Serine Protease Inhibitor Kazal Type 1 Promotes Proliferation of Pancreatic Cancer Cells through the Epidermal Growth Factor Receptor

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Abstract

Serine protease inhibitor, Kazal type 1 (SPINK1) is expressed not only in normal human pancreatic acinar cells but also in a variety of pancreatic ductal neoplasms. There are structural similarities between SPINK1 and epidermal growth factor (EGF). Hence, we hypothesized that SPINK1 binds to EGF receptor (EGFR) to activate its downstream signaling. We first showed that SPINK1 induced proliferation of NIH 3T3 cells and pancreatic cancer cell lines. We showed that SPINK1 coprecipitated with EGFR in an immunoprecipitation experiment and that the binding affinity of SPINK1 to EGFR was about half of that of EGF using quartz-crystal microbalance (QCM) technique. As expected, EGFR and its downstream molecules, signal transducer and activator of transcription 3, v-Akt murine thymoma viral oncogene homologue, and extracellular signal-regulated kinase 1/2, were phosphorylated by SPINK1 as well as EGF. To determine which pathway is the most important for cell growth, we further analyzed the effect of inhibitors. Growth stimulation by EGF or SPINK1 was completely inhibited by EGFR and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor but not by Janus-activated kinase and phosphoinositide 3-kinase inhibitors. To further analyze the clinical importance of SPINK1 in the development of pancreatic cancer, we examined the expression of SPINK1 and EGFR in pancreatic tubular adenocarcinomas and pancreatic intraepithelial neoplasm. Both SPNK1 and EGFR were coexpressed not only in the early stage of cancer, PanIN-1A, but also in advanced stages. Taken together, these results suggest that SPINK1 stimulates the proliferation of pancreatic cancer cells through the EGFR/mitogen-activated protein kinase cascade. (Mol Cancer Res 2009;7(9):1572–81)

Introduction

Serine protease inhibitor, Kazal type 1 (SPINK1; ref. 1), which is also known as pancreatic secretory trypsin inhibitor and tumor-associated trypsin inhibitor, is mainly produced in the acinar cells of the exocrine pancreas. Tumor-associated trypsin inhibitor was initially isolated from the urine of a patient with ovarian cancer (2) and was later found to be identical to SPINK1 (3). The role of SPINK1 has been postulated to be the prevention of inadvertent proteolysis in the pancreas caused by intra-acinar premature activation of trypsinogen (4-6). However, considerable amounts of SPINK1 were detected in many extrapancreatic tissues, including the stomach, colon, small intestine, liver, lung, kidney, and ovary (7-10). In addition, Ogawa et al. (11, 12) found elevated serum SPINK1 concentrations in patients with severe systemic inflammation. We also found that Spink3, a mouse homologue of human SPINK1, was expressed in several tissues, including brain or urogenital system, during embryonic development (10). These results suggest that SPINK1 has additional functions in many tissues other than the pancreas.

Various cancers, including pancreatic cancers, produce SPINK1 (8, 13-16). SPINK1 is also a marker of poor prognosis (3, 17-20). Quantitative RT-PCR identified *SPINK1* as a gene associated with early recurrence of intrahepatic cholangiocarcinoma after resection (19). Thus far, the expression of SPINK1 in tumor cells has been explained by its coexpression with tumor-associated trypsin, which is thought to participate in tumor-associated protease cascades mediating tumor invasion (13). However, the role of SPINK1 in malignant tumors remains to be elucidated (19).

The possibility that SPINK1 was a growth factor was raised when SPINK1 was shown to have some structural similarities to epidermal growth factor (EGF). They share ~50% amino acid sequence homology (21, 22) and have similar numbers of amino acid residues (56 and 53, respectively; molecular weights

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~6 kDa) and three intrachain disulfide bridges (21-23). In addition, SPINK1 has been shown to bind specifically to several cell lines (24), suggesting that SPINK1 works as a growth factor in tumor cells. SPINK1 was shown to stimulate the growth of NIH 3T3 fibroblasts (25, 26), human endothelial cells (27), intestinal epithelial cells (28), and rat pancreatic cancer cells (29). On the other hand, the identity of the receptor for SPINK1 has been a controversial matter. SPINK1 was shown to bind to a cell surface receptor, which is distinct from the EGF receptor (EGFR; refs. 24, 30). In contrast, Fukuoka et al. (31) reported that SPINK1 competed with mouse EGF for binding to EGFR on NIH 3T3 fibroblasts. We showed previously that excessive autophagy (cellular self-digestion) is induced in pancreatic acinar cells deficient in Spink3 (5). Interestingly, residual duct-like cells in the tubular complexes of the pancreas showed no sign of acinar cell regeneration in spite of the expression of EGFR.[6] Based on these findings, we hypothesized that SPINK1 is a ligand for the EGFR. To test this, we investigated the (*a*) proliferative activity of SPINK1 using various cell lines, (*b*) binding of SPINK1 to receptors including EGFR, (*c*) activation of a downstream signal cascade, and (*d*) expression of SPINK1 and EGFR in pancreatic tubular adenocarcinomas and pancreatic intraepithelial neoplasm (PanIN).

Results

Growth-Stimulating Activity of SPINK1 on NIH 3T3 Fibroblast and Pancreatic Cancer Cell Lines

To examine whether EGF or SPINK1 act as growth factors, NIH 3T3 cells, four pancreatic cancer cell lines (AsPC-1, MIAPaCa-2, PANC-1, and Capan-2), and one breast cancer cell line (BT-474) were treated with EGF (100 pg/mL and 1, 10, and 100 ng/mL) or SPINK1 (1 and 10 ng/mL; Fig. 1).

By treatment with EGF, the cell numbers of NIH 3T3 and PANC-1 cells increased in a dose-dependent manner from 100 pg/mL to 10 ng/mL and the cell numbers of AsPC-1, Capan-2, and BT-474 increased in a dose-dependent manner from 100 pg/mL to 100 ng/mL. In case of MIAPaCa-2, the cell numbers increased in a dose-dependent manner from 1 to 100 ng/mL. In treatment with SPINK1, all these cells, except BT-474, showed increase of cell number in a dose-dependent manner. Although BT-474 cells originated from breast cancer cells, they express EGFR (32). Actually, EGFR is expressed especially in a triple-negative breast cancer [estrogen receptor (-), progesterone receptor (-), and human EGFR (HER2) (-)] patient, who has high-risk breast cancer (33). SPINK1 is expressed not only in the pancreas but also in the normal breast (34). Thus, it is reasonable that SPINK1 caused proliferation of BT-474 cells. Aprotinin, a bovine pancreatic trypsin inhibitor, did not stimulate the growth of NIH 3T3 fibroblasts and AsPC-1 (Fig. 1).

Binding of SPINK1 with EGFR and Binding Affinity Analysis Using Quartz-Crystal Microbalance

We next examined whether SPINK1 can bind with EGFR using immunoprecipitation with an anti-EGFR antibody followed by Western blot analysis with an anti-SPINK1 antibody in AsPC-1 and MIAPaCa-2 cell lines. As shown in Fig. 2A, SPINK1 was clearly detected in both cell lines, suggesting that SPINK1 did bind with EGFR.

We then examined the binding affinity of SPINK1 with EGFR, HER2 (ErbB2), HER3 (ErbB3), or HER4 (ErbB4) in comparison with EGF using a quartz-crystal microbalance (QCM) technique. In this method, qualitative and quantitative changes resulting from intermolecular interactions can be monitored and frequency (Hz) decreases in proportion to the change of molecular mass caused by binding of ligands, which are immobilized on the gold electrode surface, with receptors. When the EGFR extracellular domain-IgG-Fc fusion protein was injected into the equilibrated solution containing a SPINK1-immobilized or EGF-immobilized sensor chip, the frequency decreased to 244.3 or 421.8 Hz, respectively (Fig. 2B), suggesting that the binding affinity of SPINK1 is about half that of EGF. There was no change in frequency when the neuregulin-1 (NRG1) sensor chip was used. NRG1 is known to bind to HER3 and HER4 as a specific ligand. When the HER2 extracellular domain-IgG fusion protein was injected into the equilibrated solution containing a SPINK1-immobilized, EGF-immobilized, or NRG1-immobilized sensor chip, no decrease of frequency occurred (Fig. 2C). When the HER3 extracellular domain-IgG fusion protein was injected into the equilibrated solution containing SPINK1- or EGF-immobilized sensor chip, the frequency did not decrease. However, the frequency decreased to 235.4 Hz when the NRG1-immobilized sensor chip was used (Fig. 2D). When the HER4 extracellular domain-IgG fusion protein was injected into the equilibrated solution containing SPINK1- or EGF-immobilized sensor chip, the frequency did not decrease. The frequency decreased to 112.5 Hz when the NRG1-immobilized sensor chip was used (Fig. 2E). NRG1 can bind to both HER3 and HER4, but the affinity of NRG1 for HER3 was about twice that for HER4.

SPINK1 Phosphorylates EGFR and Its Downstream Targets

We investigated whether SPINK1 phosphorylates EGFR using Western blotting with rabbit anti-phosphorylated human EGFR antibody. Incubation of AsPC-1 and MIAPaCa-2 cells with varying doses of EGF (100 pg/mL and 1, 10, and 100 ng/mL) and SPINK1 (1 and 10 ng/mL) for 10 min resulted in phosphorylation of EGFR (Fig. 3A). Intensities of bands were stronger from cells treated with EGF than those treated with SPINK1.

We then examined phosphorylation of downstream targets. There are at least three signaling pathways downstream of EGFR, including the phosphoinositide 3-kinase (PI3K)/v-Akt murine thymoma viral oncogene homologue (AKT), Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT), and mitogen-activated protein kinase (MAPK) pathways. We investigated the phosphorylation of AKT, STAT3, and extracellular signal-regulated kinase 1/2 (ERK1/2) by Western blot analysis using anti-phosphorylated protein antibodies. AKT, STAT3, and ERK1/2 were phosphorylated in a dose-dependent manner by EGF. AKT, STAT3, and ERK1/2 were also phosphorylated by stimulation with SPINK1 (Fig. 3B). In addition, we investigated the level of GTP-RAS

[6] Unpublished data.

using a RAS activation assay and Western blotting in AsPC-1 and MIAPaCa-2 cell lines. GTP-RAS was activated after stimulation with SPINK1 as well as EGF (Fig. 3B).

Cell Growth Signal Is Mainly Mediated via MAPK Cascade

As shown above, three pathways were activated by EGF and SPINK1. To determine which pathway is actually involved in cell growth, we analyzed the effects of inhibitors on the growth of AsPC-1 cells. After stimulation of AsPC-1 cells with EGF or SPINK1, the cell number increased up to three times when compared with those cultured in serum-free medium (Fig. 4A-D). However, growth stimulation by EGF or SPINK1 was completely inhibited by the addition of an EGFR inhibitor (AG1478; Fig. 4A). EGFR was strongly expressed in the group to which AG1478 was added. This may be due to feedback up-regulation of EGFR when phosphorylation of EGFR was inhibited. This feedback regulation might be varied among samples. When AsPC-1 cells were treated with the JAK inhibitor (AG490) or PI3K inhibitor (LY294002), cell numbers tended to be lower than those without inhibitors, but the difference was not statistically significant (Fig. 4B and C). In contrast, the MAPK/ERK kinase (MEK) inhibitor (U0126) also completely inhibited cell proliferation by EGF or SPINK1 (Fig. 4D). These results clearly suggest that SPINK1 as well as EGF can bind to EGFR and stimulate cell growth mainly through the MAPK (RAS/MEK/ERK) cascade.

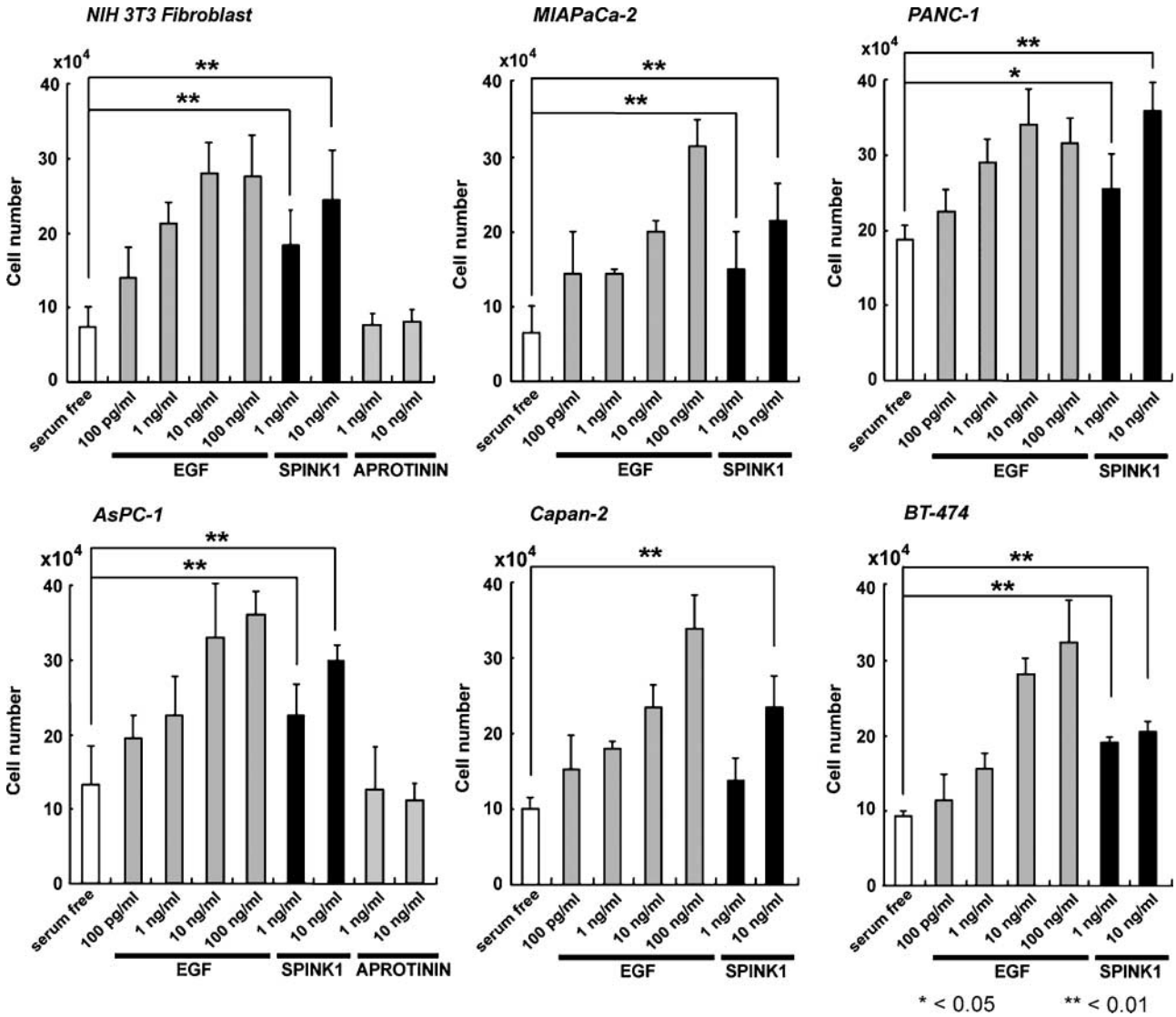

FIGURE 1. Proliferation of NIH 3T3 fibroblast and cancer cell lines. Fibroblasts were either untreated (control; FBS-free) or treated by serum-free medium containing EGF (100 pg/mL and 1, 10, and 100 ng/mL) or SPINK1 (1 and 10 ng/mL) or aprotinin (1 and 10 ng/mL) and then incubated for 72 h at 37°C. AsPC-1, MIAPaCa-2, PANC-1 Capan-2, and BT-474 cell lines were either untreated (control; FBS-free) or treated by serum-free medium containing EGF (100 pg/mL and 1, 10, and 100 ng/mL) or SPINK1 (1 and 10 ng/mL) and then incubated for 72 h at 37°C. Mean ± SE. statistical analysis was done by ANOVA followed by Student's *t* test. *, $P < 0.05$ for containing SPINK1 compared with serum-free; **, $P < 0.01$ for containing SPINK1 compared with serum-free.

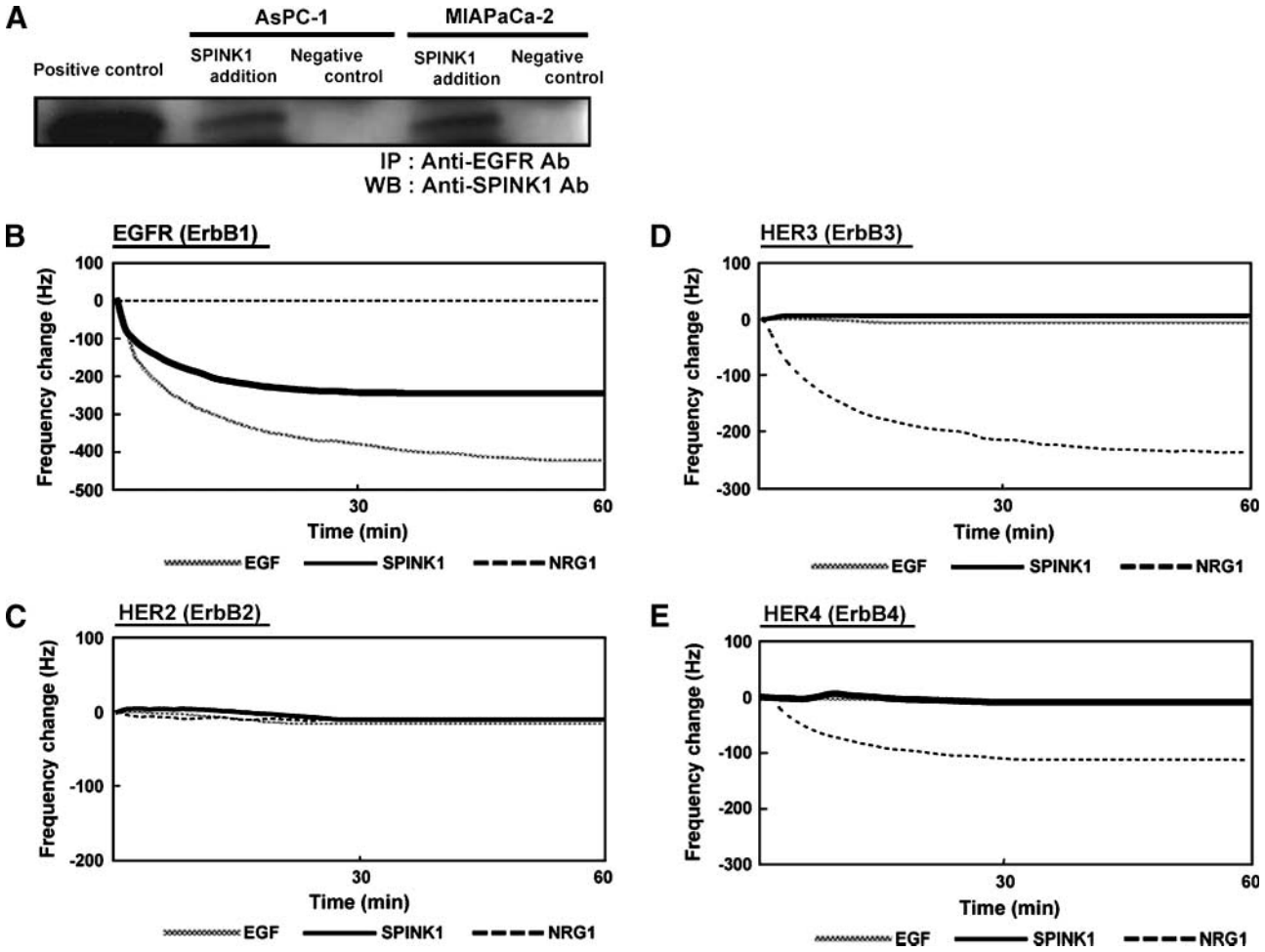

FIGURE 2. Binding of SPINK1 with EGFR. **A.** Binding of SPINK1 with EGFR. AsPC-1 and MIAPaCa-2 cell lines were either untreated (control; FBS-free) or treated by FBS-free medium containing SPINK1 (10 ng/mL) and then incubated for 10 min at 37°C. Immunoprecipitates with an anti-EGFR antibody were analyzed by Western blotting using rabbit anti-human SPINK1 antibody. **B.** QCM analysis using EGFR extracellular domain-IgG fusion protein. Decrease of frequency in SPINK1-immobilized sensor chip was about half of that in EGF-immobilized sensor chip. **C.** QCM analysis using HER2 extracellular domain-IgG fusion protein. Decrease of frequency was not observed for any sensor chip. **D.** QCM analysis using HER3 extracellular domain-IgG fusion protein. Decrease of frequency was observed in NRG1 sensor tip but not in other sensor chips. **E.** QCM analysis using HER4 extracellular domain-IgG fusion protein. Decrease of frequency was observed in NRG1 sensor tip but not in other sensor chips.

Expression of SPINK1 and EGFR in Pancreatic Tubular Adenocarcinomas and PanIN

As SPINK1 can bind to the EGFR, it is possible that SPINK1 can work as a growth factor for pancreatic cancer cells through EGFR. To test this possibility, we examined whether SPINK1 is coexpressed with EGFR. Histochemical and immunohistochemical analyses were done using normal pancreatic tissue and 22 cases of pancreatic tubular adenocarcinomas. In normal pancreas (Fig. 5A), pancreatic duct cells were negative for both SPINK1 (Fig. 5B) and EGFR immunostaining (Fig. 5C). Pancreatic acinar cells were positive for SPINK1 (Fig. 5B) but not for EGFR (Fig. 5C), as expected. However, all pancreatic tubular adenocarcinoma cases (Fig. 5D and G), which are derived from pancreatic duct cells, showed positive SPINK1 (Fig. 5E and H) and EGFR (Fig. 5F and I) immunoreactivity. We then examined the expression of SPINK1 and EGFR in PanIN. PanIN lesions were graded according to standard criteria (35). Twelve PanIN cases were positive for both SPINK1 and EGFR immunoreactivity (Fig. 6). Figure 6A to F shows early stages of intraepithelial neoplasm showing a clear boundary between the normal pancreatic duct and PanIN-1A. SPINK1 was strongly detected on a membrane of the lumenal side (Fig. 6B and E) in PanIN-1A lesion but not in normal pancreatic duct cells (Fig. 6E, *arrow*). On the other hand, EGFR was expressed in normal pancreatic duct cells as well as in the PanIN-1A region. In PanIN-1B and PanIN-2 lesions, mainly mucinous cytoplasm was stained with SPINK1. On the other hand, cytoplasm but not mucinous cytoplasm was stained with EGFR (Fig. 6H, I, K, and L). In PanIN-3 lesion, both SPINK1 and EGFR were detected in the same region under the light microscopic observation (Fig. 6N and O). Taken together, SPINK1 was expressed specifically in PanIN region from an early stage of cancer development, whereas EGFR was expressed not only in the PanIN region but also in morphologically normal duct cells from an early stage of cancer development.

Discussion

In this study, we showed that SPINK1 bound to the EGFR, mediating cell proliferation through the MAPK (RAS/MEK/ERK) cascade. We also showed that SPINK1 and EGFR were coexpressed not only in tubular adenocarcinoma cells but also in PanIN region from its early stage.

It is well documented that pancreatic adenocarcinoma and dysplasias frequently overexpress EGFR (36). In addition, there is evidence for an important contribution of autocrine EGF family signaling in pancreatic carcinoma cells (37-41). Consistent with the existence of such an autocrine loop, pancreatic adenocarcinomas overexpress EGF family ligands, such as transforming growth factor-α and EGF, and receptors including EGFR, HER2, and HER3 (39, 40). EGFR and HER2 induction also occurs in low-grade PanIN, indicating that autocrine EGF family signaling is operative at the early stages of a pancreatic neoplasm (42). Moreover, an EGFR-blocking antibody removed the promigratory effects of SPINK1 on human HT-29 cells (34). Thus far, seven ligands have been described for EGFR including EGF, transforming growth factor-α, heparin-binding EGF-like growth factor, amphiregulin, betacellulin, epiregulin, and epigen. However, ligands for EGFR in pancreatic cancer cells are still unclear. Here, we showed that SPINK1 is a new ligand for EGFR.

Fukuoka et al. (31) showed that the binding affinity of SPINK1 with EGFR was similar to that of EGF using [^{125}I]EGF radioreceptor assay. However, we showed that the binding affinity of SPINK1 with EGFR was about half of that of EGF using QCM. In a radioreceptor assay, the reaction mixture is required to contain several hundreds of microgram per milliliter of unlabeled SPINK1 or EGF, whereas we need only 0.2 μg to 2 μL. In our method, frequency (Hz) decreases in proportion to the change of molecular mass caused by binding of ligands to receptors; our method is much more sensitive and more reliable than radioreceptor assay. In addition, the conserved region in the above seven EGFR ligands is not present in SPINK1. Taken together, these results suggest that the affinity of SPINK1 for EGFR is lower than that of EGF and/or the binding site of EGFR with SPINK1 may not be the same as that of EGF. Yet, SPINK1 can phosphorylate EGFR and induce signal transduction.

PI3K/AKT, JAK/STAT, and MAPK pathways are downstream cascades of EGFR related to cell proliferation (43). Generally speaking, the MAPK pathway is an important route that regulates cell proliferation and survival (28). Activated MAPKs are imported into the nucleus where they phosphorylate specific transcription factors involved in cell proliferation (44). In our study, we showed that all three downstream targets are phosphorylated by SPINK1 and EGF. However, the MAPK pathway may play a major role in cell proliferation, because cell proliferation was completely inhibited by MEK inhibitor, like EGFR inhibitor, but not by PI3K and JAK inhibitors. In the

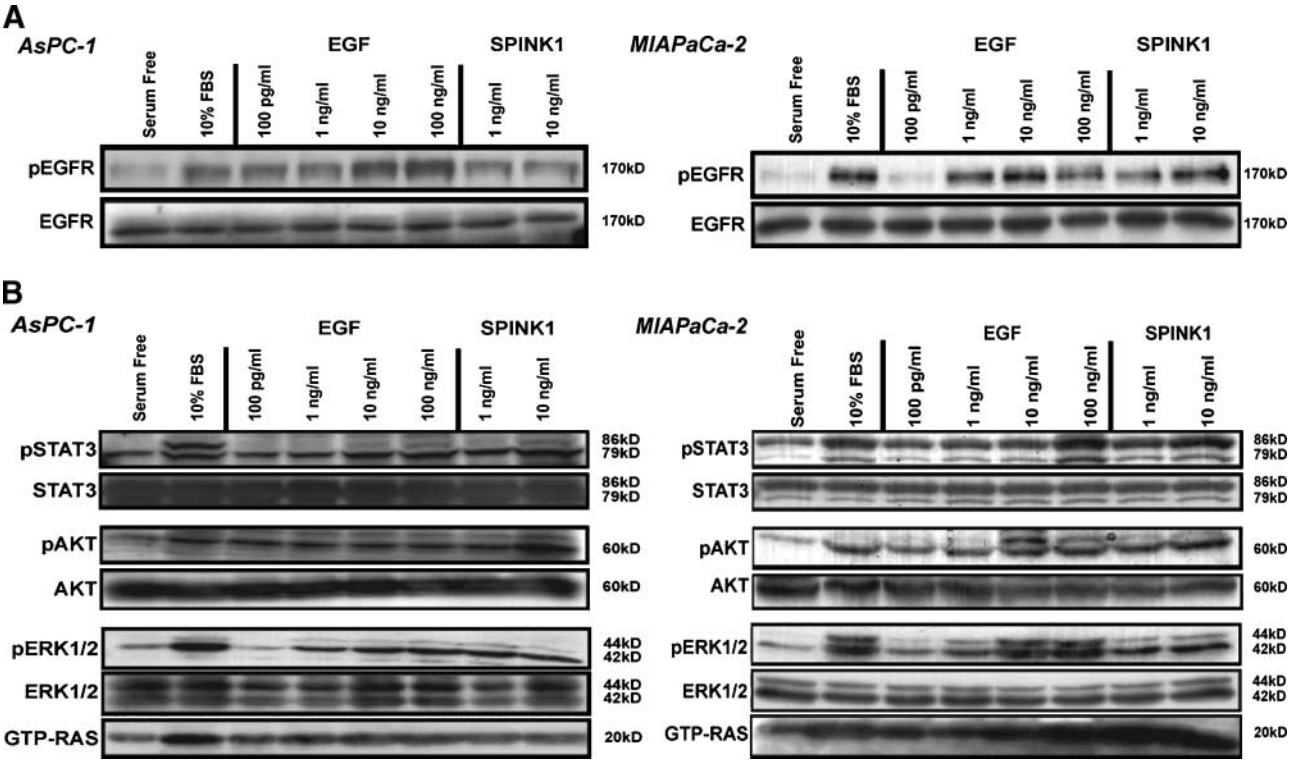

FIGURE 3. Phosphorylation of EGFR and its downstream targets. **A.** Phosphorylation of EGFR. AsPC-1 and MIAPaCa-2 cell lines were incubated for 10 min at 37°C in serum-free medium (control; serum-free) or medium containing EGF (100 pg/mL and 1, 10, and 100 ng/mL) or SPINK1 (1 and 10 ng/mL). EGFR protein (*M*$_r$ 170,000) and phosphorylated EGFR (*pEGFR*) were analyzed by Western blotting. Amounts of phosphorylated EGFR were increased by treatment with EGF and SPINK1. **B.** Phosphorylation of STAT3, AKT, and ERK1/2. Amounts of phosphorylated STAT3 (*pSTAT3*), phosphorylated AKT (*pAKT*), and phosphorylated ERK1/2 (*pERK1/2*) were increased after stimulation with EGF and SPINK1. Activated RAS (GTP-RAS) was also increased by treatment with EGF and SPINK1.

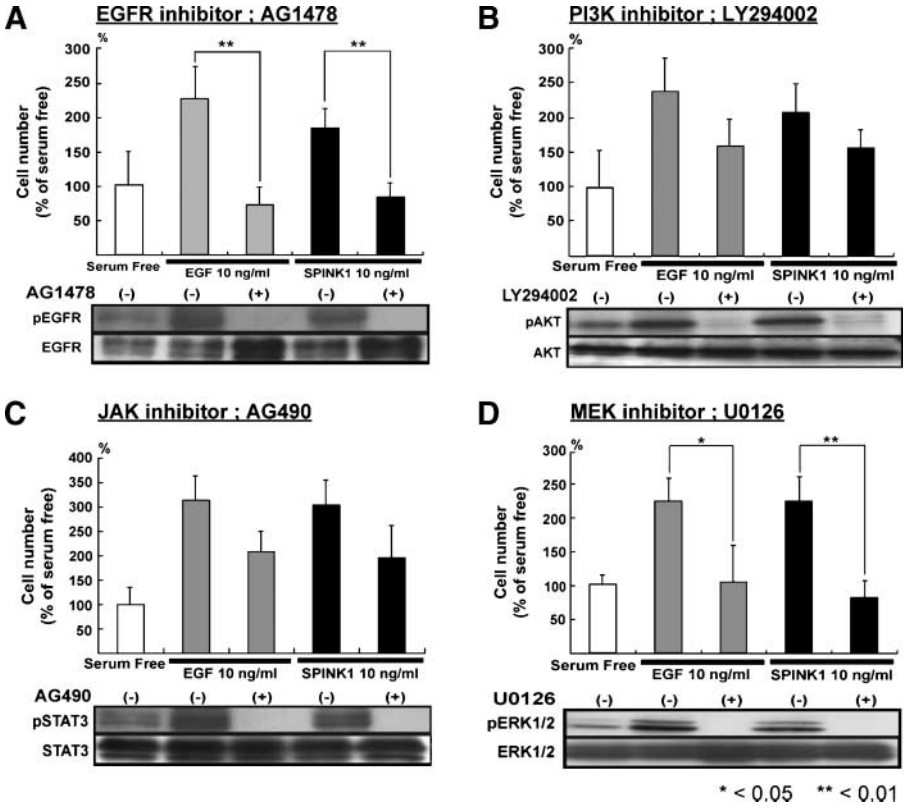

FIGURE 4. Inhibition of cell proliferation by inhibitors. **A.** Inhibition by EGFR inhibitor. Cell proliferation was completely blocked. **B.** Inhibition by PI3K inhibitor. Cell proliferation tended to be decreased. However, the difference was not statistically significant. **C.** Inhibition by JAK inhibitor. Cell proliferation tended to be decreased. However, the difference was not statistically significant. **D.** Inhibition by MEK inhibitor. Cell proliferation was completely blocked. In these experiments, AsPC-1 cell lines were either untreated (control; serum-free) or treated by serum-free medium containing EGF (10 ng/mL) or SPINK1 (10 ng/mL) and then incubated for 10 min at 37°C. Four specific inhibitors, U0126 (10 μmol/L, MEK inhibitor), LY294002 (50 μmol/L, PI3K inhibitor), AG490 (100 μmol/L, JAK Inhibitor), and AG1478 (250 no/EGFR inhibitor), were applied 30 min before EGFR (10 ng/mL) or SPINK1 (10 ng/mL) treatment. Mean ± SE. Statistical analysis was done by ANOVA followed by Student's *t* test. *, $P < 0.05$ for EGF or SPINK1 + inhibitors compared with EGF or SPINK1 alone; **, $P < 0.01$ for EGF or SPINK1 + inhibitors compared with EGF or SPINK1 alone.

mouse, Spink3 has essential roles in the maintenance of integrity and regeneration of acinar cells (5) and may play important roles in proliferation and/or differentiation of various cell types during development (10). Thus, human SPINK1 may also function as a growth factor during embryogenesis or regeneration after pancreatitis and as a maintaining factor for exocrine integrity of the pancreas.

Histochemical and immunohistochemical studies showed that SPINK1 and EGFR were coexpressed not only in pancreatic tubular adenocarcinoma but also in precancerous PanIN. This suggests that SPINK1 and EGFR play important roles in autocrine EGF signaling and that this autocrine signaling is operative at the earliest stage of pancreatic neoplasms as suggested by Bardeesy and DePinho (45). Interestingly, EGFR, but not SPINK1, was expressed in morphologically normal epithelial cells in PanIN-1A. On the other hand, SPINK1 is produced by the acinar cells and secreted into the pancreatic duct. Thus, SPINK1 may stimulate the proliferation of EGFR-positive duct cells from the luminar side. This may be the first step of the transformation. When EGFR-positive duct cells are transformed to PanIN, these cells would become to produce SPINK1 by themselves to develop autocrine stimulation system. This may be the second step of transformation.

Approximately 230,000 patients with pancreatic cancer are registered worldwide every year and the prognosis for pancreatic cancer is extremely poor, with 98% of patients expected to die from the disease (46). Because single-agent gemcitabine became the standard treatment for advanced pancreatic cancer ~10 years ago (47), most strategies to improve the management of this disease have been unsuccessful, and it is only recently that some progress has been made. Among the newer approaches, targeting human EGFR shows promise. Erlotinib (a EGFR tyrosine kinase inhibitor) combined with gemcitabine showed a statistically significant survival benefit over gemcitabine alone (48). In our study, SPINK1 was suggested to play a role as a growth factor through the EGFR pathway in pancreatic cancer cells. Thus, an inhibitor for SPINK1 could be the next choice of treatment for pancreatic cancer. Clearly, extensive further studies are necessary to develop a SPINK1 inhibitor.

Materials and Methods

Cell Culture and Growth Stimulation Study

Cultures of the NIH 3T3 fibroblast, AsPC-1 (pancreatic cancer cell line), MIAPaCa-2 (pancreatic cancer cell line), PANC-1 (pancreatic cancer cell line), Capan-2 (pancreatic cancer cell line), and BT-474 (breast cancer cell line) cell lines were seeded at 4×10^4 cells per 6-well plate in growth medium [DMEM with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin] and grown at 37°C with 5% CO_2. Cell numbers were determined using a hemocytometer (Erma). In the growth stimulation assay, after 24 h incubation, the growth medium was removed, and the cells were washed twice with TBS [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl] and then serum starved by continuing cell culture overnight in FBS-free medium (DMEM without FBS). Recombinant human SPINK1 protein was prepared as described previously (49). In briefly, human SPINK1 gene has been constructed and expressed in an *Escherichia coli* host-vector system. The recombinant cells

were grown in 1 liter of YPGal medium [2% yeast extract, 4% Bacto-peptone, 4% galactose (pH 7.2)]. After filtration with a 0.22 μm membrane, the supernatant was loaded onto a 50 mL SP-Sepharose Fast Flow column (Amersham Pharmacia Biotech) equilibrated with 20 mmol/L acetic acid (pH 3.0). The SPINK1 was eluted with a linear gradient of 50 mmol/L NaCl and precipitated by 75% saturated ammonium sulfate from active fractions. The precipitate was dissolved in a small volume of 50 mmol/L sodium acetate buffer and subjected to reverse-phase high-performance liquid chromatography using an ODS-A column (YMC). Active fractions were pooled and concentrated with a rotary evaporator. Subsequently, the concentrated sample was further purified by a second reverse-phase high-performance liquid chromatography using an ODS-A column. Active fractions were pooled, concentrated, and kept at 80°C until use. Recombinant human EGF was obtained from Peprotech EC. Aprotinin was obtained from Sigma. Cells were either untreated (controls) or treated with FBS-free medium containing EGF (100 pg/mL and 1, 10, and 100 ng/mL), SPINK1 (1 and 10 ng/mL), or aprotinin (1 and 10 ng/mL) and then incubated at 37°C.

Inhibition Assay for Cell Growth

AsPC-1 cells were used in this study. U0126 (MEK inhibitor) and LY294002 (PI3K inhibitor) were from Cell Signaling Technology. AG1478, a specific inhibitor of the tyrosine kinase activity of the EGFR, and AG490, a JAK inhibitor, were purchased from Invitrogen. Four specific inhibitors, AG1478 (250 nmol/L, EGFR inhibitor), AG490 (100 μmol/L, JAK inhibitor); LY294002 (50 μmol/L, PI3K inhibitor), and U0126 (25 μmol/L, MEK inhibitor), were used in these studies. These inhibitors were applied 30 min before EGFR (10 ng/mL) or SPINK1 (10 ng/mL) treatment.

Immunoprecipitation and Western Blotting

AsPC-1 and MIAPaCa-2 cells were either untreated (controls) or treated by removing the serum-free medium and replacing with serum-free medium containing recombinant human SPINK1 protein (10 ng/mL) and then incubated for 10 min at 37°C. Immediately following SPINK1 stimulation, the cells were washed three times with ice-cold TBS and lysed on ice with 1 mL lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 1× protease inhibitor mixture (1:100 dilution; Sigma), and 1× phosphatase inhibitor mixture (1:100 dilution (Nacalai Tesque)] per plate for 10 min, scraped, and then transferred to a tube. The clarified AsPC-1 and MIAPaCa-2 lysates were washed by 30 μL Dynabeads protein G (Invitrogen) by tumbling for 2 h at 4°C. Dynabeads protein G was removed by magnetic capture and the clarified supernatants were transferred to new tubes. For EGFR immunoprecipitation, the washed lysates from above were incubated with 50 μg rabbit anti-EGFR antibody (Cell Signaling Technology) for 2 h at 4°C with tumbling. The rabbit anti-EGFR antibody-protein G immunoprecipitated complex was isolated, washed four times with chilled lysis buffer and then twice with chilled TBS (containing protease and phosphatase inhibitors), and eluted with 1× lithium dodecyl sulfate buffer and mercaptoethanol (2% final concentration) for 10 min at 100°C. Elutes were aliquoted and prepared for Western blotting. Immunoprecipitation products were separated by SDS-PAGE and transferred onto 0.45 μm Invitrolon polyvinylidene difluoride membranes

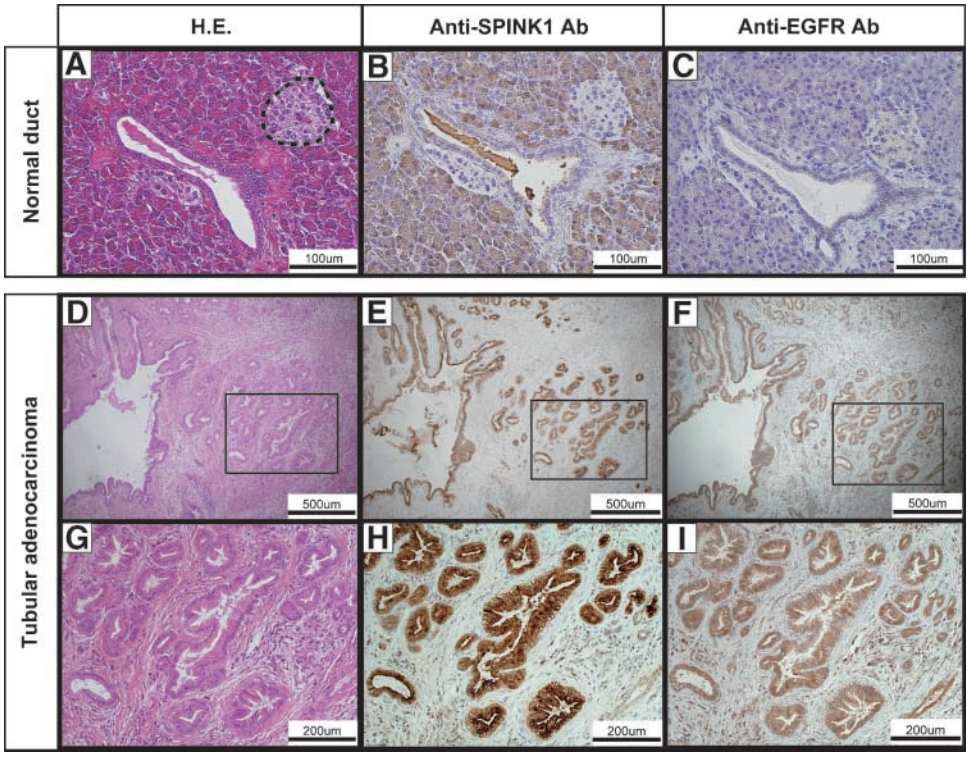

FIGURE 5. Expression of SPINK1 and EGFR in pancreatic tubular adenocarcinoma. **A.** H&E staining of normal pancreas. Area surrounded by a dotted line shows the islet. **B.** Normal pancreas showing expression of SPINK1 in acinar cells but not in pancreatic islet and normal pancreatic duct. **C.** Normal pancreas showing no EGFR expression in any type of cells. **D** and **G.** H&E staining of pancreatic tubular adenocarcinoma. **E** and **H.** Expression of SPINK1 in pancreatic tubular adenocarcinoma. **F** and **I.** Expression of EGFR in pancreatic tubular adenocarcinoma. **G** to **I.** Higher magnification of areas indicated in **D** to **F**.

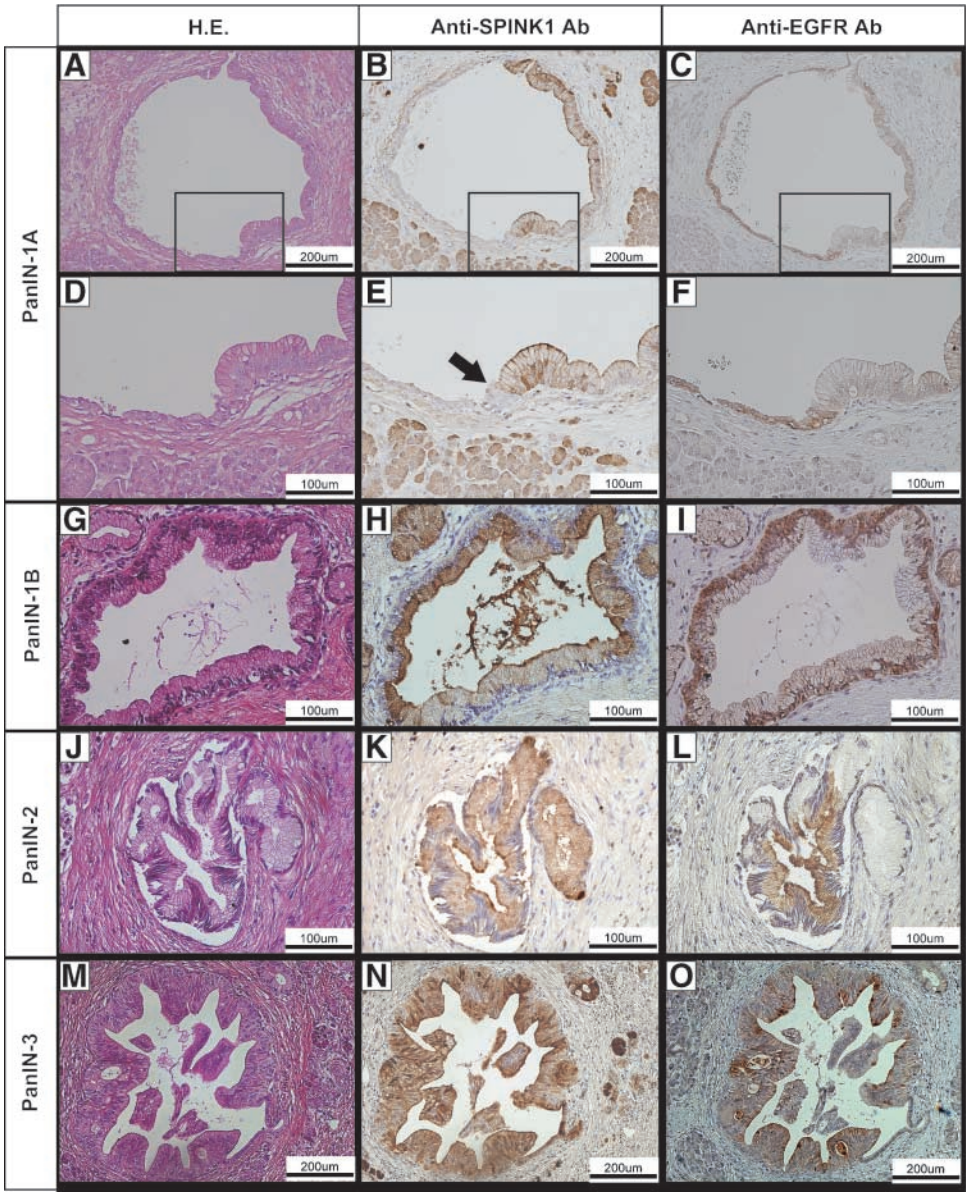

FIGURE 6. Expression of SPINK1 and EGFR in PanIN lesions. **A.** H&E staining of PanIN-1A. **B.** Expression of SPINK1 in PanIN-1A region. Note that SPINK1 was not expressed in normal epithelial cells. Arrow, boundary of normal duct and PanIN-1A. **C.** Expression of EGFR in PanIN-1A and normal epithelial cells. **D** to **F.** Higher magnification of areas indicated in **A** to **C**. **G.** H&E staining of PanIN-1B. **H.** Expression of SPINK1 in PanIN-1B region. **I.** Expression of EGFR in PanIN-1B region. **J.** H&E staining of PanIN-2. **K.** Expression of SPINK1 in PanIN-2 region. **L.** Expression of EGFR in PanIN-2 region. **M.** H&E staining of PanIN-3. **N.** Expression of SPINK1 in PanIN-3 region. **O.** Expression of EGFR in PanIN-3 region.

(Millipore). After 1 h incubation at room temperature in blocking buffer (TBS, 5% nonfat dry milk, 0.1% Tween 20), the membranes were incubated with rabbit anti-human SPINK1 antibody (1:1,000 dilution) diluted in wash buffer (TBS, 0.1% Tween 20) containing 5% nonfat dry milk overnight at 4°C. Membranes were then washed three times (5 min each) and incubated with secondary antibody (anti-rabbit IgG antibody; 1:5,000 dilution; Amersham Biosciences) diluted in wash buffer (TBS, 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature followed by three washes (10 min each). Blots were developed with ECL Plus reagents (GE Healthcare).

Binding Affinity Analysis Using a QCM

A 27-MHz QCM (Initium) was employed to analyze the affinity of recombinant human SPINK1 (0.1 μg/μL: volume 2 μL), recombinant human EGF (Peprotech EC; 0.1 μg/μL: volume 2 μL), and recombinant human NRG1 (ProSpec-Tany TecnoGene; 0.1 μg/μL: volume 2 μL) for various receptors. These ligands were immobilized directly onto the gold electrode surface of the QCM ceramic sensor chip, after which the sensor chip was soaked in a chamber containing 8 mL PBS (pH 7.5) at 25°C until frequency equilibrium was attained. Three members of a EGFR (ErbB1) family have been identified in human. Those are HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). EGF is known to bind to EGFR as a specific ligand, and NRG1 is known to bind to HER3 and HER4 as a specific ligand. No specific ligand was found for HER2. The extracellular domains of EGFR, HER2, HER3, and HER4 and human IgG-Fc fusion proteins were prepared as described previously (50). EGFR, HER2, HER3, and HER4 extracellular domain-IgG-Fc fusion protein (0.1 μg/mL: volume 80 μL) was applied into the equilibrated solution containing the

SPINK1-immobilized sensor chip. The binding of EGFR, HER2, HER3, and HER4 extracellular domain to SPINK1 was determined by monitoring the alterations in frequency resulting from changes in mass on the electrode surface. We used Block Ace and protein G as blocking agents for the gold electrode surface and human IgG-Fc chain. The stability and drift of the 27-MHz QCM frequency in the solution were ±5 Hz for 12 h at 25°C.

Phosphorylation Analysis of Receptor and Signal Transduction Molecules

AsPC-1 and MIAPaCa-2 cells were either untreated (controls) or treated with FBS-free medium containing recombinant human EGF (100 pg/mL and 1, 10, and 100 ng/mL) or recombinant human SPINK1 (1 and 10 ng/mL) and then incubated for 10 min at 37°C. Immediately following EGF or SPINK1 stimulation, the cells were washed three times with ice-cold TBS and lysed on ice with 1 mL lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% SDS, 7 mol/L urea, 2 mol/L thiourea, 1× protease inhibitor mixture (1:100 dilution), and 1× phosphatase inhibitor mixture (1:100 dilution)] per plate for 10 min, scraped, and then transferred to a tube. Pooled lysates were adjusted to 1 mL with additional lysis buffer followed by 10 passages through a 21-gauge needle, incubation on ice for 10 min, and centrifugation at 15,000 rpm for 5 min. The clarified supernatants were transferred to new tubes and normalized by total protein concentration. Protein samples (20 μg protein/lane) were mixed with 2× lithium dodecyl sulfate buffer and mercaptoethanol (2% final concentration) and then heated at 95°C for 5 min. Cell lysates and immunoprecipitation products were separated by SDS-PAGE and transferred onto 0.45 μm Invitrolon polyvinylidene difluoride membranes. After 1 h incubation at room temperature in blocking buffer (TBS, 5% nonfat dry milk, 0.1% Tween 20), the membranes were incubated with primary antibody diluted in wash buffer (TBS, 0.1% Tween 20) containing 5% nonfat dry milk overnight at 4°C. Rabbit anti-human EGFR polyclonal antibody, rabbit anti-phosphorylated human EGFR (Tyr^{1173}) monoclonal antibody (1:1,000 dilution), rabbit anti-human ERK1/2 monoclonal antibody (1:1,000 dilution), rabbit anti-phosphorylated human ERK1/2 monoclonal antibody (1:500 dilution), rabbit anti-human STAT3 polyclonal antibody (1:1,000 dilution), rabbit anti-phosphorylated human STAT3 (Tyr^{705}) polyclonal antibody (1:500 dilution), rabbit anti-human AKT polyclonal antibody (1:1,000 dilution), and rabbit anti-phosphorylated human AKT (Tyr^{308}) monoclonal antibody (1:500 dilution) were from Cell Signaling Technology. Membranes were then washed three times (5 min each) and incubated with secondary antibody (anti-rabbit IgG antibody, 1:5,000 dilution) diluted in wash buffer (TBS, 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature followed by three washes (10 min each). Blots were developed with ECL Plus reagents (GE Healthcare).

Statistical Analysis

All data are expressed as mean ± SE, and significant differences were determined by ANOVA followed by Student's *t* test. The differences were considered to be statistically significant at $P < 0.05$.

Immunohistochemistry

A total of 22 patients were made available for immunohistochemistry analysis with fully informed consent. Each sample was excised and fixed in 10% buffered formalin for histologic examination. For histologic analysis, tissue was sectioned and stained with H&E procedure. Affinity-purified rabbit anti-human SPINK1 antibody was prepared as described previously (49). Rabbit anti-human EGFR polyclonal antibody was from Cell Signaling Technology. Immunohistochemistry was done using the following primary antibodies: rabbit anti-human SPINK1 antibody (diluted 1:200) and rabbit anti-human EGFR antibody (diluted 1:100). Primary antibodies were detected with a commercial biotin-streptavidin system (Vector Laboratories).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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