MK2461 suppress progression of pancreatic cancer disrupting interaction between pancreatic cancer cells and pancreatic stellate cells

博士論文

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(MK2461 は膵癌細胞と pancreatic stellate cell の相互作用を制御し膵癌の増殖を抑制する)

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Abstract

Pancreatic cancer is characterized excessive desmoplasia, which occupies 80% of pancreatic cancer tissue and mainly consists of pancreatic stellate cells (PSCs). Desmoplasia has been shown to play an important role in the progression, invasion, and metastasis of pancreatic cancer and has been implicated in the development of resistance to chemotherapy and radiotherapy. Moreover, growth factors, which bind to receptor tyrosine kinases (RTKs) and activate downstream signaling, are one of the components modulating the interactions between pancreatic cancer cells (PCCs) and PSCs. Therefore, blocking RTKs, which are highly expressed in both PCCs and PSCs, could suppress cancer progression. In this study, I profiled the expression of RTKs with quantitative targeted absolute proteomics using liquid chromatography tandem mass spectrometry (LC-MS/MS) in PCCs and PSCs. In PCCs, epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (MET) levels were elevated compared with those in PSCs. Additionally, in PSCs, platelet-derived growth factor receptor beta (PDGFRβ) and MET were upregulated compared with other RTKs. Conditioned medium from PSCs promoted the proliferation of PCCs, and vice versa. In addition, MK2461, a multikinase inhibitor targeting MET and PDGFRβ, suppressed the effects of conditioned medium on PCCs and PSCs. Finally, MK2461 significantly inhibited tumor growth in mice co-injected with PCCs and PSCs. In conclusion, PDGFRβ and MET may play a critical role in the interaction between PCCs and PSCs, which was modulated by MK2461. Therefore, MK2461 may have therapeutic potential in the treatment of pancreatic cancer by targeting the interaction between PCCs and

PSCs.

Introduction

Pancreatic cancer is a highly aggressive disease characterized by an extremely poor prognosis. Despite recent developments in the diagnosis and therapeutic management of pancreatic cancer, the overall 5-year survival rate is less than 5% [1)], in part due to the poor response of pancreatic cancer to most chemotherapeutic agents and radiotherapy. Therefore, improving our understanding of the development and progression of pancreatic cancer is essential [2)].

Pancreatic cancer is characterized by excessive desmoplasia, which occupies 80% of pancreatic cancer tissue [3)]. However, most previous studies have focused on cancer cells themselves, and the abundant desmoplasia has been largely ignored [4)]. The desmoplasia is thought to be essential for the invasion, metastasis, and chemotherapeutic resistance of pancreatic cancer [5-10)] and has been shown to be comprised primarily of pancreatic stellate cells (PSCs), which are observed in the interlobular areas and the peri-acinar lesions of the pancreas [11)]. PSCs are transformed from a quiescent state into myofibroblast-like cells in response to cytokines and growth factors, such as transforming growth factor (TGF) β1, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), which are secreted from inflammatory cells and cancer cells. Activated PSCs are characterized by high expression of α-smooth muscle actin (SMA), and once PSCs are activated by pancreatic cancer cells (PCCs), these cells are suggested to remain in the active state via autonomous signaling loops [4, 12, 13)]. Activated PSCs produce abundant extracellular matrix (ECM), cytokines, and growth factors, and the production of ECM contributes to excessive fibrosis, thereby

leading to interstitial hypertension, inefficient drug delivery [14-16], and resistance to radiotherapy [17]. Furthermore, secreted growth factors from activated PSCs, such as PDGF, FGF, insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and TGFβ1, promote PCC proliferation, invasion, and migration, partially through induction of the epithelial-to-mesenchymal transition (EMT) [10, 18, 19]. In an *in vivo* study, PCCs subcutaneously injected with PSCs were shown to grow more rapidly than PCCs injected alone [20]. Moreover, in an orthotopic model, co-injection of PCCs with PSCs resulted in increased tumor incidence, metastasis, and tumor size [21]. In addition, PCC stimulation increases the secretion of growth factors and ECM components from PSCs [18]. Thus, reciprocal stimulation of PCCs and PSCs is essential in the progression of pancreatic cancer.

Altered expression of various receptor tyrosine kinases (RTKs) has been observed in several types of cancer, and the expression of some RTKs correlates with patient prognosis [22-24]. Growth factors mediate their effects by binding to various RTKs. Thus, profiling the expression of RTKs in both PCCs and PSCs may lead to identification of growth factors regulating the interaction between PCCs and PSCs.

MK2461 is a multikinase inhibitor that was developed as ATP-competitive inhibitor of activated HGF receptor (MET). This compound effectively inhibits constitutive or ligand-dependent phosphorylation of MET and significantly inhibits several other RTKs. Moreover, MK2461 exerts significant antitumor activities through inhibition of MET, FGF receptor 2 (FGFR2), and PDGF receptor (PDGFR) *in vitro* and

in vivo [25]. Therefore, MK2461 may have applications as a potential anticancer agent in pancreatic cancer through disruption of RTK signaling in PCCs and PSCs.

In this study, I sought to identify novel candidate targets for improving the therapeutic management of pancreatic cancer. To this end, I measured the expression levels of 15 RTKs by quantitative targeted absolute proteomics (QTAP) and analyzed the roles of these RTKs in pancreatic cancer. In addition, I examined the effects of MK2461 on the PCC-PSC interaction.

Materials and Methods

Cell culture

PANC-1 cells were obtained from RIKEN BRC CELL BANK (Tsukuba, Japan). Capan-2, HPAF-II, BxPC-3, SW1990, and AsPC-1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). SUIT-2, KLM-1, PK-1, and PK-8 cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). All of the pancreatic cancer cell lines used in this study have KRAS mutation. TIG-1-20, human fetal lung fibroblast, were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nichirei Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS) with 4.5 g/L glucose, 1.5 g/L $NaHCO_3$, 70 μg/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin at 37°C in a humidified atmosphere of 5% CO_2.

Reagents and antibodies

All of the standard peptides and stable isotope-labeled peptides used for QTAP were synthesized by Thermo Fisher Scientific (Sedantrade, Germany). Sequencing-grade modified trypsin (Promega, Madison WI, USA) was used for trypsin digestion of the targeted proteins. MK2461 was purchased from Selleck (Houston, TX, USA). All other reagents were commercial products of analytical grade unless specifically described. The antibodies used in this study included anti-phospho-MET (Tyr^{1234}/Tyr^{1235}), anti-AKT, anti-phospho-AKT (Ser^{473}), anti-extracellular signal-regulated kinase 1/2 (ERK1/2), anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-GAPDH, anti-PDGFRβ, and

anti-vimentin from Cell Signaling Technology (Danvers, MA, USA); anti-MET and anti-α-SMA from Abcam (Cambridge, MA, USA); anti-phospho-PDGFRβ (Tyr^{1021}) and anti-cytokeratin19 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Ki-67 from Nichirei Biosciences (Tokyo, Japan); and Alexa Fluor 488-conjugated anti-rabbit IgG antibody, Alexa Fluor 555-conjugated anti-mouse IgG antibody, horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody, and HRP-conjugated anti-mouse IgG antibody from Cell Signaling Technology.

Human samples

The pancreatic tissue blocks used in this study were obtained from patients undergoing surgery at Tohoku University Hospital. All patients were diagnosed with pancreatic cancer by biopsy before surgery. The samples were obtained in accordance with the policies and practices of the Ethics Committee of Tohoku University Graduate School of Medicine, and patients provided informed consent.

Primary culture of PSCs

PSCs were prepared from pancreatic cancer tissues by the outgrowth method [20]. The tissues were minced into 0.5–1 mm^3 pieces and seeded in 6-well uncoated culture plates in the presence of 10%–20% FBS in a 1:1 (v/v) mixture of DMEM and Ham's F12 medium (ATCC) containing 70 μg/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin. Tissue blocks were cultured at 37°C in a 5% CO_2/air humidified atmosphere. After incubation for 24 h, the tissue blocks were transferred to new 6-well plates, and PSCs grew out of the blocks a few days after seeding. After reaching confluence, PSCs were trypsinized and passaged. The purity of the cells was determined

by the immunofluorescence for α-SMA and vimentin, as well as morphology (spindle-shaped cells with cytoplasmic extensions) [26]. In this study, all of the established PSCs were used at passages 3–6.

Preparation of conditioned medium

Conditioned medium was prepared to evaluate the PCC-PSC interaction [21, 27]. SUIT-2 and PANC-1 cells were grown to 70%–80% confluence, and PSCs were grown to 90%–100% confluence in DMEM containing 10% FBS. The medium was then changed to serum-free medium, which was collected after 24–48 h, passed through a 0.22-μm pore size filter (Thermo Scientific, Waltham, MA, USA), and concentrated with a 3-kDa Ultrafiltration membrane (Millipore, Billerica, MA, USA). Protein concentrations were determined by the Lowry method (Bio-Rad, Hercules, CA, USA), and aliquots were stored at −80°C until use.

Immunofluorescence staining of PSCs

For immunofluorescence staining, PSCs were seeded in 2-well glass coverslips (IWAKI Scitech Division, Tokyo, Japan). PSCs were then fixed with 4% paraformaldehyde for 15 min at room temperature and blocked with 5% normal goat serum and 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 1 h at room temperature after washing with PBS three times for 5 min each wash. PSCs were then incubated with mouse monoclonal anti-α-SMA and rabbit monoclonal anti-vimentin antibodies at 4°C overnight, washed with PBS (three times for 5 min each), and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG antibodies and Alexa Fluor 555-conjugated anti-mouse IgG antibodies for 2 h at room temperature in the dark. The antibodies were

diluted in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 according to the manufacturer's instructions. PSCs were then washed with PBS (three times for 5 min each) and mounted with Prolong Anti-fade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Staining was observed using a confocal laser-scanning microscope (CLSM; C2si, Nikon, Japan).

Preparation of plasma membrane fractions from cells

Plasma membrane fractions were extracted as described previously [28, 29]. Cells were grown to confluence in 10-cm^2 dishes, scraped, and suspended in Suspension buffer (10 mM Tris-HCl [pH 7.4], 250 mM sucrose, 1 mM EGTA) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Then, cells were lysed by nitrogen cavitation at 750 psi for 15 min at 4°C and centrifuged at 10,000 × *g* for 10 min at 4°C to remove debris. The supernatants were collected and centrifuged at 100,000 × *g* for 40 min at 4°C, and the resulting pellets were resuspended in Suspension buffer. The suspensions were layered on top of a 38% (w/v) sucrose solution and centrifuged at 100,000 × *g* for 40 min at 4°C. The resulting turbid layer at the interface was collected and centrifuged at 100,000 × *g* for 40 min. The pellets were resuspended in Suspension buffer as the plasma membrane fraction. Protein concentrations were determined with the Lowry method (Bio-Rad).

Preparation of plasma membrane fractions from pancreatic tissues

Pancreatic tissues were minced into 1-mm^3 pieces and added to Hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) containing protease inhibitor cocktail (Sigma Chemical Co.) and PMSF (125 mM in isopropanol). Then, tissues were

homogenized using a Potter-Elvehjem Tissue Grinder on ice and lysed by nitrogen cavitation at 750 psi for 15 min at 4°C. The homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C to remove debris, and the supernatants were collected. The same procedures were then used as for the preparation of the plasma membrane fraction from cells.

QTAP by liquid chromatography tandem mass spectrometry (LC-MS/MS)

The absolute amount of membrane protein was quantified using a multiplex SRM/MRM method, as described previously [28-30]. The protein samples were denatured with alkylation buffer (7 M guanidine hydrochloride, 10 mM EDTA-Na, 0.5 M Tris-HCl, pH 8.5), followed by reduction and S-carbamoylmethylation. The alkylated proteins were digested with sequence-grade modified trypsin (Promega) at 37°C for 16 h. Trypsin-digested samples were mixed with a stable isotope-labeled peptide mixture as internal standard peptides and acidified with formic acid. Then, samples were centrifuged at 15,000 rpm, and the supernatants were injected into the high-performance liquid chromatography (HPLC) system (Agilent 1200 HPLC system; Agilent Technologies, Santa Clara, CA, USA), which was connected to an ESI-triple quadrupole mass spectrometer (QTRAP5500; AB SCIEX, Foster City, CA, USA). C18 columns (XBridge BEH130 C18, 130 Å, 3.5 µm, 100 × 1.0 mm; Waters, Milford, MA, USA) were used for HPLC. The mass spectrometer was set up to run an SRM/MRM experiment for peptide detection using a dwell time of 10 ms per SRM/MRM transition. The ion counts in the chromatograms were determined by using the quantitation procedures in Analyst software version 1.5 (AB Sciex). In the SRM/MRM analysis,

each peptide for a targeted protein was monitored with four SRM/MRM transitions specific for that peptide. For QTAP, the peak area ratios of the analyte and the stable isotope-labeled peptides in each SRM/MRM transition were measured, and quantitative values were calculated from the standard curve ($R^2 > 0.98$). To construct standard curves, a dilution series of standard peptide with a fixed amount of internal standard peptide was injected into an LC-MS/MS instrument. Unless otherwise indicated, at least three of the four SRM/MRM transitions needed to be measurable in order for a prototypic peptide to be judged as confirmed and for a quantitative value to be assigned. The value of the quantification limit of each protein (fmol/mg protein) was determined as described previously [28-30]. Peptide sequences for targeted proteins and MRM transitions (*m/z* values) are shown in Table 1.

Proliferation assay

SUIT-2 and PANC-1 cells were seeded at 3000 cells/well, and PSCs were seeded at 5000 cells/well in 96-well plates. Cells were incubated overnight in DMEM containing 10% FBS, after which the medium was changed to serum-free medium. Then, different concentrations of conditioned medium (0, 0.1, 0.5 mg/mL) and MK2461 (0, 0.1, 1, 3 μM) were added, and cells were incubated for 48 h. Serum-free medium was added to control wells. Following incubation, proliferation was measured using a Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) according to the manufacturer's instructions. Each assay was carried out in triplicate wells and repeated twice. Data are shown as percent change compared to the control.

Western blotting

Cells were grown to 70%–80% confluence in DMEM containing 10% FBS, then serum starved overnight. Cells were incubated with MK2461 (1 or 3 μM) for 4 h, then stimulated with conditioned medium (0.1 or 0.5 mg/mL) for 10 min. Cells were then washed with PBS, scraped, and lysed in radio-immunoprecipitation assay (RIPA) buffer (Thermo Scientific) (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche Diagnostic, East Sussex, UK) on ice. Whole cell lysates were centrifuged (20,000 × *g*; 30 min; 4°C), and the supernatants were collected. Protein concentrations were determined by BCA protein assays (Thermo Scientific), with BSA as the standard protein. Lysates were mixed with Laemmli Sample Buffer (Bio-Rad) (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 1% LDS, 0.005% Bromophenol Blue) and denatured at 95°C for 5 min. Cell lysates were then fractionated on 4%–15% SDS-polyacrylamide gels with Tris/glycine/SDS buffer (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Membranes were blocked with SuperBlock (TBS) Blocking Buffer (Thermo Scientific) for 1 h at room temperature, washed with TBST (TBS, 0.1% Tween-20) three times for 5 min, and incubated with primary antibodies diluted with TBST containing 5% BSA at 4°C overnight. Membranes were then washed with TBST three times for 10 min each and incubated with corresponding HRP-conjugated secondary antibodies for 1 h at room temperature. Detection was facilitated by Clarity Western ECL Substrate (Bio-Rad), and the protein bands were observed on an ImageQuant LAS 4000 mini (GE Healthcare, Buckinghamshire, UK).

Invasion assay

Invasion assays were carried out with 24-well BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Franklin Lakes, NJ, USA). SUIT-2 and PANC-1 cells (2×10^4) were resuspended in 500 μL of serum-free medium and seeded in the upper chambers. Lower chambers contained 750 μL of conditioned medium (0, 0.1, or 0.5 mg/mL) and MK2461 (0, 0.1, 1 μM) in serum-free medium. Cells were incubated for 48 h at 37°C/5% CO_2, and cells that had invaded through the pores to the lower surface were fixed and stained with Diff-Quick reagent (Sysmex International Reagents, Kobe, Japan). Invaded cells were counted in eight random adjacent fields using a microscope (BZ-9000, KEYENCE, Tokyo, Japan). Each experiment was repeated three times.

Migration assay

Migration assays were carried out in 24-well Transwell chambers (BD Biosciences). SUIT-2 and PANC-1 cells (2×10^4) were seeded in the upper chambers of Transwell in 500 μL of serum-free medium. Treatments were as described for the invasion assays, and analyses were carried out as described for the invasion assays at 24 h after treatment. Each experiment was repeated three times.

In vivo experiments

To evaluate the effects of MK2461 on the PCC-PSC interaction in vivo, animal experiments were performed as previously described [17, 18]. All animal experiments were reviewed and approved by the Tohoku University Institutional Animal Care and Use Committee. Seven-week-old male nude mice (BALB/cAJcl-nu/nu) were obtained from CLEA Japan (Tokyo, Japan). Mice were acclimated to the animal housing facility for 1 week before studies. SUIT-2 cells and PSCs were resuspended in 100 μL of DMEM

containing 20% Matrigel (BD Biosciences), and SUIT-2 cells (1×10^6) and PSCs (1×10^6) were subcutaneously co-injected into the right flanks, while SUIT-2 cells (1×10^6) alone were injected into the left flanks. One week later, mice were divided into two groups randomly (n = 7 per group) and administered either vehicle or MK-2461 (20 mg/kg) twice daily for 20 days by oral gavage. MK2461 was diluted in 0.9% saline containing 30% PEG400, 1% DMSO, and 1% Tween-80. Tumor sizes were determined with calipers, and tumor volumes were calculated using the formula: $\pi / 6 \times (L \times W^2)$, where L indicates the largest tumor diameter, and W indicates the smallest tumor diameter. When the experiment was terminated, subcutaneous tumors were excised and weighed.

Immunofluorescence staining of tumor tissue

The tumor tissues were resected, fixed with 10% formalin, embedded in paraffin, and cut into 5-μm-thick sections. The sections were deparaffinized and rehydrated with a series of alcohol solutions of decreasing concentrations. Antigen retrieval for α-SMA and cytokeratin19 was performed by heating the slides in a microwave for 15 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). After blocking with 1× PBS containing 3% BSA and 0.3% Triton X-100, sections were incubated with primary antibodies overnight at 4°C. The dilution ranges of primary antibodies were follows: α-SMA, 1:100; cytokeratin19, 1:100; Ki-67, 1:100. Sections were then washed with PBS three times for 5 min each and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG antibodies and Alexa Fluor 555-conjugated anti-mouse IgG antibodies for 1 h at room temperature in the dark. The antibodies were diluted in

PBS containing 1% BSA and 0.3% Triton X-100 according to the manufacturer's instructions. After incubation, sections were washed with PBS three times and counterstained with Prolong Anti-fade with DAPI to identify nuclei. Sections were then observed with a microscope (BZ-9000, KEYENCE), and the stained cells were counted in three random fields. Scale bar, 50 μm.

Statistical analysis

Data are shown as the mean ± SEM. Comparisons of paired data were analyzed by two-tailed Student's t tests, and comparisons of over three groups were analyzed by one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparisons test. Differences with *P* values of less than 0.05 were considered significant. Statistical analyses were performed using JMP Pro 11 software (SAS Institute).

Results

Isolation and identification of PSCs

PSCs were derived from fresh human pancreatic adenocarcinoma surgical specimens by the outgrowth method, and their identities were confirmed by immunofluorescence staining for α-SMA or vimentin (Fig. 1A). The isolated cells were stained with α-SMA, a marker of activated PSCs, and expressed vimentin, a marker of mesenchymal cells, indicating that they were not pancreatic cancer cells. In addition, these cells exhibited a spindle-shaped morphology with cytoplasmic extensions, characteristic of myofibroblasts. PSCs were isolated from three patients individually and named PSCI, PSCII, and PSCIII. Interestingly, α-SMA expression was increased in PSCs compared with TIG-1-20 cells, a normal fibroblast cell line, indicating that the isolated PSCs were in an activated state (Fig. 1B).

PCC-PSC interaction on cell proliferation

To investigate the effects of PSC conditioned medium on PCCs, proliferation assays were performed. Three condition media derived from PSCI, PSCII, and PSCIII promoted the proliferation of SUIT-2 and PANC-1 cells as compared to the serum-free control (Fig. 1C). For all assays, PCC proliferation was increased following exposure to PSC conditioned medium (0.5 mg/mL) as compared with the serum-free control.

Reciprocally, the conditioned medium from both SUIT-2 and PANC-1 cells significantly increased PSCI and PSCII proliferation compared to the serum-free controls in a concentration-dependent manner (Fig. 1D).

Comparison of cell proliferation induced by paracrine and autocrine secretions

To elucidate whether paracrine or autocrine effects were modulating the proliferation of PCCs in our experiment, I performed proliferation assays using conditioned medium from PCCs or PSCs. SUIT-2 and PANC-2 cell proliferation rates were significantly increased in the presence of PSCI conditioned medium as compared to SUIT-2 or PANC-1 conditioned medium at 0.5 mg/mL, respectively (Fig. 2A). Furthermore, SUIT-2 and PANC-1 conditioned media (0.1 or 0.5 mg/mL) significantly accelerated the proliferation of PSCIs compared to PSCI conditioned medium (Fig. 2B). Therefore, paracrine signaling was more effective at inducing proliferation in both PCCs and PSCs.

Expression levels of membrane RTKs in pancreatic cancer cell lines, PSCs, and pancreatic cancer tissues

Next, I analyzed the expression levels of 15 RTKs by QTAP in plasma membrane fractions from 10 human pancreatic cancer cell lines, three primary cultured PSCs (PSCI, PSCII, PSCIII), pancreatic cancer tissues obtained from Patient 1-17 (PT1-17), and three normal pancreatic tissues obtained from normal noncancerous lesion of surgical specimens from Patient 1-3 (NT1-3) (Table 2). EGFR and MET were detected in all of the pancreatic cancer cell lines, while EGFR, PDGFRβ, and MET were detected in the three PSCs. Moreover, EGFR and PDGFRβ were detected in all 17 pancreatic cancer tissues, and MET was detected in 11 pancreatic cancer tissues (64.7%). In the three noncancerous tissues, only EGFR was detected. Na^+/K^+ ATPase was detected in all of the samples.

EGFR expression was about 10–20-fold higher in most pancreatic cancer cell

lines than in PSCs and pancreatic cancer tissues (Fig. 3A). In eight pancreatic cancer cell lines, the expression levels of PDGFRβ were under the detection limit; however, this RTK was detected in all of the PSCs and pancreatic cancer tissues (Fig. 3A). Expression levels of PDGFRβ were equivalent between PSCs and pancreatic cancer tissues. MET expression levels in pancreatic cancer cell lines were higher than those in PSCs and pancreatic cancer tissues (Fig. 3A).

Next, I compared the expression levels of RTKs between pancreatic cancer tissues (PT1-3) and normal pancreatic tissues (NT1-3). Expression levels of EGFR in pancreatic cancer tissues were 2.6–4.9-fold higher than those in noncancerous tissues (Fig. 3B). The expression levels of both MET and PDGFRβ in noncancerous tissues were all under the detection limit, whereas both proteins were detected in pancreatic cancer tissues (Fig. 3B).

MK2461 inhibited the effects of PSC conditioned medium on PCC proliferation by suppressing the activation of MET and its downstream signaling

The RTK profiling experiments revealed that MET was expressed in both PCCs and PSCs, but not in normal pancreatic tissue, suggesting that inhibition of MET could effectively inhibit the interaction between PSCs and PCCs. In both SUIT-2 and PANC-1 cells, significant growth inhibition was observed at 1 μM MK2461 (Fig. 4A). Additionally, 0.1 μM MK2461 significantly inhibited SUIT-2 cell proliferation induced by PSC conditioned medium. To assess whether MK2461 actually inhibited the effects of PSC conditioned medium, I compared the effects of MK2461 in cells treated with or without conditioned medium. In SUIT-2 and PANC-1 cells, inhibition of cell growth by

1 or 3 μM MK2461, respectively, was significantly higher in cells cultured with PSC conditioned medium than in cells cultured in serum-free medium (Fig. 4B). Furthermore, I evaluated the effects of MK2461 by western blotting (Fig. 4C). In both SUIT-2 and PANC-1 cells, MK2461 inhibited the phosphorylation of MET, AKT, and ERK1/2 induced by PSC conditioned medium.

MK2461 inhibited the effects of PCC conditioned medium on PSC proliferation and suppressed activation of PDGFRβ, MET, and downstream signaling

Since PDGFRβ and MET were expressed in PSCs and PCC conditioned medium induced PSC proliferation, I next evaluated the effects of MK2461 on PSC proliferation. In both PSCIs and PSCIIs, MK2461 significantly suppressed the proliferation induced by PCC conditioned medium when used at concentrations of 0.1 and 1 μM, respectively (Fig. 5A). Moreover, the growth of PSCIs and PSCIIs was significantly inhibited in the presence of PCC conditioned medium compared to serum-free medium following treatment with 0.1 or 1 μM MK2461, respectively (Fig. 5B). In addition, phosphorylation of PDGFRβ, MET, ERK1/2, and AKT in PSCs stimulated by PCC conditioned medium was inhibited by MK2461 (Fig. 5C).

MK2461 inhibited the effects of PSC condition medium on PCC invasion and migration

Overexpression or hyperactivation of MET has been associated with increased invasiveness in several cancers [31]. As PSC conditioned medium activated MET (Fig. 4), I next sought to determine whether blocking MET activation affected PCC invasion and migration. Indeed, I observed that exposure of SUIT-2 and PANC-1 cells to PSC

conditioned medium significantly enhanced the invasion and migration of these cells compared to culture under serum-free conditions in a concentration-dependent manner (Fig. 6A, B). Furthermore, inhibition of MET with increasing concentrations of MK2461 in both SUIT-2 and PANC-1 cells decreased the number of invading (Fig 6C) or migrating (Fig 6D) cells in a concentration-dependent manner.

MK2461 regulated tumor progression in vivo

Our results indicated that MK2461 significantly affected pancreatic cancer progression by disrupting the PCC-PSC interaction. To further characterize this process, I evaluated whether MK2461 inhibited tumor growth in a mouse xenograft model. PSCs alone are not tumorigenic; therefore, I co-injected SUIT-2 cells and PSCs into the right flanks of mice and SUIT-2 cells alone into the left flanks of mice, without using a PSC-alone control. One week after inoculation, mice were treated with MK2461 (20 mg/kg twice daily) or vehicle control for 20 days. No decreases in body weights of the mice were observed, and no treatment-related deaths were observed. In the co-injection model, MK2461 significantly inhibited tumor progression compared to vehicle, whereas in the SUIT-2-alone injection model, I did not observe any differences in tumor volumes between the MK2461-treated group and the vehicle-treated group (Fig. 7A). Consistent with this, I observed significant reductions in final tumor weights following MK2461 treatment compared with vehicle treatment for the co-injection model (vehicle: 431 ± 33.2 mg vs. MK2461: 320 ± 26.3 mg, $P = 0.022$) but not for tumors containing SUIT-2 cells alone (vehicle: 259 ± 23.9 mg vs. MK2461: 247 ± 12.6 mg, $P = 0.67$). Therefore, MK2461 inhibited tumor growth only in the presence of PSCs.

Since MK2461 had antitumor effects in both SUIT-2 cells and PSCs in our *in vitro* study (Figs. 4A and 5A), I next examined whether MK2461 inhibited the proliferation of both SUIT-2 cells and PSCs using the markers Ki-67 and cytokeratin19 (Fig. 7B and C). In co-injection models, the percentage of Ki-67-positive SUIT-2 cells was significantly higher in the vehicle group than in the MK2461 group, although no significant difference was observed between the vehicle group and MK2461 group in tumors arising from injection of SUIT-2 cells alone (Fig. 7D). Additionally, the percentage of Ki-67-positive PSCs was significantly higher in the vehicle group than in the MK2461 group (Fig. 7E). Similarly, the number of α-SMA-positive cells per field was significantly higher in the vehicle group than in the MK2461 group (Fig. 7F).

Discussion

For the development of potential new therapeutic options for the treatment of pancreatic cancer, it is important to consider the tumor microenvironment. Therefore, in this study, I assessed the interactions between PCCs and PSC, which have been implicated in the progression to the malignant phenotype [20, 21, 32]. I showed that PSC conditioned medium, which include secretions from the cells, promoted the proliferation of PCCs; conversely, conditioned medium from PCCs stimulated the proliferation of PSCs. Furthermore, I found that paracrine signaling was more effective at promoting proliferation than autocrine signaling, suggesting that components secreted from PSCs are essential to the progression of pancreatic cancer. Importantly, I also found that this effect was controlled by MK2461, a multitargeted kinase inhibitor, suggesting that MK2461 may represent a novel therapeutic agent for the treatment of pancreatic cancer.

Previous studies have shown that PSCs exhibit increased secretion of growth factors, such as PDGF, HGF, CTGF, and FGF, and ECM components, including collagen type I and fibronectin, through the PCC-PSC interaction, resulting in enhancement of PCC proliferation [18]. On the other hand, PCCs secrete growth factors such as PDGF, FGF, and TGFβ1 to promote PSC proliferation and to stimulate the secretion of additional growth factors and ECM components from PSCs [10, 20]. Therefore, I hypothesized that inhibition of RTK signaling could suppress the progression of pancreatic cancer.

Using QTAP by LC-MS/MS, I revealed the expression levels of 15 RTKs

among 10 pancreatic cancer cell lines, three primary cultured PSCs, and 17 human pancreatic cancer tissues. I used purified plasma membrane fractions; therefore, the results accurately reflected the RTK expression levels presented on the cellular membrane. Importantly, my data demonstrated that EGFR, MET, and PDGFRβ were expressed in pancreatic cancer cell lines, PSCs, and/or pancreatic tissues. Thus, my data supported that these RTKs likely played an important role in the PCC-PSC interaction.

The expression levels of RTKs in pancreatic cancer tissues were almost equivalent to those of PSCs, consistent with the observation that 80% of pancreatic tissue consists of PSCs. Previous studies have shown that the expression of PDGFRβ in PSCs correlates with the prognosis of patients with pancreatic cancer [33, 34]. In this study, all of the established PSCs expressed PDGFRβ. Moreover, previous studies have shown that PCCs express MET and that MET expression is correlated with prognosis [35-37]. HGF secretion from PSCs has been shown to accelerate the progression of pancreatic cancer by increasing cell proliferation, invasion, and migration [38-40]. My RTK profiling showed that MET was expressed not only in PCCs but also in PSCs. Interestingly, despite the high expression of MET in PCC, SUIT-2 cells alone are only weakly tumorigenic and essentially insensitive to MET inhibition in the absence of PSCs or PSC conditioned medium. This suggests that MET is inactive in PCCs and requires PSCs for activation. Hence, the HGF/MET pathway is thought to be important in mediating the interaction between PCCs and PSCs.

PSCs are considered a potential target for pancreatic cancer therapy, and PSC proliferation induced by components secreted from PCCs is significantly inhibited by

PDGF-neutralizing antibodies [20]. Therefore, targeting MET and PDGFRβ should be more effective for the inhibition of PSC proliferation. Previous studies have also showed that inhibition of HGF/MET signaling contributes to the regulation of PCC progression *in vitro* and *in vivo* [41]. In my pilot study, Tivantinib, MET inhibitor, suppressed SUIT-2 proliferation treated with PSC conditioned medium and imatinib, PDGFR inhibitor, and Tivantinib suppressed PSC proliferation treated with SUIT-2 conditioned medium. Therefore, in this study, I focused on the effects of MK2461, a multikinase inhibitor targeting MET and PDGFRβ, on the interaction between PSCs and PCCs. I did not use EGFR inhibitors because, although EGFR was detected in both pancreatic cancer cell lines and PSCs, EGF is not thought to be secreted from PSCs and the role of EGF/EGFR signaling is supposed to be small in the PSC-PCC interaction. In addition, in a clinical trial examining the efficacy of an EGFR inhibitor in the treatment of pancreatic cancer, overall survival was prolonged only 2 weeks by adding EGFR inhibitor to gemcitabine compared with gemcitabine only, so EGFR inhibitor is not used in standard pancreatic cancer therapy[42]. Furthermore, in my pilot study, Gefitinib, EGFR inhibitor, had no effect on SUIT-2 and PSC proliferation.

In this study, I found that MK2461 significantly inhibited PCC proliferation induced by PSC conditioned medium. Moreover, MET and downstream signaling components, such as ERK1/2 and AKT, were activated by PSC conditioned medium, and the phosphorylation of these enzymes was suppressed by MK2461 treatment. Consistently with this, MK2461 significantly inhibited PSC proliferation in the presence of PCC conditioned medium compared to serum-free medium, and MET,

PDGFRβ, ERK1/2, and AKT were activated by PCC conditioned medium, but inactivated by treatment with MK2461. Since MET was activated by PCC conditioned medium, these data suggested that HGF was secreted from both PCCs and PSCs and contributed to autocrine signaling.

An important observation in this model was that MK2461 significantly inhibited both PCC and PSC proliferation, disrupting the PCC-PSC interaction. Previous studies have focused on the inhibition of cell growth for either PCCs or PSCs. In the studies focusing on the tumor microenvironment, inhibiting PSC proliferation and suppressing the secretion of growth factors and other components from PSCs have been shown to result in inhibition of pancreatic cancer progression or enhancement of chemotherapeutic effects [15, 16, 43]. In this study, MK2461 was found to contribute to the reduction in PSC secretion by inhibiting PSC proliferation. Furthermore, MK2461 also inhibited the activation of MET, which was highly expressed in PCCs and was activated by PSC conditioned medium. Therefore, MK2461 was thought to exert substantial inhibitory effects on pancreatic cancer progression.

PDGF and HGF have been reported to promote cancer cell invasion and migration [39, 44], and previous studies have indicated that PSC conditioned medium promotes PCC invasion and migration [18, 21, 32], which was confirmed by the results of this study. I also revealed that MK2461 inhibited the effects of PSC conditioned medium on PCC invasion and migration. Therefore, future studies should assess whether MK2461 reduces the incidence of metastasis using orthotopic xenograft models.

In this *in vivo* study, I revealed that MK2461 significantly inhibited tumor growth in co-injection models and that the effects of MK2461 were not observed in tumors derived from SUIT-2 cells alone. These findings were consistent with my *in vitro* proliferation assays. Surprisingly, tumor volumes in the MK2461 group in the co-injection model were equivalent to those in the vehicle group in the tumors derived from injection of SUIT-2 cells alone; therefore, these findings suggested that MK2461 strongly inhibited tumor growth by disrupting the PCC-PSC interaction. Targeting HGF/MET signaling with a monovalent monoclonal antibody against MET was reported to inhibit HGF paracrine-driven pancreatic tumor growth in a xenograft model [41]. However, while SUIT-2 cells secreted HGF, MK2461 did not suppress tumor progression in our mouse model in which SUIT-2 cells were injected alone. These data suggested that the paracrine pathway was essential to the growth of PCCs, consistent with the results of our *in vitro* study.

In previous study, it is reported that the PCC-PSC interaction which work as paracrine pathway plays an important role in pancreatic cancer progression[9, 10]. In contrast, latest studies showed opposite function of stroma that stroma act to restrain, rather than support in pancreatic cancer progression[45, 46]. From theses studies, stroma is supposed to have dual function. It is supposed that MK2461 regulated stromal function that promote pancreatic cancer progression, resulted in suppressing pancreatic cancer progression.

Immunofluorescence staining for Ki-67 showed that MK2461 inhibited both PCC and PSC proliferation in co-injection models, but not in tumors derived from

injection of SUIT-2 cells alone. These results confirmed the reduction in tumor volume in the co-injection model. In addition, my results suggested that MK2461 inhibited PSC proliferation more strongly than PCC proliferation. In addition, the number of α-SMA-positive cells in the MK2461 group was less than 30% that of the vehicle group, whereas the number of cytokeratin19-positive cells was equivalent between the two groups (data not shown). This indicated that the area occupied by PSCs in tumors was reduced, consistent with the results of immunofluorescence staining for Ki-67.

Clinically, our data may have implications in the field of personalized medicine. Indeed, I showed that MET exhibited differential expression in pancreatic cancer tissues, suggesting that MK2461 may be more effective in some patients (i.e., those expressing high levels of MET) than in others. These conjectures will need to be explored further in additional studies.

In conclusion, profiling of 15 RTKs showed that PDGFRβ and MET were highly expressed in PCCs and PSCs. Moreover, MK2461 treatment effectively inhibited tumor progression in pancreatic cancer by disrupting the PCC-PSC interaction *in vitro* and *in vivo*. Therefore, MK2461 may represent a novel chemotherapeutic agent for the treatment of pancreatic cancer, particularly in patients with high tumoral expression of MET.

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Figure legends

Figure 1. α-SMA expression in established PSCs and effects of the PCC-PSC interaction on cell proliferation.

Immunofluorescence staining of α-SMA and vimentin in established PSCs. Red: α-SMA; green: vimentin; blue: DAPI. Scale bar, 100 μm. (B) Protein lysates from PSCI, PSCII, or PSCIII and TIG-1-20 were analyzed for α-SMA expression by western blotting. GAPDH served as a loading control. (C) Effects of PSCI, PSCII, and PSCIII conditioned medium on PCC (SUIT-2 and PANC-1 cell) proliferation. Proliferation assays were performed 48 h after adding PSC conditioned medium. SFM, serum-free medium; CM, condition medium; *: $P < 0.05$, ***: $P < 0.001$ versus the serum-free medium control. (D) Effects of PCC (SUIT-2 and PANC-1 cell) conditioned medium on PSC (PSCI and PSCII) proliferation. Proliferation assays were performed 48 h after adding PCC conditioned medium. SFM, serum-free medium; CM, condition medium; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus the serum-free medium control.

Figure 2. Comparison of cell proliferation induced by paracrine and autocrine secretions.

(A) Proliferation assays were performed in SUIT-2 and PANC-1 cells 48 h after adding PSC conditioned medium. CM, condition medium; *: $P < 0.05$ versus PCC conditioned medium. (B) Proliferation assays were performed 48 h after adding PSC conditioned medium. CM, condition medium; *: $P < 0.05$, **: $P < 0.01$ versus PSC conditioned medium.

Figure 3. Expression levels of RTKs in plasma membrane fractions.

(A) Comparison of RTK (EGFR, PDGFRβ, and MET) expression levels between pancreatic cancer cell lines, PSCs, and pancreatic cancer tissues. PT1-17 indicates the pancreatic cancer tissue obtained from Patient 1-17. (B) Comparison of RTK (EGFR, PDGFRβ, and MET) expression levels between pancreatic cancer tissues and normal pancreatic tissues. Pancreatic cancer tissue and normal pancreatic tissue was separated from the sample given by Patient 1-3 and used for the assay. Each bar represents the protein expression level in the plasma membrane fraction (mean ± SEM).

Figure 4. MK2461 inhibited the effects of PSC conditioned medium on PCC proliferation and suppressed the activation of MET and downstream signaling.

(A) Proliferation assays were performed in SUIT-2 and PANC-1 cells 48 h after adding PSC conditioned medium (0.5 mg/mL) and MK2461 (0, 0.1, 1, and 3 μM). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus the untreated control. (B) Comparison of PCC (SUIT-2 and PANC-1 cell) growth inhibition by MK2461 in cells treated with PSC conditioned medium (0.5 mg/mL) or serum-free medium. Cell growth inhibition was calculated as the percent of absorbance differences between 0 μM and MK2461 (0.1, 1, and 3 μM). Proliferation assays were performed 48 h after adding PSC conditioned medium (0.5 mg/mL) and MK2461 (0, 0.1, 1, and 3 μM). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus the untreated control. (C) Western blotting of signaling intermediates. PCCs (SUIT-2 and PANC-1 cells) were incubated with MK2461 (1 μM) for 4 h, stimulated with PSC conditioned medium (0.5 mg/mL) for 10 min, then lysed. GAPDH served as the loading control. CM, condition medium

Figure 5. MK2461 inhibited the effects of PCC conditioned medium on PSC

proliferation and suppressed activation of PDGFRβ, MET, and downstream signaling.

(A) Proliferation assays were performed in SUIT-2 and PANC-1 cells 48 h after adding PCC conditioned medium (0.5 mg/mL) and MK2461 (0, 0.1, and 1 μM). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus the untreated control. (B) Comparison of PSC (PSCI and PSCII) growth inhibition by MK2461 in SUIT-2 and PANC-2 cells treated for 48 h with conditioned medium (0.5 mg/mL) or serum-free medium. Cell growth inhibition was calculated as a percent of absorbance differences between untreated cells and cells treated with MK2461 (0.1 and 1 μM). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus the untreated control. (C) Western blotting of signaling intermediates. PSCs were incubated with MK2461 (3 μM) for 4 h, stimulated with SUIT-2 cell conditioned medium (0.1 mg/mL) for 10 min, then lysed. GAPDH served as the loading control. CM, condition medium

Figure 6. PSC conditioned medium promoted PCC invasion and migration in an MK2461-dependent manner.

(A) Invasion assays were performed in SUIT-2 and PANC-1 cells 48 h after adding PSC conditioned medium. SFM, serum-free medium; CM, condition medium; **: $P < 0.01$, ***: $P < 0.001$ versus the serum-free medium control. Scale bar, 50 μm. (B) Migration assays were performed in SUIT-2 and PANC-1 cells 24 h after adding PSC condition medium (0.1 or 0.5 mg/mL). SFM, serum-free medium; CM, condition medium; **: $P < 0.01$, ***: $P < 0.001$ versus the serum-free medium control. Scale bar, 50 μm. (C) Invasion assays were performed in SUIT-2 and PANC-1 cells 48 h after

adding PSC conditioned medium (0.5 mg/mL) and MK2461 (0, 0.1, and 1 μM). **: $P <$ 0.01, ***: $P < 0.001$ versus the untreated control. Scale bar, 50 μm. (D) Migration assays were performed in SUIT-2 and PANC-1 cells 24 h after adding PSC condition medium (0.5 mg/mL) and MK2461 (0, 0.1, and 1 μM). *: $P < 0.05$, **: $P < 0.01$, ***: P < 0.001 versus the untreated control. Scale bar, 50 μm.

Figure 7. MK2461 regulated tumor progression in vivo.

The effects of MK2461 on tumor progression were assessed using a xenograft model. SUIT-2 cells and PSCs were subcutaneously co-injected into the right flanks of mice, while SUIT-2 cells were injected alone into the left flanks of mice. One week later, mice were administered vehicle or MK-2461 (20 mg/kg) twice daily for 20 days by oral gavage. (A) Co-injection model. ***: $P < 0.001$. SUIT-2 injection alone. (B) Immunofluorescence staining for cytokeratin19 + Ki-67 (top panel) and cytokeratin19 + DAPI (bottom panel). Red: cytokeratin19; green: Ki-67; blue: DAPI. Scale bar, 50 μm. (C) Immunofluorescence staining for α-SMA + Ki-67 (top panel) and for α-SMA + DAPI (bottom panel). Red: α-SMA; green: Ki-67; blue: DAPI. Scale bar, 50 μm. White arrows indicate PSCs stained for both α-SMA and Ki-67. (D) The percent of Ki-67-positive cells in cytokeratin19-positive cells. ***: $P < 0.001$. (E) The percent of Ki-67-positive cells in α-SMA-positive cells. ***: $P < 0.001$. (F) The number of α-SMA-positive cells in each field. ***: $P < 0.001$.

Abbreviations

α-SMA	Alpha smooth muscle actin
BSA	Bovine serum albumin
CTGF	Connective tissue growth factor
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRB2	Growth factor receptor-bound protein-2
HER2	Human epidermal growth factor receptor-2
HER3	Human epidermal growth factor receptor-3
HGF	Hepatocyte growth factor
HPLC	High performance liquid chromatography
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MRM	Multi-channel reaction monitoring
MEK	MAPK/ERK kinase

MET	Hepatocyte growth factor receptor
mTOR	Mammalian target of rapamycin
MRM	Multiple reaction monitoring
NF	Normal fibroblast
PBS	Phosphate buffered saline
PCC	Pancreatic cancer cell
PSC	Pancreatic stellate cell
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3 kinase
QTAP	Quantitative targeted absolute proteomics
SDS-PAGE	Sodium lauryl sulfate poly-acrylamide gel electrophoresis
SEM	Standard error of the mean
SRM	Selected reaction monitoring
TBS	Tris buffered saline
TGFβ	Transforming growth factor β
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Table 1 Peptide probe sequences and selected ions for quantification of each protein

name	MRM	st/is	Q1(m/z)	Q3(m/z)	DP(V)	CE(V)	Peptide sequence
EGFR	1	st	489.8	623.3	71	21	ELIIEFSK
		is	493.3	630.3	71	21	ELII*EFSK
	2	st	489.8	510.3	71	19	ELIIEFSK
		is	493.3	510.3	71	19	ELII*EFSK
	3	st	489.8	736.4	71	23	ELIIEFSK
		is	493.3	743.4	71	23	ELII*EFSK
	4	st	489.8	381.2	71	29	ELIIEFSK
		is	493.3	381.2	71	29	ELII*EFSK
HER2	1	st	391.7	570.3	91	19	VLQGLPR
		is	394.7	576.3	91	19	VLQGLP*R
	2	st	391.7	442.3	91	19	VLQGLPR
		is	394.7	448.3	91	19	VLQGLP*R
	3	st	391.7	683.4	91	21	VLQGLPR
		is	394.7	689.4	91	21	VLQGLP*R
	4	st	391.7	385.3	91	21	VLQGLPR
		is	394.7	391.3	91	21	VLQGLP*R
HER3	1	st	607.8	759.4	26	25	GVWIPEGESIK
		is	611.8	767.4	26	25	GVWIPEGESIK*
	2	st	607.8	343.2	26	27	GVWIPEGESIK
		is	611.8	343.2	26	27	GVWIPEGESIK*
	3	st	607.8	456.3	26	21	GVWIPEGESIK
		is	611.8	456.3	26	21	GVWIPEGESIK*
	4	st	607.8	872.5	26	32	GVWIPEGESIK
		is	611.8	880.5	26	23	GVWIPEGESIK*
PDGFRα	1	st	458.8	688.4	71	21	VEETIAVR
		is	461.8	694.4	71	21	VEETIAV*R
	2	st	458.8	559.4	71	23	VEETIAVR
		is	461.8	565.4	71	23	VEETIAV*R
	3	st	458.8	817.4	71	21	VEETIAVR
		is	461.8	823.4	71	21	VEETIAV*R
	4	st	458.8	345.2	71	24	VEETIAVR
		is	461.8	351.2	71	24	VEETIAV*R

PDGFRβ	1	st	690.3	834.4	70	29	EVDSDAYYVYR
		is	695.3	844.4	70	29	EVDSDAYYVYR*
	2	st	690.3	763.4	70	29	EVDSDAYYVYR
		is	695.3	773.4	70	29	EVDSDAYYVYR*
	3	st	690.3	600.3	70	29	EVDSDAYYVYR
		is	695.3	610.3	70	29	EVDSDAYYVYR*
	4	st	690.3	437.3	70	29	EVDSDAYYVYR
		is	695.3	447.3	70	29	EVDSDAYYVYR*
c-kit	1	st	582.3	965.5	66	23	VVEATAYGLIK
		is	586.3	973.5	66	23	VVEATAYGLIK*
	2	st	582.3	765.5	66	25	VVEATAYGLIK
		is	586.3	773.5	66	25	VVEATAYGLIK*
	3	st	582.3	836.5	66	25	VVEATAYGLIK
		is	586.3	844.5	66	25	VVEATAYGLIK*
	4	st	582.3	664.4	66	25	VVEATAYGLIK
		is	586.3	672.4	66	25	VVEATAYGLIK*
IGFR	1	st	639.9	753.5	81	33	AENGPGPGVLVLR
		is	644.9	763.5	81	33	AENGPGPGVLVLR*
	2	st	639.9	907.6	81	31	AENGPGPGVLVLR
		is	644.9	917.6	81	31	AENGPGPGVLVLR*
	3	st	639.9	500.4	81	35	AENGPGPGVLVLR
		is	644.9	510.4	81	35	AENGPGPGVLVLR*
	4	st	639.9	810.5	81	35	AENGPGPGVLVLR
		is	644.9	820.5	81	35	AENGPGPGVLVLR*
VEGFR1	1	st	621.8	789.4	101	27	NILLSENNVVK
		is	625.8	797.4	101	27	NILLSENNVVK*
	2	st	621.8	1015.6	101	25	NILLSENNVVK
		is	625.8	1023.6	101	25	NILLSENNVVK*
	3	st	621.8	902.5	101	27	NILLSENNVVK
		is	625.8	910.5	101	27	NILLSENNVVK*
	4	st	621.8	702.4	101	27	NILLSENNVVK
		is	625.8	710.4	101	27	NILLSENNVVK*

VEGFR2	1	st	773.4	940.5	126	31	TFEDIPLEEPEVK
		is	777.4	948.5	126	31	TFEDIPLEEPEVK*
	2	st	773.4	472.3	126	47	TFEDIPLEEPEVK
		is	777.4	480.3	126	47	TFEDIPLEEPEVK*
	3	st	773.4	601.3	126	31	TFEDIPLEEPEVK
		is	777.4	609.3	126	31	TFEDIPLEEPEVK*
	4	st	773.4	730.4	126	43	TFEDIPLEEPEVK
		is	777.4	738.4	126	43	TFEDIPLEEPEVK*
VEGFR3	1	st	706.9	903.5	41	33	GPILEATAGDELVK
		is	710.9	911.5	41	33	GPILEATAGDELVK*
	2	st	706.9	1032.5	41	31	GPILEATAGDELVK
		is	710.9	1040.5	41	31	GPILEATAGDELVK*
	3	st	706.9	660.4	41	31	GPILEATAGDELVK
		is	710.9	668.4	41	31	GPILEATAGDELVK*
	4	st	706.9	832.4	41	31	GPILEATAGDELVK
		is	710.9	840.4	41	31	GPILEATAGDELVK*
MET	1	st	580.8	819.5	76	23	DLIGFGLQVAK
		is	584.8	827.5	76	23	DLIGFGLQVAK*
	2	st	580.8	615.4	76	23	DLIGFGLQVAK
		is	584.8	623.4	76	23	DLIGFGLQVAK*
	3	st	580.8	932.6	76	27	DLIGFGLQVAK
		is	584.8	940.6	76	27	DLIGFGLQVAK*
	4	st	580.8	762.5	76	23	DLIGFGLQVAK
		is	584.8	770.5	76	23	DLIGFGLQVAK*
FGFR1	1	st	623.3	788.4	101	27	DDVQSINWLR
		is	628.3	798.4	101	27	DDVQSINWLR*
	2	st	623.3	588.3	101	25	DDVQSINWLR
		is	628.3	598.3	101	25	DDVQSINWLR*
	3	st	623.3	916.5	101	25	DDVQSINWLR
		is	628.3	926.5	101	25	DDVQSINWLR*
	4	st	623.3	701.4	101	29	DDVQSINWLR
		is	628.3	711.4	101	29	DDVQSINWLR*

FGFR2	1	st	495.8	804.5	81	23	DAAVISWTK
		is	499.8	812.5	81	23	DAAVISWTK*
	2	st	495.8	634.4	81	17	DAAVISWTK
		is	499.8	642.4	81	17	DAAVISWTK*
	3	st	495.8	733.4	81	19	DAAVISWTK
		is	499.8	741.4	81	19	DAAVISWTK*
	4	st	495.8	357.2	81	19	DAAVISWTK
		is	499.8	357.2	81	19	DAAVISWTK*
FGFR3	1	st	673.4	819.5	61	31	VGPDGTPYVTVLK
		is	677.4	827.5	61	31	VGPDGTPYVTVLK*
	2	st	673.4	977.6	61	25	VGPDGTPYVTVLK
		is	677.4	985.6	61	25	VGPDGTPYVTVLK*
	3	st	673.4	460.3	61	39	VGPDGTPYVTVLK
		is	677.4	468.3	61	39	VGPDGTPYVTVLK*
	4	st	673.4	1092.6	61	27	VGPDGTPYVTVLK
		is	677.4	1100.6	61	27	VGPDGTPYVTVLK*
FGFR4	1	st	649.3	744.4	86	27	YNYLLDVLER
		is	654.3	754.4	86	27	YNYLLDVLER*
	2	st	649.3	631.3	86	27	YNYLLDVLER
		is	654.3	641.3	86	27	YNYLLDVLER*
	3	st	649.3	441.2	86	27	YNYLLDVLER
		is	654.3	441.2	86	27	YNYLLDVLER*
	4	st	649.3	1020.6	86	27	YNYLLDVLER
		is	654.3	1030.6	86	27	YNYLLDVLER*
Na+/K+ ATPase	1	st	414.2	685.4	50	17	AAVPDAVGK
		is	417.2	691.4	50	17	AAVPDAV*GK
	2	st	414.2	586.3	50	17	AAVPDAVGK
		is	417.2	592.3	50	17	AAVPDAV*GK
	3	st	414.2	489.3	50	27	AAVPDAVGK
		is	417.2	495.3	50	27	AAVPDAV*GK
	4	st	414.2	374.2	50	27	AAVPDAVGK
		is	417.2	380.2	50	27	AAVPDAV*GK

Conditions of MRM were optimized for high signal intensity by direct injection of peptide solution into the mass spectrometer through a turbo ion spray source. Theoretical m/z values of doubly charged ions of intact peptides (Q1) were postulated as precursor ions. Four singly charged fragment ions (Q3) were derived from each precursor ion. In peptide sequence, letters with asterisks and under bars indicate amino acid residues labeled with stable isotope (^{13}C and ^{15}N). MRM, Multi-channel reaction monitoring; st, standard peptide; is, internal peptide; DP, declustering potential; CE, collusion energy

Table 2. Expression profile of RTKs in plasma membrane fraction

	Pancreatic cancer cell lines									
	SUIT-2	PANC-1	AsPC-1	KLM-1	PK-1	PK-8	SW1990	BxPC-3	HPAF-Ⅱ	Capan-2
Recetor tyrosine kinase										
EGFR	115±2.37	21.1±0.767	16.9±0.107	16.6±0.352	38.5±0.478	13.6±0.109	9.55±0.322	30.1±0.313	31.1±0.146	24.8±0.261
HER2	2.91±0.224	0.763±0.014	6.27±0.135	2.26±0.076	2.79±0.068	1.37±0.012	1.90±0.046	0.958±0.041	2.63±0.088	1.03±0.077
HER3	1.08±0.016	U.L.Q.(<0.226)	2.32±0.014	0.939±0.005	0.535±0.012	0.509±0.012	0.451±0.018	U.L.Q.(<0.226)	U.L.Q.(<0.227)	U.L.Q.(<0.226)
PDGFRα	U.L.Q.(<0.172)	U.L.Q.(<0.179)	U.L.Q.(<0.148)	U.L.Q.(<0.160)	U.L.Q.(<0.161)	U.L.Q.(<0.172)	U.L.Q.(<0.179)	U.L.Q.(<0.326)	U.L.Q.(<0.195)	U.L.Q.(<0.214)
PDGFRβ	U.L.Q.(<0.497)	U.L.Q.(<0.504)	1.56±0.157	U.L.Q.(<0.523)	1.36±0.143	U.L.Q.(<0.518)	U.L.Q.(<0.535)	U.L.Q.(<0.426)	U.L.Q.(<0.443)	U.L.Q.(<0.450)
c-kit	U.L.Q.(<0.855)	U.L.Q.(<0.847)	U.L.Q.(<0.864)	U.L.Q.(<0.851)	U.L.Q.(<0.853)	U.L.Q.(<0.837)	U.L.Q.(<0.844)	3.89±1.58	U.L.Q.(<0.938)	U.L.Q.(<0.798)
IGFR	1.16±0.035	1.85±0.072	3.93±0.126	1.40±0.052	1.35±0.058	2.00±0.111	U.L.Q.(<0.246)	1.72±0.082	0.963±0.046	U.L.Q.(<0.380)
VEGFR1	0.787±0.059	U.L.Q.(<0.367)	0.578±0.018	0.893±0.030	0.620±0.009	0.753±0.049	U.L.Q.(<0.363)	U.L.Q.(<0.368)	U.L.Q.(<0.353)	U.L.Q.(<0.371)
VEGFR2	U.L.Q.(<0.823)	U.L.Q.(<0.829)	U.L.Q.(<0.823)	U.L.Q.(<0.840)	U.L.Q.(<0.831)	U.L.Q.(<0.838)	U.L.Q.(<0.857)	U.L.Q.(<0.504)	U.L.Q.(<0.814)	U.L.Q.(<0.776)
VEGFR3	U.L.Q.(<0.665)	1.12±0.103	U.L.Q.(<0.671)	U.L.Q.(<0.656)	0.834±0.039	U.L.Q.(<0.676)	U.L.Q.(<0.656)	U.L.Q.(<0.698)	U.L.Q.(<0.691)	U.L.Q.(<0.704)
MET	10.8±0.118	4.72±0.229	12.7±0.142	6.94±0.313	14.3±0.233	6.35±0.169	5.53±0.282	17.5±0.627	10.6±0.307	10.3±0.094
FGFR1	U.L.Q.(<0.435)	U.L.Q.(<0.453)	U.L.Q.(<0.469)	U.L.Q.(<0.438)	U.L.Q.(<0.463)	U.L.Q.(<0.439)	U.L.Q.(<0.433)	U.L.Q.(<0.433)	U.L.Q.(<0.478)	U.L.Q.(<0.437)
FGFR2	U.L.Q.(<0.120)	U.L.Q.(<0.118)	U.L.Q.(<0.118)	U.L.Q.(<0.110)	U.L.Q.(<0.126)	U.L.Q.(<0.110)	U.L.Q.(<0.123)	U.L.Q.(<0.111)	U.L.Q.(<0.120)	U.L.Q.(<0.124)
FGFR3	U.L.Q.(<0.405)	U.L.Q.(<0.409)	U.L.Q.(<0.410)	U.L.Q.(<0.413)	U.L.Q.(<0.411)	U.L.Q.(<0.405)	U.L.Q.(<0.411)	U.L.Q.(<0.578)	U.L.Q.(<0.527)	U.L.Q.(<0.440)
FGFR4	U.L.Q.(<0.516)	U.L.Q.(<0.517)	U.L.Q.(<0.550)	U.L.Q.(<0.511)	U.L.Q.(<0.508)	U.L.Q.(<0.513)	U.L.Q.(<0.504)	U.L.Q.(<0.508)	U.L.Q.(<0.523)	U.L.Q.(<0.523)
Membrane marker protein										
Na+/K+ ATPase	55.2±1.62	33.9±1.60	63.3±0.689	58.6±1.47	58.9±0.924	70.8±0.996	50.3±1.13	57.3±1.66	120±2.97	35.1±0.970

	Primary cultured PSCs			Non-pancreatic cancer tissues		
	PSCI	PSCII	PSCIII	NT1	NT2	NT3
Recetor tyrosine kinase						
EGFR	1.97±0.043	1.79±0.038	2.5±0.035	1.86±0.087	0.707±0.022	0.190±0.033
HER2	0.524±0.047	0.874±0.043	0.774±0.056	U.L.Q.(<0.926)	U.L.Q.(<0.867)	U.L.Q.(<0.071)
HER3	U.L.Q.(<0.083)	U.L.Q.(<0.086)	U.L.Q.(<0.083)	U.L.Q.(<0.351)	U.L.Q.(<0.344)	U.L.Q.(<0.358)
PDGFRα	0.940±0.051	U.L.Q.(<0.271)	U.L.Q.(<0.271)	U.L.Q.(<0.154)	U.L.Q.(<0.171)	U.L.Q.(<0.125)
PDGFRβ	3.98±0.270	5.01±0.095	3.55±0.163	U.L.Q.(<1.26)	U.L.Q.(<0.992)	U.L.Q.(<0.441)
c-kit	U.L.Q.(<0.284)	U.L.Q.(<0.279)	U.L.Q.(<0.278)	U.L.Q.(<0.621)	U.L.Q.(<0.656)	U.L.Q.(<0.847)
IGFR	U.L.Q.(<0.332)	U.L.Q.(<0.326)	U.L.Q.(<0.308)	U.L.Q.(<0.208)	U.L.Q.(<0.229)	U.L.Q.(<0.417)
VEGFR1	U.L.Q.(<0.345)	U.L.Q.(<0.352)	U.L.Q.(<0.335)	U.L.Q.(<0.320)	U.L.Q.(<0.303)	U.L.Q.(<0.325)
VEGFR2	U.L.Q.(<3.58)	U.L.Q.(<2.69)	U.L.Q.(<2.91)	U.L.Q.(<1.87)	U.L.Q.(<2.17)	U.L.Q.(<3.45)
VEGFR3	U.L.Q.(<0.776)	U.L.Q.(<0.812)	U.L.Q.(<0.784)	U.L.Q.(<0.748)	U.L.Q.(<0.716)	U.L.Q.(<0.631)
MET	1.2±0.023	1.17±0.055	1.01±0.060	U.L.Q.(<0.644)	U.L.Q.(<0.725)	U.L.Q.(<0.504)
FGFR1	U.L.Q.(<0.213)	U.L.Q.(<0.226)	U.L.Q.(<0.208)	U.L.Q.(<0.281)	U.L.Q.(<0.285)	U.L.Q.(<0.406)
FGFR2	U.L.Q.(<0.067)	U.L.Q.(<0.067)	U.L.Q.(<0.062)	U.L.Q.(<0.170)	U.L.Q.(<0.161)	U.L.Q.(<0.172)
FGFR3	U.L.Q.(<0.381)	U.L.Q.(<0.434)	U.L.Q.(<0.367)	U.L.Q.(<0.308)	U.L.Q.(<0.313)	U.L.Q.(<1.40)
FGFR4	U.L.Q.(<0.199)	U.L.Q.(<0.196)	U.L.Q.(<0.161)	U.L.Q.(<0.672)	U.L.Q.(<0.785)	U.L.Q.(<0.429)
Membrane marker protein						
Na+/K+ ATPase	9.86±0.228	19.8±0.786	22.4±0.984	18.1±0.729	21.3±0.391	21.0±0.601

	Pancreatic cancer tissues									
	PT1	PT2	PT3	PT4	PT5	PT6	PT7	PT8	PT9	PT10
Recetor tyrosine kinase										
EGFR	7.58±0.134	1.80±0.260	0.935±0.082	0.959±0.306	3.24±0.106	0.602±0.034	1.14±0.245	0.841±0.042	1.67±0.066	1.09±0.047
HER2	2.03±0.027	1.54±0.293	U.L.Q.(<0.070)	U.L.Q.(<0.104)	1.47±0.042	U.L.Q.(<0.071)	8.18±0.096	U.L.Q.(<0.090)	1.71±0.074	0.915±0.055
HER3	0.757±0.027	0.771±0.017	U.L.Q.(<0.360)	U.L.Q.(<0.365)	0.450±0.014	U.L.Q.(<0.360)	U.L.Q.(<0.323)	U.L.Q.(<0.358)	U.L.Q.(<0.353)	U.L.Q.(<0.332)
PDGFR α	U.L.Q.(<0.114)	1.22±0.159	U.L.Q.(<0.125)	U.L.Q.(<0.142)	U.L.Q.(<0.109)	U.L.Q.(<0.130)	1.21±0.078	U.L.Q.(<0.167)	2.06±0.032	U.L.Q.(<0.321)
PDGFR β	7.15±0.367	6.71±0.678	3.12±0.110	0.958±0.081	2.77±0.104	1.71±0.122	4.07±0.232	3.99±0.107	10.6±0.483	4.31±0.078
c-kit	U.L.Q.(<0.706)	7.64±2.26	U.L.Q.(<0.756)	U.L.Q.(<0.578)	1.39±0.092	U.L.Q.(<0.752)	U.L.Q.(<0.466)	U.L.Q.(<0.313)	U.L.Q.(<0.540)	U.L.Q.(<0.450)
IGFR	U.L.Q.(<0.265)	U.L.Q.(<0.336)	U.L.Q.(<0.336)	U.L.Q.(<0.334)	U.L.Q.(<0.191)	U.L.Q.(<0.333)	U.L.Q.(<0.350)	0.525±0.041	U.L.Q.(<0.235)	U.L.Q.(<0.156)
VEGFR1	U.L.Q.(<0.337)	1.04±0.451	U.L.Q.(<0.319)	U.L.Q.(<0.264)	U.L.Q.(<0.229)	U.L.Q.(<0.321)	U.L.Q.(<0.088)	U.L.Q.(<0.226)	U.L.Q.(<0.307)	U.L.Q.(<0.099)
VEGFR2	U.L.Q.(<2.60)	U.L.Q.(<3.09)	U.L.Q.(<3.43)	U.L.Q.(<2.28)	U.L.Q.(<2.15)	U.L.Q.(<3.43)	U.L.Q.(<3.47)	U.L.Q.(<2.09)	U.L.Q.(<1.80)	U.L.Q.(<3.56)
VEGFR3	U.L.Q.(<0.932)	3.68±1.03	U.L.Q.(<0.625)	U.L.Q.(<0.860)	U.L.Q.(<0.805)	U.L.Q.(<0.483)	U.L.Q.(<0.551)	U.L.Q.(<0.791)	U.L.Q.(<0.730)	U.L.Q.(<0.770)
MET	5.71±0.252	4.62±0.893	U.L.Q.(<0.529)	3.56±0.483	2.98±0.193	U.L.Q.(<0.550)	U.L.Q.(<0.496)	U.L.Q.(<0.541)	U.L.Q.(<0.628)	1.55±0.275
FGFR1	U.L.Q.(<0.287)	U.L.Q.(<0.336)	U.L.Q.(<0.434)	U.L.Q.(<0.503)	U.L.Q.(<0.421)	U.L.Q.(<0.436)	U.L.Q.(<0.426)	U.L.Q.(<0.440)	U.L.Q.(<0.293)	U.L.Q.(<0.434)
FGFR2	U.L.Q.(<0.181)	U.L.Q.(<0.212)	U.L.Q.(<0.173)	U.L.Q.(<0.195)	U.L.Q.(<0.165)	U.L.Q.(<0.164)	U.L.Q.(<0.160)	U.L.Q.(<0.171)	U.L.Q.(<0.160)	U.L.Q.(<0.182)
FGFR3	U.L.Q.(<0.343)	U.L.Q.(<1.27)	U.L.Q.(<1.60)	U.L.Q.(<1.82)	U.L.Q.(<1.23)	U.L.Q.(<1.38)	U.L.Q.(<1.20)	U.L.Q.(<1.04)	U.L.Q.(<0.288)	U.L.Q.(<1.48)
FGFR4	U.L.Q.(<0.679)	U.L.Q.(<0.976)	U.L.Q.(<0.220)	U.L.Q.(<0.266)	U.L.Q.(<0.179)	U.L.Q.(<0.212)	U.L.Q.(<0.388)	U.L.Q.(<0.420)	U.L.Q.(<0.667)	2.70±0.086
Membrane marker protein										
Na+/K+ ATPase	59.1±1.27	68.8±5.38	47.5±0.473	13.2±0.145	33.1±0.780	28.3±0.517	23.0±0.601	16.9±0.437	90.7±3.83	16.6±0.573

	Pancreatic cancer tissues						
	PT11	PT12	PT13	PT14	PT15	PT16	PT17
Recetor tyrosine kinase							
EGFR	1.40±0.012	1.02±0.023	1.06±0.068	1.58±0.021	0.828±0.039	0.796±0.064	0.838±0.015
HER2	1.50±0.029	1.24±0.070	1.20±0.029	1.35±0.141	0.801±0.031	0.966±0.068	0.707±0.020
HER3	0.589±0.017	0.505±0.029	0.534±0.019	0.811±0.044	U.L.Q.(<0.359)	0.426±0.011	U.L.Q.(<0.347)
PDGFRα	U.L.Q.(<0.308)	1.26±0.034	U.L.Q.(<0.312)	1.51±0.069	1.68±0.067	U.L.Q.(<0.353)	U.L.Q.(<0.337)
PDGFRβ	5.01±0.320	5.37±0.092	6.13±0.429	3.89±0.516	3.85±0.214	2.95±0.082	5.36±0.584
c-kit	U.L.Q.(<0.499)	U.L.Q.(<0.556)	U.L.Q.(<0.529)	U.L.Q.(<0.468)	U.L.Q.(<0.506)	U.L.Q.(<0.486)	U.L.Q.(<0.528)
IGFR	4.60±0.182	U.L.Q.(<0.148)	U.L.Q.(<0.137)	U.L.Q.(<0.150)	U.L.Q.(<0.162)	U.L.Q.(<0.158)	U.L.Q.(<0.161)
VEGFR1	U.L.Q.(<0.100)	U.L.Q.(<0.088)	U.L.Q.(<0.082)	U.L.Q.(<0.098)	U.L.Q.(<0.099)	U.L.Q.(<0.086)	U.L.Q.(<0.078)
VEGFR2	U.L.Q.(<3.51)	U.L.Q.(<3.71)	U.L.Q.(<3.69)	U.L.Q.(<4.06)	U.L.Q.(<3.51)	U.L.Q.(<2.93)	U.L.Q.(<2.56)
VEGFR3	U.L.Q.(<0.739)	U.L.Q.(<0.704)	U.L.Q.(<0.730)	U.L.Q.(<0.824)	U.L.Q.(<0.809)	U.L.Q.(<0.723)	U.L.Q.(<0.761)
MET	3.93±0.171	1.76±0.076	1.82±0.366	1.30±0.025	1.92±0.121	1.50±0.122	U.L.Q.(<0.486)
FGFR1	U.L.Q.(<0.424)	1.91±0.023	2.66±0.101	U.L.Q.(<0.424)	U.L.Q.(<0.438)	U.L.Q.(<0.434)	U.L.Q.(<0.402)
FGFR2	U.L.Q.(<0.192)	U.L.Q.(<0.201)	U.L.Q.(<0.201)	U.L.Q.(<0.183)	U.L.Q.(<0.201)	U.L.Q.(<0.186)	U.L.Q.(<0.196)
FGFR3	U.L.Q.(<1.27)	U.L.Q.(<1.16)	U.L.Q.(<1.36)	U.L.Q.(<1.33)	U.L.Q.(<1.32)	U.L.Q.(<1.20)	U.L.Q.(<1.35)
FGFR4	U.L.Q.(<0.380)	U.L.Q.(<0.417)	U.L.Q.(<0.421)	U.L.Q.(<0.408)	U.L.Q.(<0.438)	1.31±0.110	U.L.Q.(<0.361)
Membrane marker protein							
Na+/K+ ATPase	39.6±0.360	29.9±1.65	28.2±1.40	101±2.65	18.5±0.508	23.7±0.181	13.3±0.193

The quantitative values were calculated from peak area ratio of analyte to stable isotope-labeled peptides as internal standards. The expression levels of each protein was determined as the average of three to four quantitative values from four SRM/MRM transitions of one analysis (fmol / μg protein). Each sample was measured three times and the value represents the mean ± SEM (n=3). U.L.Q., under the limit of quantification