Oncogenes and Tumor Suppressors

**SPINK1 Status in Colorectal Cancer, Impact on Proliferation, and Role in Colitis-Associated Cancer**

Satoshi Ida, Nobuyuki Ozaki, Kimi Araki, Kotaro Hirashima, Yoko Zaitsu, Katsunobu Taki, Yasuo Sakamoto, Yuiji Miyamoto, Eiji Oki, Masaru Morita, Masayuki Watanabe, Yoshihiko Maehara, Ken-ichi Yamamura, Hideo Baba, and Masaki Ohmuraya

Abstract

Colorectal cancer is a major cause of deaths due to cancer; therefore, research into its etiology is urgently needed. Although it is clear that chronic inflammation is a risk factor for colorectal cancer, the details remain uncertain. Serine protease inhibitor, Kazal type 1 (SPINK1) is mainly produced in pancreatic acinar cells. However, SPINK1 is expressed in various cancers and in inflammatory states, such as colon cancer and inflammatory bowel disease. There are structural similarities between SPINK1 and epidermal growth factor (EGF). Hence, it was hypothesized that SPINK1 functions as a growth factor for tissue repair in inflammatory states, and if prolonged, acts as a promoter for cell proliferation in cancerous tissues. Here, immunohistochemical staining for SPINK1 was observed in a high percentage of colorectal cancer patient specimens and SPINK1 induced proliferation of human colon cancer cell lines. To clarify its role in colon cancer in vivo, a mouse model exposed to the colon carcinogen azoxymethane and nongenotoxic carcinogen dextran sulfate revealed that Spink3 (mouse homolog of SPINK1) is overexpressed in cancerous tissues. In Spink3 heterozygous mice, tumor multiplicity and tumor volume were significantly decreased compared with wild-type mice. These results suggest that SPINK1/Spink3 stimulates the proliferation of colon cancer cells and is involved in colorectal cancer progression.

Implications: Evidence suggests that SPINK1 is an important growth factor that connects chronic inflammation and cancer.

**Introduction**

Colorectal cancer is a major cause of death in Japan. According to 2012 statistics, colorectal cancer accounted for the largest number of deaths from malignant neoplasms in women and the third largest number in men. Research into the etiology and development of new treatment methods are needed to improve patient outcome. Colorectal cancer is one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease. The relative risk of colorectal cancer in patient with colitis is two to eight times that of the general population (1). Although it is clear that chronic inflammation is a risk factor of colorectal cancer, the pathogenesis of colitis-associated cancer (CAC) is still uncertain. Serine protease inhibitor, Kazal type 1 (SPINK1; mouse homolog: Spink3; ref. 2), which is also known as pancreatic secretory trypsin inhibitor and tumor-associated trypsin inhibitor (3), is mainly produced in the acinar cells of the exocrine pancreas. The role of SPINK1 is postulated to be the prevention of inadvertent proteolysis in the pancreas caused by intra-acinar premature activation of trypsinogen (4-6). However, it is known that SPINK1 is widely expressed in extrapancreatic tissues, especially in the gastrointestinal and urinary tracts (7, 8), suggesting that SPINK1 has additional functions in many tissues other than the pancreas. In the normal gastrointestinal tract, SPINK1 is believed to play a protective role in both gastric (9) and colonic (10) mucosa. However, when the gastrointestinal tract is inflamed, SPINK1 is involved in the regenerative process at the gastric ulcer edge and is strongly expressed within the ulcer-associated cell lineage in patients with IBD (11, 12). In addition, we also observed Spink3 in the large intestine at 11.5 days postconception, an earlier stage of development, thus Spink3 influences the proliferation of colonic epithelial cells (8). Furthermore, Ogawa and colleagues (13, 14) found elevated serum SPINK1 levels in patients with severe systemic inflammation.

SPINK1 is also produced in cancers of the colon (15, 16), lung (17, 18), liver (19), breast (20), prostate (21), and pancreas (20, 22). In colon cancer, Gouyer and colleagues (23) identified and characterized SPINK1 as a major proinvasive secreted factor from the conditioned medium of HT-29 SM21 human colon cancer cell line. However, when the gastrointestinal tract is in inflammation and cancer, the role of SPINK1 is still uncertain. Serine protease inhibitor, Kazal type 1 (SPINK1; mouse homolog: Spink3; ref. 2), which is also known as pancreatic secretory trypsin inhibitor and tumor-associated trypsin inhibitor (3), is mainly produced in the acinar cells of the exocrine pancreas. The role of SPINK1 is postulated to be the prevention of inadvertent proteolysis in the pancreas caused by intra-acinar premature activation of trypsinogen (4-6). However, it is known that SPINK1 is widely expressed in extrapancreatic tissues, especially in the gastrointestinal and urinary tracts (7, 8), suggesting that SPINK1 has additional functions in many tissues other than the pancreas. In the normal gastrointestinal tract, SPINK1 is believed to play a protective role in both gastric (9) and colonic (10) mucosa. However, when the gastrointestinal tract is inflamed, SPINK1 is involved in the regenerative process at the gastric ulcer edge and is strongly expressed within the ulcer-associated cell lineage in patients with IBD (11, 12). In addition, we also observed Spink3 in the large intestine at 11.5 days postconception, an earlier stage of development, thus Spink3 influences the proliferation of colonic epithelial cells (8). Furthermore, Ogawa and colleagues (13, 14) found elevated serum SPINK1 levels in patients with severe systemic inflammation.

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cancer cells, which express a spontaneous invasive phenotype. However, the role of SPINK1 in malignant tumors remains to be elucidated, especially in tumor progression.

Here, we hypothesized that SPINK1/Spink3 is an important factor that connects chronic inflammation and cancer. In other words, we predicted that SPINK1/Spink3 would play a role as a growth factor in tissue repair in inflammatory states, and if prolonged, function as a promoter of cell proliferation in cancerous tissues.

We showed the function of SPINK1 as a growth factor for colorectal cancer: (i) expression of SPINK1 in human colon cancer; (ii) the proliferative activity of SPINK1 using human colon cancer cell lines; and (iii) the role of SPINK1 in the proliferation of CAC in vivo.

Materials and Methods

Immunohistochemistry

A total of 226 patients were made available for immunohistochemical analysis with fully informed consent. All resections for colorectal cancer were performed between 1994 and 2007 at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University Hospital, Fukuoka, Japan. Immunohistochemical staining was performed according to a previously described protocol (22). Each sample was excised and fixed in 10% buffered formalin for histologic examination. Immunohistochemistry was performed using rabbit anti-human SPINK1 primary antibody, which was detected with a commercial biotin–streptavidin system (Vector Laboratories).

SPINK1 expression was scored by two independent investigators (S. Ida and K. Hirashima). Immunoreactivity scoring of SPINK1 was performed using proportion scoring (24) and intensity scoring (25). First, a proportion score was assigned, which represented the estimated proportion of positively stained tumor cells (score: 0, none; 1, <10%; 2, 10%–50%; 3, >50%). Next, an intensity score was assigned a value of score 0, none; 1, weak; 2, moderate; 3, strong. The proportion and intensity scores were then multiplied to obtain a total score, with a range of 0 to 9. For further analysis, the patients were divided into two groups: negative with scores 0 to 1 and positive with scores 2 to 9. Concordance between the two investigators was assessed using k coefficients.

Cell culture and growth stimulation study

Colo205, HCT116, HT29, and SW620 human colon cancer cell lines were obtained from the Riken Bioresource Center Cell Bank, Japanese Collection of Research Bioresources Cell Bank, American Type Culture Collection, and DS Pharma Biomedical, respective-ly. They were seeded at 5 × 10^4 cells in 6-well plates. HCT116 and SW620 cells were grown in DMEM, Colo205 cells were grown in RPMI 1640, and HT29 cells were grown in McCoy’s 5A (Modified) Medium; all were supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, and incubated at 37°C in 5% CO2. Cell numbers were determined using a hemocytometer (Erma). In the growth stimulation assay, growth medium was removed after 24-hour incubation, and the cells were washed twice with PBS and then serum starved by culturing overnight in FBS-free medium.

RNA interference

Two different siRNA duplexes targeting SPINK1 were purchased from Invitrogen (Carlsbad). The sequences were as follows: siRNA #1: sense, UUJACCAGAUJAGACUACAGGGCCA, antisense, UGGCCCUUGUJGAGJGCHiAiJGUGUAA, and siRNA #2: sense, AIAJUGGJUGJUGJUGJUGJUGCJUJC, antisense, GACJHJ- AAIUGJUGCJACCAJUJAAJ. A total of 50 nmol/l siRNA was transfected using Lipofectamine 2000 (Invitrogen). The cells were incubated at 37°C for 48 hours and subsequently harvested for the following studies. Negative control siRNA was also purchased from Invitrogen.

SPINK1 minigene construction and transfection

The human SPINK1 gene is approximately 7.5 kb long and consists of four exons (26). The SPINK1 minigene contained all four exons from the human gene; for ease of handling, the first intron was shortened from 1.9 to 0.5 kb, the second from 1.5 to 0.6 kb, and the third from 3.3 to 0.5 kb by deleting sequences from the center of the introns. We generated SPINK1-overexpressing transient cells in which a minigene composed of the CAG promoter was coupled to the human SPINK1 gene. A total of 50 nmol/L DNA was transfected using Lipofectamine 2000 (Invitrogen). The cells were incubated at 37°C for 48 hours and were subsequently harvested for the following studies. Empty vector was inserted in cells as a negative control.

Reverse-transcription PCR

Total RNA was obtained from each cell line using an RNeasy Mini kit (Qiagen). cDNA was synthesized using a Thermoscript one-step RT-PCR system (Invitrogen). RT-PCR analysis was performed using the primers 5′-GAAGGTAAACGGCATTTCTTC-TTCTC-3′ and 5′-TTGAAATGGATACGAATCTCGGCTT-3′ for GAPDH (221 bp) and 5′-GAGTCAACGGATTTGGTCGT-3′ and 5′-GCTGTATCATCTTGAGGCTG-3′ for GAPDH (426 bp). The temperature protocol for PCR amplifications was as follows: initial denaturation at 95°C for 15 minutes, followed by 30 amplification cycles of 30 seconds at 95°C, then 30 seconds at 63°C and 30 seconds at 72°C, followed by final extension at 72°C for 7 minutes. Amplified PCR products were separated by 2.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Saline was used as a negative control in each analysis.

Animals and chemicals

Male C57BL/6N mice (2 months old and weighing 20–25 g) were obtained from Charles River Japan. Mice were housed in a climate-controlled room on a 12-hour light–dark cycle. Azoxymethane (AOM), a colonic carcinogen, was purchased from Sigma-Aldrich (St Louis). Dextran sodium sulfate (DSS), a nongenotoxic carcinogen with a molecular weight of 36,000 to 50,000, which is widely used to produce colitis in rodents, was purchased from ICN Biochemicals. All procedures were approved by the Animal Care and Use Committee of Kumamoto University.

Experimental procedure

Colitis and colonic neoplasia were induced using previously established methods (27, 28). Colitis was induced by 2% DSS in drinking water for 7 days. Mice were sacrificed on days 0, 1, 3, 5, 7, 10, 14, 21, and 60.

Colonic neoplasia was induced by a single intraperitoneal injection of AOM (10 mg/kg) at the beginning of the experiment and followed by administration of 2% DSS in drinking water for 7 days starting at 1 week after AOM injection. Mice were killed at 12 weeks, and colon tissues were rapidly extracted and prepared for the following studies. The number of tumors was counted macroscopically. Measurements of the longest perpendicular tumor...
diameters were made using a digital caliper, and tumor volumes were estimated using the following formula: \( V = \frac{L \times W \times D}{6} \), where \( V \) is the tumor volume, \( L \) the length, \( W \) the width, and \( D \) the depth (29).

Evaluation of clinical colitis

Disease activity index was scored according to changes in weight, hemoccult positivity, or gross bleeding, and stool consistency on days 0, 1, 3, 5, 7, 10, 14, 21, and 28 as described previously (30).

Histologic analysis

The large intestine was divided into three equal segments (proximal, middle, and distal). The distal colon was cut open longitudinally along the main axis and washed with PBS. It was fixed by 10% buffered formalin for 24 hours, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Colitis was evaluated on H&E-stained sections, according to the morphologic criteria described by Cooper and colleagues (30).

Western blot analysis

Conventional Western blot analysis was performed as previously described (22). Rabbit anti-mouse Spink3 antibody (Cell Signaling Technology) and rabbit anti-mouse actin antibody (Sigma-Aldrich) were used at 1:1,000 and 1:2,000, respectively. Anti-rabbit IgG antibody conjugated with horseradish peroxidase (Amersham Biosciences) was used. The band intensities were quantified by densitometry using ImageJ software (http://imagej.nih.gov/ij/), and normalized by the actin signals (n = 3).

X-gal staining for β-galactosidase activity

We previously generated a Spink3lacZ knock-in mouse using a Cre recombination strategy (8). To determine Spink3 promoter activity, we detected the activity of β-galactosidase produced by the knock-in lacZ reporter gene in colon tissues. The protocol of X-gal staining has been described previously (8). For each experiment, rigorous controls were carried out simultaneously in Spink3 wild-type mice to assay background endogenous β-galactosidase activity.

Table 1. Characteristics of patients with colorectal cancer and clinicopathological factors related to recurrence after surgical resection

<table>
<thead>
<tr>
<th></th>
<th>SPINK1 (−)</th>
<th>SPINK1 (+)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>66.2 ± 1.3</td>
<td>65.3 ± 1.2</td>
<td>0.63</td>
</tr>
<tr>
<td>Sex (M; F)</td>
<td>52; 50</td>
<td>83; 41</td>
<td>*&lt;0.05</td>
</tr>
<tr>
<td>Tumor location (colon; rectum)</td>
<td>58; 44</td>
<td>61; 63</td>
<td>0.22</td>
</tr>
<tr>
<td>Tumor (T) stage</td>
<td>0 0 (0)</td>
<td>4 (3.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 4 (3.9)</td>
<td>7 (5.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 12 (11.8)</td>
<td>21 (16.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 57 (55.9)</td>
<td>78 (62.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 29 (28.4)</td>
<td>14 (11.3)</td>
<td></td>
</tr>
<tr>
<td>Lymph node (N) stage</td>
<td>0 0 (0)</td>
<td>45 (44.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>1 36 (35.3)</td>
<td>37 (27.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 17 (16.7)</td>
<td>29 (23.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 4 (3.9)</td>
<td>1 (0.8)</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td>0 0 (0)</td>
<td>2 (1.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 9 (8.8)</td>
<td>18 (14.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 28 (27.5)</td>
<td>41 (33.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3a 32 (31.4)</td>
<td>24 (19.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3b 10 (9.8)</td>
<td>28 (22.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 23 (25.6)</td>
<td>11 (8.9)</td>
<td></td>
</tr>
<tr>
<td>Histologic type</td>
<td>Well, Mod</td>
<td>80 (78.4)</td>
<td>118 (95.2)</td>
</tr>
<tr>
<td></td>
<td>Por, Sig. Muc</td>
<td>22 (21.6)</td>
<td>6 (4.8)</td>
</tr>
</tbody>
</table>

Abbreviations: Mod, moderately differentiated adenocarcinoma; Muc, mucinous adenocarcinoma; Por, poorly differentiated adenocarcinoma; Sig, signet ring cell carcinoma; TNM, tumor–node–metastasis; Well, well-differentiated adenocarcinoma.
Statistical analysis

All data are expressed as mean ± SE. The survival curves were plotted according to the Kaplan–Meier method. Statistical analyses were performed using JMP version 10.0 statistical software package (SAS Institute). A two-sided significance level of \( P < 0.05 \) was used for all the statistical analyses.

Results

SPINK1 expression in human colon cancer tissues

We examined expression of SPINK1 in human colorectal cancer. SPINK1 expression in normal human colon tissues was observed at the basal side of normal crypts (Fig. 1A). In colorectal cancer tissues, SPINK1 was expressed in the cell membrane and cytoplasm of the mucosa (Fig. 1C–E, no staining in B). In immunohistochemical staining of colorectal cancer tissues, positive expression (scores 2–9) of SPINK1 in the primary tumor was observed in 124 (51.1%) of the 226 samples. SPINK1 immunoreactivity was negative in 102 (45.1%), with scores 0 and 1 in 75 (33.2%) and 27 (11.9%), respectively. The concordance between the two observers was 0.86 (\( k = 0.71 \)), indicating substantial agreement. SPINK1 was positively associated significantly with histologic type. The value of SPINK1 immunoreactivity in well/moderately differentiated adenocarcinoma and undifferentiated tumors was 2.8 ± 0.2 and 1.3 ± 0.5, respectively (\( P < 0.01 \)). Significantly higher expression of SPINK1 was observed in advanced tumors (\( P < 0.01 \); Table 1). We analyzed the relationship between overall survival and expression of SPINK1. SPINK1 was not significantly associated with overall survival (log rank, \( P = 0.34 \); Supplementary Fig. S1).

SPINK1 plays a role in proliferation of colon cancer cell lines

To analyze the growth stimulation in colorectal cancer of SPINK1, we used four typical human colon cancer cell lines: Colo205, HCT116, HT29, and SW620. Under normal conditions, SPINK1 was clearly detected by RT-PCR in Colo205 and HT29 cells. However, SPINK1 expression was weak in HCT116 and SW620 cell lines (Fig. 2A). We examined the growth-stimulating activity of SPINK1 using Colo205 cells. To assay the effects of silencing of SPINK1, Colo205 and HT29 cells were transiently transfected with two different types of siRNAs (SPINK1 siRNA #1 and #2) that target SPINK1 mRNA for degradation. siRNA#1 and siRNA#2 both inhibited SPINK1 mRNA in the respective cell lines. The number of Colo205 cells transfected with SPINK1 siRNA#1 and #2 was significantly reduced as compared with mock-transfected cells (\( P < 0.05 \); Fig. 2B). Otherwise, HT29 cells were also transfected with SPINK1 siRNA #1 and #2 tended to reduce as compared with mock-transfected cells. To assay the effects of overexpression of SPINK1 on cell proliferation, Colo205 cells were transiently transfected either with empty vector or SPINK1 expression (SPINK1 was driven by CAG promoter) vector. Compared with empty vector, the number of transfected cells with SPINK1 expression vector was significantly increased (\( P < 0.05 \); Fig. 2C). Furthermore, we transfected SPINK1 expression vector into HT116 cells, which showed weak expression of SPINK1. The number of transfected cells with SPINK1 expression vector was also significantly increased (\( P < 0.01 \); Fig. 2D). These results suggest that SPINK1 functions in the proliferation of human colon cancer cells.
Spink3 expression is overexpressed at the regenerative stage in colitis

We examined whether the expression of Spink3 differed according to each segment of the colon in normal conditions. In the Western blotting analysis, Spink3 expression in the proximal colon tissue was weak, and was strongest in the distal colon (Fig. 3A). The tumor tends to arise in the distal colon in the AOM-DSS colon cancer model; therefore, we used distal colon tissue for subsequent studies.

To analyze the reaction with DSS, we examined the severity of the colitis \( (n = 3) \). The disease activity index was highest at day 7 \( (8.7 \pm 0.3) \), and this score gradually decreased during the study (Fig. 3B). Histologically, infiltration of inflammatory cells and shortening and loss of the basal one third of the actual crypts were observed from day 3 (Fig. 3D), and entire crypts were destroyed by day 7 (Fig. 3E), compared with the normal colon tissue (Fig. 3C). Formation of new surface epithelium and crypts was observed after day 10 (Fig. 3F-H), especially on days 10 and 14. Thus, we defined days 1 to 7 as the acute colitis stage, and after day 8 as the regenerative stage. The level of Spink3 expression reached its peak at days 10 to 14 and declined by day 60 (Fig. 3I). These results suggest that Spink3 is overexpressed at the regenerative stage in colitis induced by DSS, and therefore might play a role in the cell proliferation of the colonic epithelium during the healing process.

Spink3 expression is upregulated in colon cancer

Using the AOM-DSS model, flat, nodular, or polypoid colonic tumors were identified macroscopically in the distal colon of mice that received AOM and DSS \( (n = 6) \); Fig. 4A). Histopathologically, they were tubular adenocarcinoma (Fig. 4B). None of the mice...
given AOM alone \((n = 6)\), DSS alone \((n = 6)\), or tap water \((n = 6)\) had any colonic tumors (data not shown). Compared with the untreated colon (control), Spink3 was increased in the tumor tissues and surrounding non-neoplastic tissues (Fig. 4C). Spink3 expression was increased significantly in tumors compared with the untreated tissues and surrounding non-neoplastic tissues \((P < 0.05; \text{Fig. } 4D)\).

Localization of Spink3 in colon cancer epithelium

We examine the localization of Spink3 in colon tissues. β-Galactosidase activity was little observed by X-gal staining in the control (untreated colon of Spink3lacZ/+ mice; \(n = 3\); Fig. 5A). In other words, endogenous β-galactosidase activity in the colon could be excluded. In AOM+DSS–treated mice \((n = 3)\), Spink3lacZ/+ was expressed highly in the colon cancer epithelium (Fig. 5B and AOM alone \((n = 6)\), DSS alone \((n = 6)\), or tap water \((n = 6)\) had any colonic tumors (data not shown). Compared with the untreated colon (control), Spink3 was increased in the tumor tissues and surrounding non-neoplastic tissues (Fig. 4C). Spink3 expression was increased significantly in tumors compared with the untreated tissues and surrounding non-neoplastic tissues \((P < 0.05; \text{Fig. } 4D)\).

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Although expression of Spink3 also increased in the surrounding non-neoplastic tissue, the expression did not occur in the entire crypt of the colon but was confined to particular areas (Fig. 5B and D).

**Spink3 promotes development of colorectal cancer**

To examine the role of Spink3 *in vivo*, we addressed whether the absence of Spink3 altered the susceptibility to develop CAC. Previously, we generated Spink3 knockout mice; however, these mice did not survive beyond 15 days postpartum because of acute pancreatitis (5). Hence, Spink3 heterozygote (Spink3<sup>+/−</sup>) and wild-type (Spink3<sup>+/+</sup>) mice were treated with AOM and DSS. The level of Spink3 in Spink3<sup>+/−</sup> mice was decreased by up to 10% compared with that of wild-type mice (Fig. 6A). In wild-type mice, tumor incidence, multiplicity, and tumor volume were significantly decreased (P < 0.05) in Spink3<sup>+/−</sup> mice compared with wild-type mice. Only four of seven (57%) Spink3<sup>+/−</sup> mice had tumors, and the multiplicity and volume of tumors were 1 ± 0.4 per mouse and 16.8 ± 1.9 mm<sup>3</sup>, respectively (Fig. 6B and C). These results suggest that Spink3 plays a role in the progression of CAC.

**Discussion**

In this study, we showed that SPINK1/Spink3 is overexpressed in colon cancer and mediates the proliferation of colon cancer cells. This is believed to be the first study to show that SPINK1/Spink3 acts as a cell proliferative factor in colon cancer using an *in vivo* model.

Previously, we showed that SPINK1/Spink3 is overexpressed in acute (31, 32) and chronic pancreatitis (33). An interesting question is the cause of the increased SPINK1 expression in the inflammatory state. The SPINK1 gene contains an IL6-responsive element in hepatoma cells, and expression of SPINK1 is induced by IL6 and IL1, which are inducers of acute-phase reactions (34). SPINK1 protein is secreted from the liver in the systemic circulation as one of the acute-phase proteins to inhibit trypsin activity in tissues. In addition, it has already been reported that IL6 is induced in DSS colitis mice (35). In the current study, we showed that Spink3 was overexpressed at the regenerative stage in DSS colitis and that it may have contributed to the proliferation and repair of the colonic cells.

Several investigators have revealed that SPINK1 and trypsin, which actively contribute to tumor invasion and metastasis, are coexpressed in colorectal adenocarcinoma. This supports a protective role of SPINK1 against tumor invasion. However, SPINK1 has been noted to play a role as a protease inhibitor and a growth factor. There are some structural similarities between SPINK1 and the potent growth factor EGF; both have similar numbers of amino acid residues (56 and 53, respectively) and molecular weights (~6 kD), and both have three intrachain disulfide bridges (7, 36). There is a 50% gene sequence homology between SPINK1 and EGF (37–39). Recently, we showed that SPINK1 binds to EGF receptor (ERGFR) to activate its downstream signaling, resulting in the proliferation of pancreatic and breast cancer cells (22). In the current study, we showed that tumor number and volume were significantly decreased in Spink3<sup>+/−</sup> mice compared with wild-type mice. These findings revealed that SPINK1/Spink3 has potential as growth factors for colorectal cancer.

In the gastrointestinal tract, Spink3 is most strongly expressed in the sigmoid colon and rectum at the embryonic stage (8). In addition, we previously revealed that the epithelial of the duodenum and small intestine in Spink3<sup>−/−</sup> mice were degenerated (5). Furthermore, SPINK1 expression in the human colon tissues was observed at the basal side of the normal crypt, an important site for proliferation of the epithelium. Therefore, SPINK1/Spink3 has a crucial role in the proliferation and integrity of the gastrointestinal tract. Spink3 expression was increased in colon cancer tissues and surrounding non-neoplastic tissues in Spink3<sup>+/−</sup> mice and in Spink3<sup>−/−</sup> and Spink3<sup>+/−</sup> mice (ref. 40, data not shown). Furthermore, its expression showed punctate distribution in the crypt. Although the mechanisms by which SPINK1 is associated...
with tumor progression in colorectal cancer are not clear, SPINK1/Spink3 may work as an autocrine- and/or paracrine-transforming factor, potentially involved in cancer progression. The identity of the receptor for SPINK1 is controversial. SPINK1 can now be classified as an EGFR ligand, but no specific SPINK1 receptor has been identified. Niinobu and colleagues (41) reported that the binding of human 125I-labeled SPINK1 to the same cells could be displaced by cold SPINK1 but not EGF, suggesting a separate receptor. Recently, the monoclonal EGFR antibody cetuximab was introduced to treat colon cancer successfully in wild-type KRAS patients. In prostate cancer, cetuximab is effective for SPINK1-positive and ETS-negative populations (42). In the current study, the rate of positive immunohistochemical staining for SPINK1 was 54.9%. It was almost equivalent to the result of SPINK1-positive rate, 65% to 75%, reported in other studies (43, 44). These results provide a rationale for the development of a human monoclonal antibody against SPINK1 and its use in combination with other tools for EGFR inhibition for the treatment of selected colon cancer patients.

Increased expression of SPINK1 in tumor tissue has been reported in patients with poor survival (21, 25, 45–48) and favorable prognosis (9, 24, 43). As shown above, SPINK1/Spink3 acts as a growth factor in colorectal cancer. However, it was shown that high tissue expression of SPINK1 correlates with better prognosis in colorectal cancer. This discrepancy could be explained by the fact that SPINK1/Spink3 works as promoting factors in the early period of tumor formation and may have an inhibitory influence on tumor in the late period. Koskensalo and colleagues (24) revealed that concomitant expression of EGFR and SPINK1 is an even better marker for improved survival. They postulated that a favorable prognosis in colorectal cancer may result from binding of SPINK1, leading to inhibition of the EGFR signaling cascade. SPINK1 binding to EGFR may inhibit the binding of stronger activating ligands. The relationship between SPINK1 function and prognosis has many obscure points.

In conclusion, SPINK1/Spink3 promotes the proliferation of colon cancer cells. Further studies are necessary to identify the specific colon receptor for SPINK1 and to analyze its signal transduction.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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