations were higher in melanoma patients than HC individuals.
Serum CD63 levels measured by the exosome ELISA were indeed higher in PDAC cases than in HC individuals and colon polyp cases (Supplementary Fig. S5B). These results suggest that low levels of CKAP4 are expressed in exosomes of HC individuals and that most exosomes are derived from cancer cells that little express CKAP4 in CKAP4-negative PDAC cases. Therefore, serum CKAP4 levels in CKAP4-negative PDAC cases may be relatively lower than those in HC individuals.

In the immunohistochemically CKAP4-positive PDAC cases, a positive correlation between the values measured by the exosome ELISA and sandwich ELISA ($R=0.477$) was also obtained (Supplementary Fig. S5C). When HC individuals and colon polyp cases are defined as one control group, serum CKAP4 levels of immunohistochemically CKAP4-positive PDAC cases were significantly higher than those of the control group in exosome ELISA and sandwich ELISA ($p=0.0045$ and $<0.0001$, respectively) (Supplementary Fig. S5D). Comparing immunohistochemically CKAP4-positive PDAC cases with the control group, ROC analysis showed that AUCs were 0.785 (95% confidence interval: 0.603-0.966) and 0.804 (95% confidence interval: 0.642-0.965) in exosome ELISA and sandwich ELISA, respectively (Supplementary Fig. S5E) and they were not significantly different ($p=0.737$). Thus, there is no clear difference in diagnosis capacities between two ELISAs.

When serum CKAP4 was positive (OD > 0.1, 10 cases) in the exosome ELISA, all cases were immunohistochemically CKAP4-positive. In contrast, only one case out of 10 serum CKAP4-negative cases showed high immunohistochemical staining of CKAP4 (Fig. 3H). In 47 PDAC cases including 27 resectable and 20 unresectable cases, serum CKAP4 levels were significantly higher in unresectable cases than in resectable cases ($p=0.002$), using the exosome ELISA (Fig. 3I). Consistent with the previous reports (22, 34), serum DKK1 values of unresectable PDAC cases were significantly higher than those of resectable PDAC cases and HC individuals ($p=0.023$ and 0.019, respectively) (Fig. 3J).
In six cases out of 20 unresectable PDAC cases, presence of CKAP4 in exosomes was examined using Western blotting. CKAP4 in exosomes was strongly detected in two patients (#E and #F) whose serum CKAP4 level was positive in two ELISAs, or weakly detected in patient #D by the exosome ELISA; by contrast CKAP4 in exosomes were barely detectable in patients (#A - #C) whose serum CKAP4 was negative in two ELISAs (Supplementary Fig. S5F). Four cases (#1 - #4) out of 27 resectable cases exhibited high expression of CKAP4 in tumor lesions immunohistochemically (Supplementary Fig. S5G). CKAP4 levels were high in preoperative sera, whereas it was greatly reduced in postoperative sera (Fig. 3K and L). Taken together, detection of CKAP4 in PDAC patient sera is possible by the ELISAs, and serum CKAP4 levels can reflect the expression of CKAP4 in PDAC tumor lesions.

**CKAP4 mAbs inhibit DKK1-CKAP4 signaling and suppress tumor formation**

mAbs 52, 73, 1G, 3F, and 5A inhibited *in vitro* binding of DKK1 and CKAP4 in a dose-dependent manner, whereas mAb 83 did not (Fig. 4A). CKAP4 mAbs did not induce the CKAP4 internalization in S2-CP8/DKK1 KO cells (Supplementary Fig. S6A). mAbs 52, 73, 1G, 3F, and 5A inhibited DKK1-dependent CKAP4 internalization, AKT activity, proliferation, and migration of S2-CP8 cells, whereas mAb 83 did not (Fig. 4B-D). On the other hand, mAb 3F did not affect AKT activity or proliferation of BxPC-3 cells (Supplementary Fig. S6B and C), in which the expression of DKK1 and PM-localized CKAP4 was low (see Fig. 1A).

Consistent with the results of *in vitro* experiments, mAbs 52, 73, 1G, 3F, and 5A, but not mAb 83, suppressed xenograft tumor formation induced by S2-CP8 cells to various degrees (Fig. 5A and Supplementary Fig. S7A). mAbs 3F and 73 also inhibited xenograft tumor formation induced by HPAF-II PDAC and A549 lung cancer cells (Fig. 5B and Supplementary Fig. S7B). mAb 3F exhibited a stronger antitumor effect than the same or higher dose of other mAbs (Fig. 5A and C and Supplementary Fig. S7C). The antitumor effect of combination of 50 μg/body mAb 3F and 400
μg/body GEM was equivalent to that of mAb 3F alone, but the combination of 50 μg/body mAb 3F and 1000 μg/body GEM showed the strongest inhibition of tumor growth (Fig. 5C).

GEM-R S2-CP8 cells were generated by exposing to high concentrations of GEM. S2-CP8/GEM-R cells were able to grow in the presence of 1 μM GEM, whereas IC₅₀ of GEM in parent S2-CP8 cells was 10-50 nM (Fig. 5D). Expression levels of DKK1 and PM-localized CKAP4 in GEM-R cells were similar to those of parent cells (Fig. 5E). mAb 3F inhibited the AKT activity and proliferation of GEM-R cells (Fig. 5F and G). Thus, anti-CKAP4 mAb may be useful for GEM-resistant PDAC.

**CKAP4 mAbs inhibit metastasis of PDAC cells and extend survival**

Effects of anti-CKAP4 mAbs were tested in different types of mouse models. Inoculation of S2-CP8 into the peritoneal cavity caused the peritoneal dissemination and accumulation of ascites, resulting in death within 30 days after inoculation; intraperitoneal injection of CKAP4 mAb 3F extended survival (Fig. 6A). As another assay, S2-CP8 cells were transplanted into the pancreas of immunodeficient mice. Pancreatic tumor metastasized to lymph nodes at 20 days after transplantation, resulting in death within 35 days. CKAP4 mAb 3F inhibited tumor formation in the pancreas and lymph nodes metastasis (Fig. 6B) and extended survival (Fig. 6C). Thus, anti-CKAP4 mAbs showed tumor suppressive activity in mouse models via the inhibition of DKK1-CKAP4 signaling.
Discussion

**CKAP4 is detected in exosomes from PDAC patient sera**

CKAP4 was internalized upon DKK1 binding. Internalized CKAP4 was found to take two routes: one is a recycling pathway to the PM to be re-used as a receptor (9) and the other is a MVB pathway to the extracellular space to be released with SEVs. The obtained results in this study indicate that CKAP4-containing SEVs are exosomes and that CKAP4-containing exosomes originate from the PM of cancer cells.

Exosomes are potential biomarkers for cancer diagnosis (16). To confirm the ability of CKAP4-exosomes to act as the biomarkers, two different types of ELISAs were developed to detect CKAP4 in PDAC patient sera. Similar results were obtained using two assays. Serum CKAP4 levels were higher in PDAC patients immunohistochemically positive for CKAP4 than in PDAC patients immunohistochemically negative for CKAP4, HC individuals, or colon polyp patients. CKAP4 was highly detected in sera from mice bearing CKAP4-expressing PDAC cells and pre-operative patients with CKAP4-positive PDAC, but not in sera from WT mice or post-operative patients. These results suggest that CKAP4 is released as exosomes from PDAC cells. Two out of 47 cases showed discrepancy in CKAP4 values in the exosome ELISA and sandwich ELISA (serum CKAP4 values were 2.2 (OD) and 86.7 ng/ml in one case and 0.2 (OD) and 444.8 ng/ml in another case for the exosome ELISA and sandwich ELISA, respectively). Currently we do not know the reason; It is necessary to confirm the presence of such a case by measuring serum CKAP4 in more cases including other types of cancer.

Proteomics analyses of exosomes from cultured colon cancer cells and human urine identified CKAP4 as one of many exosomal proteins (1000~2000 candidate proteins) (35, 36). It was recently reported that serum CKAP4 levels in lung cancer patients were higher than in HC individuals (37). Our exosome ELISA and sandwich ELISA showed that serum CKAP4 levels in PDAC patients are higher in unresectable cases than resectable cases. Therefore, CKAP4 can be detectable in the sera of
unresectable PDAC cases without immunohistochemical data. Taken together with the previous observation that high DKK1 levels in the serum are associated with poor prognosis for patients with lung and pancreatic cancer (15), serum levels of CKAP4 and DKK1 may be able to determine responders for anti-CKAP4 mAb therapy. On the other hand, it was reported that CKAP4 expression is associated with favorable prognosis in intrahepatic cholangiocarcinoma and hepatocellular carcinoma (38, 39). It is necessary to investigate whether expression of both CKAP4 and DKK1 correlates with poor prognosis in various types of cancer.

**CKAP4 represents a potential molecular target for PDAC**

Although CKAP4 is expressed in various cells and is involved in ER functions and receptor activities (5-7), CKAP4 KO mice survived for at least 18 months and were fertile. Therefore, loss of CKAP4 function by anti-CKAP4 mAb should not affect critical cellular functions and should have few side effects. In addition, since anti-CKAP4 mAbs generated from CKAP4 KO mice have a stronger ability to neutralize DKK1-CKAP4 signaling than those generated in WT mice, CKAP4 KO mice were valuable for producing good antibodies. mAb 3F, generated in CKAP KO mice, was especially efficacious in recognizing three dimensional structures containing aa 503-524 and aa 588-590 and thus most efficiently immunoprecipitated endogenous CKAP4 and exhibited the highest antitumor effects *in vivo*.

Anti-CKAP4 mAbs inhibited the binding of DKK1 to CKAP4 and DKK1-induced internalization of CKAP4 from the PM, thereby suppressing AKT activity as well as the proliferation and migration of S2-CP8 cells. Therefore, the direct action of anti-CKAP4 mAb on DKK1-CKAP4 signaling could be involved in tumor-suppressive activity. This possibility is supported by the results that mAb 83, which did not inhibit the binding of DKK1 and CKAP4, failed to suppress AKT activity, proliferation, and migration of cancer cells. The tumor-suppressive ability of anti-CKAP4 mAbs was confirmed in xenograft models using S2-CP8, HPAF-II, and A549 cells and in S2-CP8 cell-induced...
peritoneal dissemination model and orthotopic transplantation model, indicating that anti-CKAP4 mAbs are effective for both PDAC and lung cancer. Moreover, the combination of mAb 3F and GEM completely inhibited S2-CP8 cell-induced tumor formation. Combination therapy with anti-CKAP4 mAb and GEM may offer a potential new approach for the treatment of PDAC. In PDAC, chemoresistance to GEM is directly linked to poor patient outcomes (40). Since anti-CKAP4 mAb inhibited AKT activity and proliferation of GEM-R cells, anti-CKAP4 mAb may be applicable to chemo-resistant tumors.

The PI3K-AKT pathway is a major therapeutic target in PDAC and a clinical trial with an AKT inhibitor MK-2206 alone or in combination with a MEK inhibitor was conducted, but the dose was limited due to toxicity of MK-2206 and the results showed insufficient effects (41, 42). If anti-CKAP4 Ab enhances the effects of inhibitor(s) of the PI3K-AKT pathway or reduces the doses of the inhibitor(s), the combination therapy would be useful as the novel therapeutic strategy from the viewpoint of toxicity reduction.
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References


**Figure legends**

**Figure 1. Secretion of CKAP4 with SEVs.**

(A and D) Top panels, SEVs were prepared by 100,000 x g precipitation from CM of PDAC cells and non-tumor cells (A) or from CM of S2-CP8 cells transfected with control (scramble) or clathrin siRNAs (D). These cells were biotinylated, then the PM proteins were precipitated with NeutrAvidin Agarose beads. SEVs, cell lysates, and precipitates were probed with the indicated antibodies. Bottom panel, the CKAP4 band intensities of SEVs, PM, and lysates were quantified. AU, arbitrary unit.

(B) SEVs prepared from CM of S2-CP8 cells was subjected to discontinuous sucrose gradient analysis; fraction samples were probed with the indicated antibodies.

(C and E) SEVs prepared from CM of WT S2-CP8, S2-CP8/DKK1 KO, S2-CP8/DKK1 KO/DKK1-FLAG, and S2-CP8/DKK1 KO/DKK1ΔCRD1-FLAG cells (C) and CM of S2-CP8 cells transfected with the indicated siRNAs (E) or lysates of these cells were probed with the indicated antibodies.

(F) Exomes were purified from serum of BALB/cAnNCrj-nu immunodeficient mice (n = 2) or mice with xenograft tumors of S2-CP8 or HPAF-II cells (n = 2) using a MagCapture™ Exosome Isolation Kit PS; subsequently, they were probed with the indicated antibodies.

**Figure 2. Determination of epitopes of anti-CKAP4 mAbs.**

(A) Schematic illustration of epitopes recognized by anti-CKAP4 mAbs used in this study are shown. E, ER anchoring domain; MB, microtubule binding domain; M, transmembrane domain; T, tyrosine sulfation region; LZ, leucine zipper domain; α, α helix domain.

(B) Pepspot membranes, in which 49 different 17-amino acid peptides from the extracellular domain of CKAP4 were spotted, were probed with various anti-CKAP4 mAbs. Bound antibodies were detected using an HRP-conjugated antibody.
(C) Various deletion mutants of CKAP4 were transiently expressed in X293T cells; cell lysates were probed with various anti-CKAP4 mAbs. Black triangles indicate the position of endogenous CKAP4.

(D) Binding of the indicated concentrations of anti-CKAP4 mAbs to 1 nM GST-CKAP4-extracellular domain (ECD) and GST proteins was measured by ELISA. Results are shown as means of three independent experiments. As a control, whether mAb 1G recognizes GST was tested.

(E) Lysates (input) of S2-CP8 cells were immunoprecipitated with 1 µg/ml anti-CKAP4 mAbs or control IgG. The immunoprecipitates (IP) were probed with anti-CKAP4 polyclonal antibody.

Figure 3. Detection of human serum CKAP4 by the ELISA.

(A and B) CKAP4 in 25-fold and 50-fold concentrated CM from S2-CP8, S2-CP8/CKAP4 KO, or S2-CP8/CKAP4-HA cells (A) or in sera from BALB/cAnNCrj-nu immunodeficient mice or mice with xenograft tumor of S2-CP8 cells (B) was measured using exosome ELISA.

(C and D) CKAP4 in the sera from pancreatic ductal adenocarcinoma (PDAC) patients was measured by exosome ELISA (C) or sandwich ELISA (D).

(E) The correlation of serologic concentration of CKAP4 measured by the two ELISAs was analyzed by Pearson correlation coefficient.

(F and G) CKAP4 in the sera from healthy control individuals, colon polyp patients, immunohistochemically CKAP4-positive (IHC(+)) or negative (IHC(-)) PDAC patients was measured by exosome ELISA (F) or sandwich ELISA (G). Results are indicated by dot plot with box plot. The median is represented with a line, the box represents the 25th-75th percentile and error bars show the 5th-95th percentile.

(H) Classification of immunohistochemical-staining patterns of CKAP4 were divided into two groups depending on whether CKAP4 was positive or negative using exosome ELISA.
(I and J) Serum CKAP4 (I) and DKK1 (J) from healthy control (HC) individuals (n=18) or resectable (n=27) or unresectable (n=20) PDAC patients were analyzed using exosome ELISA (I) and sandwich ELISA (J).

(K and L) Serologic concentrations of CKAP4 before and after surgery from the PDAC cases were measured using sandwich ELISA (K). SEVs were purified from serum using a MagCapture™ Exosome Isolation Kit PS; samples were probed with the indicated antibodies (L). *, P < 0.05; **, P < 0.01 (Wilcoxon rank sum test).

Figure 4. Characterization of anti-CKAP4 mAbs.

(A) After 2 nM GST-CKAP4-ECD was pre-treated with the indicated concentrations of anti-CKAP4 mAbs or control IgG; then, the mixtures were incubated with 2.5 nM DKK1 for 1 hour. The protein mixtures were precipitated with glutathione agarose and the precipitates were probed with anti-DKK1 antibody.

(B) Top panels, after S2-CP8/DKK1 KO cells were pretreated with 3 µg/ml anti-CKAP4 mAbs or control IgG for 1 hour, the cells were stimulated with 4 nM DKK1-FLAG for the indicated times. PM proteins were biotinylated (at each time point) and precipitated with NeutrAvidin Agarose beads. The precipitates and cell lysates were probed with anti-CKAP4 antibody. Bottom panel, the CKAP4 band intensities of PM and lysates were quantified.

(C) Lysates from S2-CP8 cells treated with 20 µg/ml anti-CKAP4 mAbs or control IgG for 4 hours were probed with the indicated antibodies.

(D) Left panels, S2-CP8 cells treated with 20 µg/ml anti-CKAP4 mAbs or control IgG were cultured for 5 days in 3D Matrigel. Right panel, the total area of spheres per field (n = 5 fields) expressed as a ratio to those of non-treated spheres.

(E) Left panels, S2-CP8 cells treated with 50 µg/ml anti-CKAP4 mAbs or control IgG were subjected to the migration assay for three hours and stained with DRAQ5 DNA dye. Migration was
expressed as a ratio to non-treated cells. *, $P < 0.05$; **, $P < 0.01$ (Student’s t test). Scale bars, 200 μm (D and E).

**Figure 5. Inhibition of tumor growth by anti-CKAP4 mAbs.**

(A and C) S2-CP8 cells were subcutaneously implanted into immunodeficient mice. Anti-CKAP4 mAbs or control IgG (200 μg/body) (A) and mAb 3F, gemcitabine (GEM), the combination of mAb 3F and GEM, or control IgG (50 μg/body) (C) were injected into the intraperitoneal cavity of the mice twice per week. Extirpated xenograft tumors (left top panels) and tumor weights (left bottom panel) an tumor volumes (middle panel) are shown.

(B) HPAF-II cells were subcutaneously implanted, then mAb 3F or control IgG (200 μg/body) was injected twice per week.

(D) S2-CP8 WT and GEM-R cells treated with the indicated concentration of GEM were subjected to the 2D cell proliferation assay for 3 days. The fluorescence intensity of the CyQUANT NF dye was measured (n =3). The relative fluorescence intensities of counted cells were expressed as arbitrary units when compared with the values for non-treated cells in WT and GEM-R, respectively. Results are shown as means ± s.d.

(E) S2-CP8/GEM-R cells were biotinylated, then the PM proteins were precipitated with NeutrAvidin Agarose beads. Cell lysates and precipitates were probed with the indicated antibodies.

(F) Lysates from S2-CP8/GEM-R cells treated with 20 μg/ml mAb 3F or control IgG for 4 hours were probed with the indicated antibodies.

(G) Top panels, S2-CP8/GEM-R cells treated with 20 μg/ml mAb 3F or control IgG were cultured for 5 days in 3D Matrigel. Bottom panel, the total area of spheres per field (n = 3 fields) expressed as a ratio to those of non-treated spheres. *, $P < 0.05$; **, $P < 0.01$ (Student’s t test); scale bars, 10 mm (A, B, and C) and 200 μm (G).

**Figure 6. Inhibition of tumor metastasis and extension of survival by anti-CKAP4 mAb**
(A) S2-CP8 cells were injected intraperitoneally into immunodeficient mice. After 2 days, mAb 3F or control IgG (50 µg/body) was injected into the intraperitoneal cavity twice per week. The Kaplan-Meier survival curve for the mice is shown. Statistical significance was determined by log-rank test.

(B and C) S2-CP8 cells were transplanted into the pancreas of immunodeficient mice. After 2 days, mAb 3F or control IgG (200 µg/body) was injected into the intraperitoneal cavity twice per week. Four mice in each group were sacrificed at 20 days after transplantation to examine the following parameters. Mesenteric lymph node metastasis (B, left panel) and extirpated pancreatic tumors (B, right top panels) and tumor weights (B, right bottom panel) are shown. The Kaplan-Meier survival curve for the mice is shown (C). Statistical significance was determined by log-rank test. *, $P < 0.05$; **, $P < 0.01$; scale bars, 10 mm (B).
CKAP4, a DKK1 receptor, is a biomarker in exosomes derived from pancreatic cancer and a molecular target for therapy

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