Hepatocyte Growth Factor Activator Inhibitor Type 1 Is a Suppressor of Intestinal Tumorigenesis

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Abstract

Hepatocyte growth factor activator inhibitor type 1 (HAI-1/SPINT1) is a membrane-bound serine protease inhibitor expressed on the surface of epithelial cells. Although HAI-1/SPINT1 is abundantly expressed in the intestinal epithelium, its role in intestinal tumorigenesis is not known. In this study, we investigated the role of HAI-1/Spint1 in intestinal tumorigenesis using mouse models. The membranous Hai-1/Spint1 immunoreactivity was decreased in murine ApcMin/+ tumors and also in carcinogen (azoxymethane treatment followed by dextran sodium sulfate administration)-induced colon tumors compared with the adjacent non-neoplastic epithelium. The decreased immunoreactivity appeared to be due to sheddase activity of membrane-type 1 matrix metalloprotease. Then, we examined the effect of intestine-specific deletion of Spint1 gene on ApcMin/+ mice. The loss of Hai-1/Spint1 significantly accelerated tumor formation in ApcMin/+ mice and shortened their survival periods. Activation of HGF was enhanced in Hai-1/Spint1-deficient ApcMin/+ intestine. Gene expression profiling revealed upregulation of the Wnt/β-catenin signaling circuit, claudin-2 expression, and angiogenesis not only in tumor tissue but also in the background mucosa without macroscopic tumors in Hai-1/Spint1-deficient ApcMin/+ intestine. Intestinal deletion of Spint1 also enhanced the susceptibility to carcinogen-induced colon tumorigenicity of wild-type Apc mice. Our findings suggest that HAI-1/SPINT1 has a crucial role in suppressing intestinal tumorigenesis, which implies a novel link between epithelial cell surface serine protease inhibitors and protection from carcinogenic stimuli.

Introduction

The intestinal mucosal surface area represents the most significant anatomic and physiologic portal for systemic exposure to environmental toxins and microorganisms (1). The systemic compartment is protected from these exposures by the mucosal epithelial barrier, with barrier dysfunction contributing to local and systemic diseases including inflammatory bowel disease, allergic disorders and malignancies (1, 2). Regarding the epithelial barrier, the role for the junctional complexes that restrict paracellular macromolecular exchange between mucosal and systemic compartments has been studied extensively and the molecular components of tight junction proteins described (1, 2). However, how the cell surface proteolysis systems participate in maintaining epithelial barrier function remains incompletely defined. Recent evidence indicates that normal functioning of the cell surface serine protease matriptase is critically required for epithelial integrity (3–6). Matriptase, encoded by the ST14 gene, belongs to the type II transmembrane serine protease superfamily and is expressed on the epithelial cell surface of most epithelial tissues (6). Moreover, recent studies indicate that its cognate cell surface inhibitor, hepatocyte growth factor activator inhibitor (HAI), also has crucial roles in epithelial functions (7–10).

HAI belongs to the type I family of transmembrane serine protease inhibitors that consists of 2 members: HAI type 1 (HAI-1)/serine protease inhibitor Kunitz type 1 (SPINT1) and HAI-2/SPINT2, which both have 2 extracellular Kunitz domains and a transmembrane domain and are encoded by the SPINT1 and SPINT2 genes, respectively (11). Unlike HAI-2/SPINT2, HAI-1/SPINT1 also has an N-terminal cysteine-rich domain and a low-density lipoprotein receptor type A-like domain (11). HAI-1/SPINT1 is expressed in most epithelial cells, with significant expression in enterocytes (8, 12). HAI-1/SPINT1 was initially discovered as an endogenous inhibitor of HGF activator, a serum-derived serine protease (13), but its membrane association may also allow it to regulate cell surface...
serine proteases. Indeed, ample evidence indicates that HAI-1/SPINT1 is a cognate inhibitor of matriptase on epithelial cell surfaces (10, 14–17). Other TTSPs, such as hepsin, human airway trypsin-like protease, TMPRSS4 and TMPRSS13, as well as the glycosylphosphatidylinositol-anchored cell surface serine protease prostatin, may also be regulated by HAI-1/SPINT1 (6, 18–20). Therefore, HAI-1/SPINT1 must have a critical regulatory role in pericellular proteolysis of epithelial cells. In fact, HAI-1/SPINT1 has essential functions in placental trophoblasts, epidermal keratinocytes, and hair follicle epithelium (7, 21). Recently, we reported that conditional deletion of the Spint1 gene in intestinal epithelial cells results in enhanced intestinal permeability and susceptibility to dextran sulfate sodium (DSS)-induced colitis (8). As intestinal epithelial integrity has an important protective role against mucosal inflammation and neoplastic progression (1, 2, 22), HAI-1/SPINT1 may have an important protective role against mucosal inflammation and neoplastic progression (1, 2, 22), HAI-1/SPINT1 may have a significant role in intestinal tumorigenesis. Moreover, all major physiologic HGF activators (matriprote, hepsin, HGF activator) are sensitive to HAI-1/SPINT1, and previous studies have revealed that colorectal cancer cells show decreased cell surface HAI-1/SPINT1 immunoreactivity with enhanced HGF activation in the cancer tissue (23). However, little is known about how HAI-1/SPINT1 may participate in intestinal tumorigenesis.

In this study, we evaluated the role of HAI-1/SPINT1 in intestinal tumorigenesis using intestinal specific HAI-1/Spint1 knockout mice (8) in 2 intestinal carcinogenesis mouse models: an Apc mutant mouse and 2-step chemical carcinogenesis in mouse colon. Our results from both models confirm that HAI-1/Spint1 has a critical role in suppressing intestinal tumorigenesis.

Materials and Methods
Animal experiments
All animal studies were reviewed and approved by the Committee on the Ethics of Animal Experiments of the University of Miyazaki Animal Research Committee, in accordance with international guidelines for biomedical research involving animals. All mice were housed in a pathogen-free barrier environment for the duration of the study. ApcMin/+ mice were obtained from The Jackson Laboratory. Mice with intestinal epithelial cell–specific deletion of the Spint1 gene (Spint1fl/fl/Vil-Cre) were generated by interbreeding mice carrying Spint1fl/fl homozygous alleles (Spint1fl/fl) with Cre transgenic mice under the control of the intestine-specific villin promoter (8). ApcMin/+ mice were then interbred with Spint1fl/fl/Vil-Cre mice, generating ApcMin/+ mice with intestine-specific deletion of the Spint1 gene (ApcMin/+ /Spint1fl/fl/Vil-Cre). All mice had a C57BL/6 genetic background and were fed a semipurified AIN-76 diet under specific pathogen-free condition. For ApcMin/+ tumorigenesis assays, age- and sex-matched mice were selected to assess tumor formation in the small and large intestine. At 15 weeks of age, all mice (male, n = 19 and 15 for ApcMin/+ /Spint1fl/fl/Vil-Cre and ApcMin/+ /Spint1fl/fl, respectively) were sacrificed by diethyl ether inhalation, and the numbers and major diameters of intestinal polyps were determined. The polypoid tumor lesion size was scored as follows: <1 mm, score 1; <2 mm, score 2; <3 mm, score 3; ≥3 mm, score 4. For the chemical carcinogenesis study, Spint1fl/fl/Vil-Cre mice and Spint1fl/fl mice were used for combined treatment with azoxymethane (AOM) and DSS (24). At 8 weeks of age all mice (male, n = 18 and 23 for Spint1fl/fl/Vil-Cre and Spint1fl/fl, respectively) were treated with a single intraperitoneal injection of AOM (Sigma-Aldrich; 10 mg/kg body weight). One week after the AOM injection, mice were exposed to 1% DSS (molecular weight 36–50 kDa; MP Biomedicals) in the drinking water for 5 days and thereafter received no further treatment. All mice were sacrificed 20 weeks after the AOM treatment. For histologic analysis, formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) or processed for immunohistochemistry.

Immunohistochemistry, immunofluorescence, immunoblotting, and gene expression analysis
The following antibodies were used: anti-human HAI-1 monoclonal antibody 1N7 (12), anti-mouse HAI-1 goat polyclonal antibody raised against recombinant HAI-1 lacking transmembrane and intracytoplasmic domains (R&D Systems), anti-mouse/human HAI-1 rabbit polyclonal antibody raised against intracytoplasmic domain (Pro484-Leu507; ref. 25), anti-mouse/human matriptase (AnaSpec), anti-mouse Hgf (R&D Systems), anti-mouse β-catenin (Sigma-Aldrich), anti-phosphorylated c-MET (Y1235; ref. 26), and anti-mouse CD31 (AnaSpec) rabbit polyclonal antibodies. The protocol for immunohistochemistry of paraffin-embedded sections of surgically resected colorectal tumors (4 cases) was approved by the ethical board of the Faculty of Medicine, University of Miyazaki (Miyazaki, Japan). The immunohistochemical methods used were described previously (12, 26, 27). Immunofluorescence and immunoblot analyses were conducted as described (8). Preparation of Hgf-enriched tissue extract and analysis of Hgf processing by immunoblot were conducted as described previously (23). Reverse transcription (RT)-PCR and quantitative real-time RT-PCR (qRT-PCR) were carried out using the primer sequences and methods described in Supplementary Table S1. Microarray analysis was conducted as described previously (27) using a GeneChip Mouse Genome 430 2.0 Array (Affymetrix).

Cell culture, gene knockdown, and forced expression
The MT1rev2 cell line was established from Mmp14-null mice intestine as described previously (28). MT1rev2 cells carry an exogenous mouse Mmp14 gene whose expression is suppressed in the presence of doxycycline (1 μg/mL; Sigma) so that cells express MT1-MMP/Mmp14 in doxycycline-free medium. To detect secreted HAI-1/Spint1, cells were washed 3 times with PBS and cultured in serum-free medium without doxycycline for the indicated time period. The culture supernatant was then concentrated in an Amicon-Ultra-4 (Millipore) and used for immunoblotting.

Statistics
Statistical analysis was done using GraphPad Prism, version 5.04 (GraphPad Software Inc.).
Results

Decreased membranous HAI-1/SPINT1 immunoreactivity accompanies neoplastic progression of intestinal epithelium

In accordance with previous observations (23, 25), the membranous immunoreactivity of HAI-1/SPINT1 was markedly reduced in colon adenocarcinoma cells compared to adjacent adenoma cells and non-neoplastic epithelium (Fig. 1A). This trend was also observed in a murine model of intestinal tumorigenesis mediated by mutation of the Apc gene (ApcMin/+ mice; Fig. 1B). The neoplastic epithelium also showed significantly decreased membranous HAI-1/SPINT1 immunoreactivity compared with the adjacent non-neoplastic epithelium. Notably, similar to human colon cancer tissue (25), the Spint1 mRNA level was essentially preserved in ApcMin/+ tumor tissue (Fig. 1B), suggesting that the decreased immunoreactivity may be caused by enhanced shedding of membrane-bound HAI-1/SPINT1 from the neoplastic cell surface. Given that HAI-1 shedding is reported to be mediated by metalloprotease activity (29) and in human oral carcinoma cells MT1-MMP/MMP14 is responsible for HAI-1/SPINT1 ectodomain shedding (30), we compared Mmp14 expression in tumor tissue with corresponding normal mucosa in ApcMin/+ intestine and found that the Mmp14 mRNA level was significantly increased in tumor tissue (Fig. 1C). The effect of MT1-MMP/Mmp14 on HAI-1/Spint1 shedding was then examined using the immortalized mouse intestinal epithelial cell line MT1rev2, which was established from Mmp14-knockout mice and carries an inducible mouse Mmp14 gene that drives exogenous Mmp14 expression under tetracycline off conditions (28). In the MT1rev2 cells, MT1-MMP/Mmp14 expression did indeed result in enhanced HAI-1/Spint1 shedding into the culture supernatant generating 58- and 42-kDa secreted forms as previously reported (ref: 30; Fig. 1D). In accordance with these observations, mature membrane bound HAI-1 was markedly decreased in extracts of ApcMin/+ tumors accompanying increased amounts of 58- and 42-kDa forms and further degraded fragments (Fig. 1E). Consequently, C-terminus fragments smaller than 15 kDa were also increased in the tumor tissue (Fig. 1E).

ApcMin/+ mice deficient in intestinal HAI-1/Spint1 have shorter survival times

To test whether the loss of membrane-bound HAI-1/SPINT1 is simply an epiphenomenon of the neoplastic transformation process in intestinal epithelial cells or has a causal role in carcinogenesis and progression, we tested the effect of HAI-1/Spint1 defect on tumorigenesis in Apc mutant mice. By inter-breeding intestine-specific Spint1-deleted mice (Spint1floxflox/Vil-Cre ref. 8) with ApcMin/+ mice, we generated ApcMin/+ mice having a conditional deletion of the Spint1 gene in the intestinal epithelium (ApcMin/+Spint1floxflox/Vil-Cre). HAI-1/Spint1 immunoreactivity was lost in most intestinal epithelial cells in ApcMin/+Spint1floxflox/Vil-Cre mice, although focal residual immunoreactivity in a small group of cells was occasionally observed (Fig. 2A). The intestinal Spint1-deleted ApcMin/+ mice showed decreased body weight gain compared with control ApcMin/+Spint1floxflox mice, which became apparent at 7 weeks of age (Supplementary Fig. S1) and significantly shorter survival times (P = 0.0009, log-rank test; Fig. 2B). The ApcMin/+ mice with heterozygous Spint1 (ApcMin/+Spint1+/−) had an intermediate survival curve (Fig. 2C). The major cause of death was severe anemia probably due to increased bleeding from the intestinal tract, which was more evident in ApcMin/+Spint1floxflox/Vil-Cre mice (mean hemoglobin concentration, 5.1 g/100 mL) than in ApcMin/+Spint1floxflox mice (9.3 g/100 mL) at 15 weeks of age (Supplementary Table S2).

Loss of intestinal HAI-1/Spint1 accelerates tumor formation in ApcMin/+ mice

We then analyzed the effect of intestinal epithelium Spint1 deletion on intestinal tumorigenesis. Mice were sacrificed at 15 weeks of age, and the number of visible neoplastic polyps was counted. Notably, intestinal HAI-1/Spint1−deficient ApcMin/+ mice showed significantly enhanced tumor formation in the small intestine (Fig. 3A and B), a major site of tumor formation in ApcMin/+ mice but not in the colon (Fig. 3C). The mean multiplicity (no. of tumors/mouse) in ApcMin/+Spint1floxflox/Vil-Cre male mice was 95.0, which was significantly higher than that of the control ApcMin/+Spint1floxflox male mice (38.5 per mouse), and the difference was particularly evident in the proximal small intestine (Fig. 3C). The sizes of tumors formed by the 15th week tended to be larger in the intestinal Spint1-deleted mice than those in the control mice (Fig. 3D). Histologically, significant morphologic differences were not observed between Spint1-deleted tumors and control tumors (Fig. 3B).

Absence of HAI-1/Spint1 enhances activation of HGF

We compared the mRNA levels of HAI-1/Spint1 and its important target membrane proteases, matriptase, by qRT-PCR. While matriptase (Sit14) mRNA levels were not significantly altered in the intestinal tumor tissue of ApcMin/+ mouse (Fig. 4A), its subcellular localization was altered in the tumor cells, showing a decreased membranous localization and increased intracellular immunoreactivity with punctate staining (Fig. 4A). It should be noted that in normal epithelium, matriptase localization was not altered even in the absence of HAI-1/Spint1 as long as the epithelial and villous architectures were retained.

HGF is secreted as an inactive proform (proHGF) and requires proteolytic activation to induce c-MET–mediated signaling (11). As all major HGF-activating proteases (HGF activator, matriptase, and hepsin) are inhibited by HAI-1/SPINT1 (11, 13), we examined the activation of proHGF in Spint1-deleted mouse intestinal tissues in the early stage (10 weeks old) of ApcMin/+ tumorigenesis (n = 4). Even at 10th week, visible tumors are found in small intestine and the number was apparently higher in Spint1-deleted intestine. As shown in Fig. 4B, processing of HGF was enhanced in Spint1-deleted ApcMin/+ tumor tissue compared with control tumor tissue. Notably, background mucosa without visible tumor (hereafter nontumor mucosa) also showed enhanced HGF activation in response to Spint1 deletion. In accordance with these observations, the immunoreactivity of phosphorylated c-
Met was increased in Spint1-deleted ApcMin+/+ tumor cells compared with control cells (Fig. 4B).

**Wnt signaling circuit is augmented in response to intestinal deletion of Spint1 in ApcMin+/+ mice**

Comprehensive gene expression profiles of intestinal mucosa and tumors from ApcMin+/+ /Spint1fl/fl/Vil-Cre mice were compared with those of control ApcMin+/+ /Spint1fl/fl mice using microarray, and the expression of selected genes was further confirmed by qRT-PCR. The entire raw data set for the microarray analyses is available at Gene Expression Omnibus (GEO). Compared with control tumors from mice of matched age, Hai-1/Spint1–deficient ApcMin+/+ tumors from 15-week-old animals showed upregulation of 22 genes that were related to...
the Wnt signal pathway, including ligands, receptors, transcription factors, and downstream genes (Table 1). Among these genes, 8 (Apcdd1, Proxl, Sox17, Wyf1, Fzd3, Igfbp4, Ptk7, Tcf4) were also upregulated in the nontumor mucosa of Spint1-deleted ApcMin/− intestine compared with control ApcMin/− intestine (Table 1). In addition to these 22 genes, 11 Wnt signal-related genes, including Fzd6, Wnt11, Wnt5A, Ror2, and Tcf7 (Tcf-1), were upregulated in nontumor mucosa in response to Spint1 deletion (Table 1). Notably, Wnt11, Apcdd1, and Sox17 mRNA levels in the Spint1-deleted nontumor mucosa were comparable with those in tumor tissue of control ApcMin/− mice (Supplementary Fig. S2). Immunohistochemically, tiny foci of β-catenin nuclear translocation, suggesting preneoplastic lesion or microadenoma, were frequently observed in Spint1-deleted nontumor mucosa but not in control nontumor mucosa (Fig. 4C).

**Gene expression signature suggests enhanced mucosal permeability and angiogenesis in Spint1-deleted ApcMin/− intestine**

The loss of Hai-1/Spint1 also induced upregulation of several genes possibly related to tumor progression and angiogenesis (Supplementary Table S3). While MT1-MMP/Mmp14 was upregulated in control ApcMin/− tumors compared with corresponding nontumor mucosa (Fig. 1C), it was also upregulated in Hai-1/Spint1-deficient ApcMin/− tumors (6.66-fold). Among these genes, upregulation of Flt1, Cdhl3, Cdhl5, and Tnfrsf12a strongly suggested enhanced angiogenesis in Spint1-deleted tumor and nontumor mucosa compared with control tumor and nontumor mucosa, respectively (Supplementary Table S3 and Fig. 4D; refs. 31–33). Furthermore, other genes related to DNA damage and/or repair were upregulated in Spint1-deleted nontumor mucosa, although their upregulation was not apparent in tumor tissue (Supplementary Table S4). Pml, a tumor suppressor with antiangiogenic function (34), was decreased (Supplementary Table S4 and Fig. 4D). In fact, CD31 immunostain revealed increased vascular density in Spint1-deleted tumors (P = 0.0005), which may result in enhanced bleeding (Supplementary Fig. S3). Cldn2 was also upregulated in nontumor mucosa in response to Spint1 deletion as well as in tumor tissues (Fig. 4D). Cldn2 encodes claudin-2 that enhances tight junction permeability (4), which may allow increased permeation of genotoxic substances. In fact, several genes related to DNA damage and/or repair were upregulated in the nontumor mucosa of ApcMin/−/Spint1lox/lox/Vil-Cre mice compared with that of control mice (Supplementary Table S5).

In contrast, Spint1-deleted small intestine without Apc mutation (Spint1lox/lox/Vil-Cre) did not show significantly upregulated expression of Cldn2, Flt1, Apcdd1, and Sox17, compared with control Spint1lox/lox mouse intestine, and only Tnfrsf12a showed statistically significant upregulation (Supplementary Fig. S2). Consequently, no tumor formation was observed in the intestine of Spint1lox/lox/Vil-Cre mice with wild-type Apc gene during the observation period (15 weeks; data not shown).

**Intestinal Hai-1/Spint1 is required for resistance to chemical carcinogen-induced colonic carcinogenesis**

To test whether a similar phenomenon can be reproduced under other carcinogenic conditions without germline mutation of Apc, we examined the effect of Spint1 deletion on chemical carcinogenesis of the colon using mice with a wild-type Apc gene. Spint1lox/lox/Vil-Cre and control Spint1lox/lox mice received an injection of AOM followed by administration of 1% DSS in the drinking water for 5 days (Fig. 5A). This carcinogenesis treatment was not efficient for the control Spint1lox/lox mice, where only 17% of treated mice (4 of 23) showed tumor formation (Fig. 5B). However, defects in epithelial Hai-1/Spint1 conferred significant susceptibility to AOM/DSS-induced tumorigenesis, as 67% (12 of 18) of Spint1lox/lox/Vil-Cre mice showed tumor formation in the colon with increased multiplicity compared with the control mice (Fig. 5B). The mean size of the formed tumors was similar between the 2 groups (3.73 and 3.58 mm for Spint1-deleted colon and control colon, respectively; Fig. 5B). The Spint1lox/lox/Vil-Cre mice that had sham treatment did not show tumor formation. It should be noted that similar to the ApcMin/− mouse model described above, the intestinal site of tumor formation was not altered by the absence of Hai-1/Spint1 with predominant tumor formation occurring in the distal colon in both groups (Fig. 5C).

While tumors in both groups showed differentiated tubular adenocarcinoma, Spint1-deleted tumors showed less differentiated features than control tumors (Fig. 5D).

**Discussion**

Using mouse knockout technology, we explored the function of the membrane-associated serine protease inhibitor, Hai-1/Spint1, in intestinal carcinogenesis and found that Hai-1/Spint1 has a novel role as a suppressor of carcinogenesis in the intestinal tract. The absence of Hai-1/Spint1 in enterocytes resulted in significantly accelerated tumor formation in ApcMin/− mice and conferred susceptibility to chemical carcinogenesis in the colons of wild-type Apc mice. In accordance with these findings, cell surface Hai-1/Spint1 expression was decreased in ApcMin/− tumor cells in mice, and importantly, human colorectal cancer cells also showed decreased cell surface HAI-1/SPINT1 levels compared with adjacent normal and adenoma epithelia. Our data also suggest that the decreased amount of membrane-associated Hai-1/Spint1 in the intestinal tumor cells may be due to proteolytic ectodomain shedding induced by MT1-MMP/Mmp14, which implies a novel pathway for MT1-MMP/Mmp14 contribution to early neoplastic progression of intestinal epithelium via the depletion of membrane-anchored Hai-1/SPINT1.

It should be noted that in contrast to the mouse ApcMin/− tumor in this study, previous studies of human colon cancer showed decreased SPINT1 mRNA in cancer tissues (23, 35). However, given the nearly complete loss of cell surface HAI-1/SPINT1 immunoreactivity, the reduction levels of SPINT1 mRNA were not sufficient in most cases: 20% and 40% reduction in well-differentiated carcinomas and overall, respectively (23, 35). In situ hybridization study also revealed a substantial amount of SPINT1 mRNA in tumor cells that showed loss of cell
surface HAI-1/SPINT1 (25). Therefore, in human colon cancer, we suggest that both decreased transcription and enhanced ectodomain shedding contribute in a coordinated manner to the observed significant reduction of cell surface HAI-1/SPINT1. Recent genome-wide analyses for mutated genes in human colon cancer did not identify relevant mutation or significant change of gene expression level of SPINT1, and only one point mutation, which results in R390H (R406H in HAI-1B, a common splicing variant), was found (36). Collectively, posttranslational enhanced ectodomain shedding might be an important mechanism underlying the loss of cell surface HAI-1/SPINT1 in tumor cells.

Multiple mechanisms could potentially contribute to the suppressive effect of the membrane-associated Hai-1/Spint1 on intestinal tumorigenicity, but so far, the evidence suggests that its roles for epithelial integrity to maintain the barrier function and regulation of Hgf processing are responsible. A previous study indicated that although mice lacking intestinal

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**Figure 2.** Generation of intestinal Hai-1/Spint1-deficient ApcMin/+ mice and their survival curves. A, Hai-1/Spint1 immunostaining of small intestine from control (ApcMin/+ /Spint1fl/fl) and intestinal epithelium–specific Spint1-knockout mice (ApcMin/+ /Spint1fl/fl/Vil-Cre). Focal residual Hai-1/Spint1 immunoreactivity is indicated by arrows. Bars, 100 (top) and 20 (bottom) μm. RT-PCR for Spint1 and matriptase mRNAs in proximal small intestine (pSI), distal small intestine (dSI), proximal large intestine (pLI), and distal large intestine (dLI) are also shown. B, Kaplan–Meier survival curves of ApcMin/+ /Spint1fl/fl/Vil-Cre (n = 12, all males) and control mice (ApcMin/+ /Spint1fl/fl, n = 10, all males). The Spint1-mutant mice showed shorter survival times (P = 0.0009, log-rank test). C, survival curves of ApcMin/+ /Spint1fl/fl/Vil-Cre (n = 20, 12 males and 8 females), ApcMin/+ /Spint1fl/fl/Vil-Cre (spint1 heterozygous; n = 5, one male and 4 females), and ApcMin/+ /Spint1fl/fl/Vil-Cre (wild-type Spint1; n = 20, 8 males and 12 females) mice. ApcMin/+ /Spint1fl/fl/Vil-Cre mouse showed shorter survival times compared with ApcMin/+ /Spint1fl/fl (P = 0.0008, log-rank test) and ApcMin/+ /Spint1fl/fl (P < 0.0001, log-rank test) mice.
Hai-1/Spint1 showed normal development and growth, intestinal permeability was increased, and these mice had enhanced susceptibility to DSS-induced colitis (8). In this study, Hai-1/Spint1 loss induced acceleration of tumor formation in ApcMin+/ mice or when mice received a carcinogenic insult but not in mice with wild-type Apc or untreated mice. Therefore, the pivotal function of Hai-1/Spint1 likely emerges when epithelial cells encounter pathologic stimuli and/or cellular stress due to genetic abnormalities. Under adverse cellular conditions, the loss of Hai-1/Spint1 may result in increased epithelial permeability that could amplify these pathologic stimuli and accelerate accumulation of genetic abnormalities. Upregulation of claudin-2 expression would further enhance epithelial permeability in Spint1-deleted ApcMin+/ mice, as this particular claudin correlates with epithelial leakiness (4, 5). Moreover, claudin-2 is reported to be involved in colon tumorigenesis (37). Enhanced activation of Hgf and its specific receptor c-Met may also be an important mechanism for tumorigenesis enhanced by Hai-1/Spint1 loss, as HAI-1/SPINT1 is a potent inhibitor of major proHGF-activating proteases, HGF activator, matriptase, and hepsin (6, 13, 16), and important roles of HGF/c-MET in cancer progression have been established (38). The enhanced Hgf activation may also be involved, at least partly, in enhanced angiogenesis in ApcMin+/ tumors (38).

The cross-talk between HGF/c-MET signaling and WNT/β-catenin signaling has been reported. The activated HGF secreted by stromal myofibroblasts reportedly activates β-catenin–dependent transcription and subsequent colon cancer stem cell clonogenicity and also restores the cancer stem cell phenotype in more differentiated tumor cells (39). HGF-induced activation of WNT pathway via transcriptional activation of lymphoid enhancer–binding factor 1 (LEF1) was also reported in human cancer cells (40). Indeed, comprehensive gene expression profiling revealed that the Spint1-deleted ApcMin+/ intestine showed significant upregulation of proteins involved in the Wnt signaling circuit and enhanced angiogenesis even in nontumor mucosa, which would provide a cancer-promoting microenvironment. T-cell factor (Tcf) 4, part of the Tcf4–LeF1 complex, was upregulated in Spint1-deleted mucosa. In addition, recent study suggested that Tcf7 (also known as Tcf-1) and LeF1 are obligate partners in the oncogenic activities of β-catenin in Apc-mutant mice (41), so it is of interest that...
Figure 4. Enhanced Hgf processing and upregulation of Wnt signal–related genes. A, qRT-PCR for Spint1 and matriptase (St14) and matriptase localization (immunofluorescence) in small intestine. Nontumor mucosa (Non-T) and tumor (T) from small intestine of ApcMin/þ/Spint1flox/flox (C, control) or ApcMin/þ/Spint1flox/flox/Vil-Cre (KO, Spint1 deletion) mice were analyzed. qRT-PCR data are means ± SD (n = 3). Green fluorescence, matriptase; blue, nuclei. N, non-neoplastic epithelium; T, neoplastic epithelium. Bar, 20 μm. B, processing of proHgf in nontumor mucosa (top) and tumor tissues (bottom; 10 weeks old). Representative immunoblot photos and bar graph data of relative processing rate (n = 4) are shown. *, P < 0.05. Immunostaining photos of phosphorylated c-Met in intestinal neoplastic lesions are also shown. Bar, 20 μm. C, immunostaining of β-catenin (b-cat) of nontumor mucosa (top) and tumor mucosa (bottom). Arrows indicate nuclear translocation of β-catenin in epithelial cells of nontumor mucosa (top) and tumor mucosa (bottom). Bars indicate nuclear translocation of β-catenin in epithelial cells of nontumor mucosa from Spint1–deleted intestine and enlarged image is also shown as inset. In the last panel, phosphorylated c-Met (pMet) was also immunostained using a serial section. Bar, 20 μm. D, RT-PCR of angiogenesis–related genes and Cldn2 and qRT–PCR analysis of Cldn2 expression. *, P = 0.0008. P values were analyzed by Mann–Whitney U test.
Tcf7 gene expression was also upregulated in Spint1-deleted mucosa. In accordance with these findings, Sox17 and Apcdd1 genes were markedly upregulated not only in tumor tissue but also in the Hai-1/Spint1–deficient nontumor mucosa. Sox17 is induced by Wnt signal activation in the early stage of gastrointestinal tumorigenesis (42), and Apcdd1 is also regulated by the β-catenin/Tcf complex with its elevated expression contributing to colorectal carcinogenesis (43). As these alterations in gene expression in the nontumor mucosa were not observed in the Spint1-deleted intestine with wild-type Apc, microadenomas intermingling with normal epithelium may contribute to the upregulation of these genes. However, the degrees of upregulation of some genes, such as Apcdd1 and Sox17, in Spint1-deleted nontumor mucosa were comparable with tumor tissue itself from control Apcmin/+ mice, indicating that Spint1 deletion amplified Wnt signaling. Taken altogether, the function of intestinal Hai-1/Spint1 as a tumor suppressor retards the formation of a tumor-promoting microenvironment under cancer-prone epithelial conditions and does not act as a gatekeeper in carcinogenesis. This notion is supported by the fact that enhanced tumor formation was observed only in its proper site in each of the mouse models examined in this study: small intestine in Apcmin/+ mice or distal large intestine in Apcmin/+ mice, indicating that matriptase activity may be involved in the phenotypes on Min/+ mice as well as intestinal epithelial integrity (3–5). Therefore, deregulated matriptase activity may be involved in the phenotypes observed here for Hai-1/Spint1 deletion. In fact, the ratio of matriptase to HAI-1/SPINT1 is higher in colorectal cancers

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Table 1. Effects of Spint1 deletion on Wnt signaling–related gene expression

NOTE: Bold, gene symbol.
than in normal tissue (44) and cancer cells show increased matriptase activity (45). However, there is an apparent paradox for the intestinal tumorigenesis in the matriptase:Hai-1/Spint1 balance. Matriptase hypomorphic mice showed enhanced susceptibility to DSS colitis and increased claudin-2 expression in intestinal epithelial cells (4). Conditional deletion of the mouse matriptase gene (St14) in the intestinal tract impairs colonic mucosal integrity and induces colonic cancer development (3, 46). Therefore, similar to Hai-1/Spint1, matriptase is a tumor suppressor in the intestinal tract. We hypothesize that membranous Hai-1/Spint1 may be critical for "normal" matriptase function that is required for epithelial integrity, and loss of Hai-1/Spint1 may cause abnormal subcellular localization or enhanced release of matriptase under adverse cellular conditions. In fact, HAI-1/SPINT1 is required for intracellular matriptase trafficking (15, 16, 47), and in tumor cells, loss of membrane-associated HAI-1/SPINT1 resulted in decreased membrane-associated matriptase and increased pericellular matriptase activity (27, 30) that might activate pericellular molecules involved in tumor progression such as proHGF. In the current study, intestinal tumor cells with decreased membranous Hai-1/Spint1 showed abnormal matriptase subcellular localization. However, in nonneoplastic epithelial cells, the cell surface localization pattern of matriptase was preserved in Spint1-deleted cells as long as the epithelium retained a normal histologic architecture. In this regard, Hai-2/Spint2 is also expressed in the intestinal epithelium and may compensate for the loss of Hai-1/Spint1 in normal epithelial cells (8).

A potential limitation of this study is the question whether total absence of Hai-1/Spint1 in the Spint1-deleted intestinal tumor cells exactly represents the pathobiology of the tumor cells with ectodomain shedding-induced loss of cell surface.

Figure 5. Effect of Spint1 deletion on AOM/DSS-induced colon carcinogenesis. A, AOM/DSS treatment protocol. B, tumor multiplicity and size. The number of visible polyps/mouse was counted and the maximum diameter of each polyp measured. cont, Spint1<sup>fl<sub>ox</sub>/fl<sub>ox</sub>; KO, Spint1<sup>fl<sub>ox</sub>/fl<sub>ox</sub>/Vil-Cre. Bold vertical line indicates the mean value. *, P = 0.0002 (Mann-Whitney U test). C, representative photographs of macroscopic findings of distal colon tissues from Spint1<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> (Cont) and Spint1<sup>fl<sub>ox</sub>/fl<sub>ox</sub>/Vil-Cre (KO) mice. Bar, 1 cm. D, histology (H&E) and Hai-1/Spint1 immunostaining of colon tumors. Photographs in top are tumor formed in Spint1<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> (Cont) mouse, showing decreased Hai-1/Spint1 immunoreactivity in tumor cells. Dotted line indicates boundary between tumor (T) and non-neoplastic epithelium (N). Middle, low-magnification histology (H&E) of Spint1<sup>fl<sub>ox</sub>/fl<sub>ox</sub>/Vil-Cre (KO) mouse tumor that lacks Hai-1/Spint1 expression (inset). Photographs in the bottom indicate comparative histology of cont and KO tumors. Tumor cells in KO mouse show decreased mucin production. Bar, 50 μm.
Hai-1/Spint1. Although known functional domain of HAI-1/Spint1 is extracellular N-terminal Kunitz domain and no function has been reported for C-terminal intracytoplasmic domain, remnant C-terminus of HAI-1/Spint1 after ectodomain shedding may acquire unexpected biologic functions. This possibility should be clarified in a future study. Another important issue in this study that requires further clarification is whether a novel protumorigenic protease regulated by Hai-1/Spint1 may exist in intestinal epithelial cells. Deregulated activity of this putative protease in the absence of Hai-1/Spint1 may contribute to accelerated tumorigenesis. In this regard, several Hai-1/Spint1–sensitive membrane serine proteases, such as TMPRSS13, TMPRSS4, hepsin, and prosasin, are also expressed in the intestinal tract (8). Prostasin activates epithelial sodium channel and protease-activated receptor 2 (6), which may be involved in intestinal tumorigenesis (48). While the biologic function of TMPRSS4 is poorly understood, this TTSP does induce an invasive phenotype in cancer cells (20, 49).

In conclusion, our data suggest that the epithelial cell surface protease–inhibitor interaction may impact mucosal carcinogenesis, and the loss of membranous HAI-1/SPINT1 in epithelial cells confers susceptibility to tumorigenesis in the intestinal tract. Further analysis of the molecular mechanisms that underlie enhanced intestinal tumor formation in the Spint1–deleted mouse carcinogenesis models described in this study may reveal novel targets for therapeutic intervention.

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

Authors’ Contributions
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Hoshiko, M. Kawaguchi, Y. Haruyama, M. Seki
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Hoshiko, M. Kawaguchi, K. Yorita, H. Tanaka, H. Kataoka
Writing, review, and/or revision of the manuscript: S. Hoshiko, M. Kawaguchi, H. Kataoka
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Fukushima, M. Seki, H. Kataoka
Study supervision: M. Seki, H. Inatsu, K. Kitamura, H. Kataoka

Acknowledgments
The authors thank Yumiko Nomura and Ai Kanemaru for preparation of histologic specimens.

Grant Support
This work was supported by Grant-in-Aid for Scientific Research no. 24390099 (H. Kataoka) and no. 23790250 (M. Kawaguchi) from the Ministry of Education, Science, Sports and Culture, Japan.

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Received August 22, 2012; revised January 15, 2013; accepted February 4, 2013; published OnlineFirst February 27, 2013.

References


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Cancer Res  Published OnlineFirst February 27, 2013.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-3337

Supplementary Material  Access the most recent supplemental material at:
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