

Supplementary Materials and methods

RNA isolation, reverse-transcription polymerase chain reaction (RT-PCR), or quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. An ABI7300 instrument (Applied Biosystems, Foster City, CA, USA) was used for RT-PCR analyses. qRT-PCR was performed using a SYBR PrimeScript RT-PCR Kit (Applied Biosystems) according to the manufacturer's instructions. β -Actin was used as an internal control. The primers were as follows: cavin-1-f, 5'-TCAGCGTCAACGTGAAGACCGT-3' and cavin-1-r, 5'-CTCCTCAATAACCTCCTCAACC-3'; cavin-2-f, 5'-GTGCAAGAGTTTGTTCCTTCAGCCC-3' and cavin-2-r, 5'-GCCTGAAGAAAGTGGATAGCCTCA-3'; cavin-3-f, 5'-GGAGCAGAGCGGTCAGGGATC-3' and cavin-3-r, 5'-GGACCGTTTGAGGTCAGTAC-3'; caveolin-1-f, 5'-ACACCAAGGAGATCGACCT-3' and caveolin-1-r, 5'-GCATGGTACAACCTGCCAGATG-3'; β -catenin-f, 5'-CCTCTGATAAAGGCTACTGTTGGAT-3' and β -catenin-r, 5'-CTGATGTGCACGAACAAGCA-3'; Snail-f, 5'-TGCAGGACTCTAATCCAAGTTTACC-3' and Snail-r, 5'-GTGGGATGGCTGCCAGC-3'; Slug-f, 5'-GGTCAAGAAGCATTTCAAC-3' and Slug-r, 5'-CTGAGCCACTGTGGTCCTTG-3'; Twist-f, 5'-TGTCCGCGTCCCCTAGC-3' and Twist-r, 5'-TGTCCATTTTCTCCTTCTCTGGA-3'; and β -actin-f, 5'-TTGTTACAGGAAGTCCCTTGCC-3' and β -actin-r, 5'-ATGCTATCACCTCCCCTGTGTG-3'. Relative mRNA levels were calculated based on Ct values, corrected for β -actin expression, according to the equation: $2^{-\Delta Ct}$ [$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (}\beta\text{-actin)}$] [1]. All experiments were performed in triplicate.

Gelatin zymography, immunoblotting, co-immunoprecipitation (co-IP), and glutathione S-transferase (GST) pull-down assays

Matrix metalloproteinase (MMP)-2 and -9 activities in conditioned media were visualized by electrophoresis on gelatin-containing polyacrylamide gels, as previously described [2]. Total cellular protein was isolated in immunoprecipitation buffer. Cytoplasmic and nuclear extracts from cultured cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA). Immunoblotting for caveolin-1 (1:1000; Sigma-Aldrich, St. Louis, MO, USA), cavin-1 (1:500; BD Biosciences, Franklin Lakes, NJ, USA), cavin-2 (1:500; Abcam Biosciences, Cambridge, UK), cavin-3 (1:800; BD Biosciences), cavin-4 (1:500; BD Biosciences), E-cadherin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), vimentin (1:1000, Sigma), and β -catenin (1:1000, BD Biosciences) were performed according to standard procedures [2]. IP was accomplished using a Universal Magnetic Co-IP kit (Active Motif, Tokyo, Japan). Cell extracts were first incubated with protein magnetic beads. The cleared supernatants were incubated with caveolin-1-specific or cavin-1-specific antibodies overnight before the addition of protein A/G agarose beads. Normal rabbit IgG was used as a control. After washing, immunoprecipitated materials were eluted and immunoblotted with human anti-caveolin-1 and anti-cavin-1 antibodies. For GST pull-down assays, GST and GST-caveolin-1 immobilized by GSH-sepharose beads (GE Healthcare, Fairfield, USA) were incubated with cavin-1 for 2 h at 4°C, followed by washing three times with NETN buffer (50 mM NaCl, 5 mM EDTA, 50 mM Tris pH 8.0, 1% NP40). Bound proteins were visualized by immunoblotting.

Immunostaining and immunofluorescence analysis

Formalin-fixed and paraffin-embedded xenograft tissues were cut into 4- μ m-thick sections for

histological studies, hematoxylin and eosin staining, and immunostaining. The antibodies used were as follows caveolin-1 (1:600), cavin-1 (1:400), cavin-2 (1:200), cavin-3 (1:600), E-cadherin (1:500), N-cadherin (1:400), and vimentin (1:600). For immunofluorescence, cells were incubated with primary antibodies to E-cadherin (1:100), vimentin (1:50), and β -catenin (1:100) at 4°C overnight, followed by the anti-mouse tetramethyl rhodamine isothiocyanate-conjugated secondary antibody (1:50, Invitrogen) for 1 h at 36°C. Fluorescent images were visualized using a confocal microscope (FV-1000, Olympus, Tokyo, Japan).

Patient inclusion criteria and follow-up

The entrance criteria for all patients were: distinctive pancreatic cancer diagnosis by pathology based on the WHO criteria; no prior anticancer treatment; all patients were assessed to meet the computed tomography (CT) criteria for respectability (absence of extrapancreatic disease; no evidence of tumor encasement of superior mesenteric artery, celiac axis, or hepatic artery as defined by the presence of a normal fat plane between the tumor and these arteries; a patent superior mesenteric vein or portal vein; tumor stage was determined according to the 7th edition of the American Joint Committee on Cancer TNM classification system) [3].

Patients were followed up in our clinic every 2 months during the first postoperative year and at least every 3~4 months thereafter. CA 19-9 and pancreas and liver ultrasonography were performed during each visit by independent doctors with no knowledge of the study. A CT scan of the abdomen was performed every 6 months. If recurrence was suspected, CT scan or magnetic resonance imaging of the abdomen was performed immediately. Bone scans were performed in patients with symptoms of bone pain. Of 69 patients, 48 had died by the last follow-up and the median survival time was 14.75 months. The 1- and 2-year postsurgical survival rates were 56.52%

and 25.98%, respectively. Sixty-three patients had disease recurrence and the median recurrence time was 8.75 months. The 1- and 2-year postoperative recurrence rates were 65.22% and 95.58%, respectively. Twenty-one patients received postoperative chemotherapy and radiation therapy and 41 patients received only postoperative chemotherapy, because of local recurrence or distant metastasis. Thirty-eight patients in cohort II had died at the last follow-up and the median survival time was 11.39 months. The 1- and 1.5-year postsurgical survival rates were 48.30% and 26.84%, respectively. A total of 53 patients had disease recurrence and the median recurrence time was 8.75 months. The 1- and 1.5-year postoperative recurrence rates were 72.64% and 85.45%, respectively. Twenty patients received postoperative chemotherapy and radiation therapy and 42 patients received only postoperative chemotherapy. Overall survival (OS) and time to recurrence (TTR) were defined as the interval between surgery and death or recurrence, respectively. If disease recurrence was not diagnosed, cases were censored on the date of death or last follow-up.

Tissue microarray analyses (TMAs) and immunostaining

TMAs were constructed and immunostaining was performed using a two-step protocol, as previously described [2]. Caveolin-1 and cavin-1, -2, and -3 expression was detected using the corresponding specific antibodies. Reaction products were visualized by incubation with 3, 3'-diaminobenzidine. Negative controls were treated identically, but with omission of the primary antibodies. The intensity of positive staining was measured as previously described [2].

Reference

- [1] Peinnequin A, Mouret C, Birot O, Alonso A, Mathieu J, Clarencon D, Agay D, Chancerelle Y, and Multon E (2004). Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. *BMC Immunol* 5, 3.
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- [3] Vincent A, Herman J, Schulick R, Hruban RH, and Goggins M (2011). Pancreatic cancer. *Lancet* 378, 607-620.