Tumor Suppressor Activity and Epigenetic Inactivation of Hepatocyte Growth Factor Activator Inhibitor Type 2/SPINT2 in Papillary and Clear Cell Renal Cell Carcinoma

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Abstract

Following treatment with a demethylating agent, 5 of 11 renal cell carcinoma (RCC) cell lines showed increased expression of hepatocyte growth factor (HGF) activator inhibitor type 2 (HAI-2/SPINT2/Bikunin), a Kunitz-type protease inhibitor that regulates HGF activity. As activating mutations in the MET proto-oncogene (the HGF receptor) cause familial RCC, we investigated whether HAI-2/SPINT2 might act as a RCC tumor suppressor gene. We found that transcriptional silencing of HAI-2 in RCC cell lines was associated with promoter region methylation and HAI-2/SPINT2 protein expression was down-regulated in 30% of sporadic RCC. Furthermore, methylation-specific PCR analysis revealed promoter region methylation in 30% (19 of 64) of clear cell RCC and 40% (15 of 38) of papillary RCC, whereas mutation analysis (in 39 RCC cell lines and primary tumors) revealed a missense substitution (P111S) in one RCC cell line. Restoration of HAI-2/SPINT2 expression in a RCC cell line reduced in vitro colony formation, but the P111S mutant had no significant effect. Increased cell motility associated with HAI-2/SPINT2 inactivation was abrogated by treatment with extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phospholipase C-γ inhibitors, but not by an inhibitor of atypical protein kinase C. These findings are consistent with frequent epigenetic inactivation of HAI-2/SPINT2, causing loss of RCC tumor suppressor activity and implicating abnormalities of the MET pathway in clear cell and papillary sporadic RCC. This information provides opportunities to develop novel targeted approaches to the treatment of RCC. (Cancer Res 2005; 65(11): 4598-606)

Introduction

Renal cell carcinoma (RCC) is a heterogeneous disorder with the majority (~75%) of cases classified as clear cell (conventional) and the next most frequent subtype is papillary RCC (~15% of all cases; ref. 1). Germline activating mutations in the MET proto-oncogene and inactivating mutations in the VHL tumor suppressor gene cause hereditary type 1 papillary RCC (HPRC1) and von Hippel-Lindau disease, respectively (2, 3). HPRC1-associated MET mutations activate MET signaling to promote cell growth and motility (4–7). The VHL tumor suppressor gene is inactivated by somatic mutation or promoter methylation in most sporadic clear cell RCC (8–11). However, somatic MET-activating mutations are uncommon (~10%) in sporadic papillary RCC and are not a feature of sporadic clear cell RCC (3). Thus, despite reports of increased expression of hepatocyte growth factor (HGF) and MET in RCC and synergy between the effect of VHL inactivation and increased MET signaling (12), direct genetic evidence for importance of the HGF/MET signaling in the pathogenesis of RCC is only present in a small minority of cases.

Methylation of CpG dinucleotides in the promoter regions of tumor suppressor genes producing transcriptional silencing plays a major role in many human cancers (13–16). The frequency of tumor suppressor gene inactivation by de novo methylation varies between tumor suppressor genes and between cancers. Thus, the 3p21.3 renal tumor suppressor gene, RASSF1A, is usually inactivated by epigenetic silencing and only rarely by somatic mutations (17–22), whereas the VHL tumor suppressor gene is inactivated more commonly by somatic mutation than by epigenetic silencing (8–11). For certain cancers, notably colorectal cancer, a subset of tumors may show extensive tumor suppressor gene promoter methylation (23). However, in a methylation profile analysis of RCC, we found promoter methylation of only a minority of tumor suppressor genes that were known to be inactivated in other tumor types (24, 25). This observation prompted us to investigate whether gene expression profiling of RCC cell lines treated with the demethylated agent 5-azacytidine might identify novel RCC tumor suppressor genes (26–28). During our investigations, we found silencing or down-regulation of HAI-2/SPINT2 expression in a number of RCC cell lines. HAI-2/SPINT2 (also known as bikunin) encodes Kunitz-type protease inhibitor that regulates HGF activity. Thus, HGF is secreted as an inactive proform and needs to be activated by HGF activator enzyme to initiate MET signaling. HAI-2/SPINT2 is an endogenous inhibitor of HGF activator enzyme and has been implicated in the pathogenesis of ovarian and hepatocellular carcinoma (29–31). In view of these findings, we proceeded to investigate whether epigenetic inactivation of SPINT2 might play a role in renal tumorigenesis.

Materials and Methods

Patients and samples. DNA from a total of 102 primary RCCs were analyzed and subdivided into 64 clear cell and 38 papillary RCCs. Protein
lysates from 31 additional RCC tumors (29 clear cell RCC and 2 nonclear cell RCC) was also analyzed. Local ethics committees approved the collection of samples and informed consent was obtained from each patient.

**Cell lines, 5-azaeducationxytidine treatment, and microarray analysis.** RCC cell lines KTCL 26, RCC4, UMR2, UMRC3, SKRC18, SKRC39, SKRC45, SKRC47, SKRC54, 786-0, and Caki-1 were routinely maintained in DMEM (Invitrogen, San Diego, CA) supplemented with 10% FCS at 37°C, 5% CO2. The demethylating agent 5-azaeducationxytidine (Sigma, Gillingham, Dorset, United Kingdom) was freshly prepared in double-distilled H2O and filter sterilized. Cell lines were plated in 75 cm2 flasks in DMEM supplemented with 10% FCS at differing densities, depending upon their replication factor, to ensure that both control and 5-azaeducationxytidine–treated lines reached ~75% confluence at the point of RNA extraction. Twenty-four hours later, cells were treated with 5 μM/L 5-azaeducationxytidine. The medium was changed 24 hours after treatment and then changed again after 72 hours. RNA was prepared 5 days after treatment using RNA Bee (AMS Biotechnology, Abingdonoxon, United Kingdom). RNA extracted from KTCL26, SKRC39, SKRC45, and SKRC47 ± 5-azaeducationxytidine was analyzed by microarray as previously described (32).

**HAI-2/SPINT2 expression** was detected by reverse transcription-PCR (RT-PCR) using the following primers: 5′-GAGGTCCACCAC-3′ and 5′-GGGCGGTGCAGTATTCTTCATAG-3′. Expression of GAPDH was used as a control. The GAPDH primers were 5′-TGAAGTTTGGACGTAACGAGGTTG-3′ and 5′-CATGTTGGGCCATGAGGTTCCAC-3′. The PCR cycling conditions for this reaction consisted of 5 minutes at 95°C followed by 30 cycles of 45 seconds of denaturation at 95°C, 45 seconds of annealing at 59°C, and 45 seconds of extension at 72°C. Semiquantitative analysis of HAI-2/SPINT2 expression was done using LabWorks software (UltraViolet Products, Cambridge, United Kingdom).

**Bisulfite modification and methylation analysis.** Bisulfite DNA sequencing was done as described previously (24). Briefly, 0.5 to 1.0 μg of genomic DNA was denatured at 0.3 mol/L NaOH for 15 minutes at 37°C, and then unmethylated cytosine residues were sulfonated by incubation in 3.12 mol/L sodium bisulfite (pH 5.0; Sigma)/5 mmol/L hydroquinone (Sigma) in a thermocycler (Hybaid, Basingstore, Hampshire, United Kingdom) for 20 cycles of 30 seconds at 99°C and 15 minutes at 50°C. The sulfonated DNA was recovered using the Wizard DNA cleanup system (Promega, Southampton, United Kingdom) in accordance with the manufacturer’s instructions. The conversion reaction was completed by desulfonating in 0.3 mol/L NaOH for 10 minutes at room temperature. The DNA was ethanol-precipitated and resuspended in water.

The HAI-2/SPINT2 Cpg island was identified on the human genome browser and the putative promoter region was predicted by Promoter Inspector software (Genomatix, Munich, Germany). The region was amplified from RCC cell lines and primary tumors using the following primers: SPINT2F (5′-CACCATCAGACGTGCTG-3′) and SPINT2R (5′-GGGCGGTGCAGTATTCTTCATAG-3′). The PCR cycling conditions for this reaction consisted of 5 minutes at 95°C followed by 30 cycles of 45 seconds of denaturation at 95°C, 45 seconds of annealing at 95°C, and 45 seconds of extension at 72°C. The PCR cycling conditions for the first PCR reaction, 0.1 volume; SPINT2F, SPINT2R, annealing temperature: 58°C) was used in a second, nested reactions (SPINT2F, SPINT2R and SPINT2F, SPINT2R, annealing temperature: 58°C) The PCR conditions for both the first and second PCR were 95°C for 5 minutes, followed by 35 cycles of 45 seconds of denaturation at 95°C, 45 seconds of annealing at 58°C, and 45 seconds of extension at 72°C. The methylation-specific primers were designed based on the sequencing data of the PCR-amplified bisulfite-modified cell-line genomic DNA. The methylated alleles were amplified using SPINT2MSP-F (5′-GGGGCGGTGTTGTTTATTTGAAGGCTC-3′) and SPINT2MSP-R (5′-ACGCCACCAAGGTTCCATACCCTG-3′). The PCR cycling conditions for this reaction consisted of 5 minutes at 95°C followed by 30 cycles of 45 seconds of denaturation at 95°C, 45 seconds of annealing at 57°C, and 45 seconds of extension at 72°C. The unmethylated allele was amplified using primers Spint2USP-F (5′-GGGTGGGTGTTTATTATTTGAAGGCTC-3′) and SPINT2USP-R (5′-TCAACACCCAACCAACCATTTAATCTCA-3′). The PCR cycling conditions were the same as for condition-specific PCR.

**Mutation analysis of primary tumors and cell lines.** Intron-exon boundaries were determined by matching the cDNA sequence for HAI-2/SPINT2 with the working draft sequence of the human genome, the UCSC assembly 5 (http://genome.ucsc.edu/). Mutation screening was done by direct sequencing on an ABI 3730 automated sequencer. The sequences of the primers used along with the annealing temperature are available upon request.

**Immunoblotting.** Protein extraction and blotting was done essentially as described previously (33). Sections of frozen tissue were fractionated, weighed, and homogenized into 20-fold excess of extraction buffer [7 mol/L urea/10% glycerol/10 mmol/L Tris-HCI (pH 6.8)/1% SDS/5 mmol/L DTT/0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1 mg/L of aprotinin, pepstatin, and leupeptin] with an electric homogenizer (Ultra-Turrax; IKA, Staufen, Germany). Extracts were quantified with the Bio-Rad detergent assay (Promipore, Bedford, MA) for 2 hours and probed with anti-HAI-2 goat antibody (R&D Systems, Minneapolis, MN) at 0.2 μg/mL. Signals were detected with horseradish peroxidase–conjugated goat antibody diluted 1:2000 (Dako, Ely, United Kingdom) and enhanced chemiluminescence (Amersham, Little Chalfont, Buckinghamshire, UK). Blots were scanned, and quantified with ImageJ (Wayne Rasband, National Institutes of Health, USA) with the help of the Fiji plugins for ImageJ.

**Plasmid constructs and colony formation assay.** The HAI-2/SPINT2 expression constructs were made by cloning the full-length human HAI-2/SPINT2 coding region from the SKRC 45 cell line, and a nonsense mutation found in the SKRC47 cell line, into the EcoRI-BamHI sites of pCDNA3.1 vector (Invitrogen). Plasmid constructs were verified by sequencing. Ten micrograms of empty vector or expression vector were transfected, by calcium phosphate method, into 5 × 104 cells (either SKRC39, UMR2, SKRC45, or COS-7). Forty-eight hours after transfection, cells were seeded in a serial dilution and maintained in DMEM and 10% fetal bovine serum supplemented with 1 mg/mL G418 (Life Technologies). Surviving colonies were stained with 0.4% crystal violet (Sigma) in 50% methanol, 14 to 21 days after initial seeding, and counted. Each transfection was carried out in triplicate. Additionally, replicate experiments were carried out to obtain further clones for expression analysis and further experiments.

**HAI-2/SPINT2–conditioned media.** COS-7 cells were transfected with wtSPINT2, mtSPINT2, or vector-only plasmids. Twenty-four hours later, the media was replaced with serum-free DMEM. Forty-eight hours after transfection, the media were cleared by centrifugation.

**Wound healing assay.** Three clones of SKRC39 and SKRC45 expressing high levels of exogenous wtSPINT2, mtSPINT2 (as verified by Western blotting), and control clones transfected with the empty vector were seeded at 5 × 104 in six-well plates, resulting in a confluent monolayer, and maintained in serum-free media. Each well of cells was scratched with the tip of a 200 μL pipette tip. Twenty-four hours following the scratch, the extent of “wound healing” was observed microscopically.

In a similar manner, untransfected SKRC39 cells were scratched and incubated in conditioned media from COS-7 cells transiently transfected (see above) with either wtSPINT2 or mtSPINT2. We also investigated whether treatment with signal transduction pathway inhibitors would modify the response to HAI-2/SPINT2 in the scratch assay: MAPK inhibitors PD98059 (30 μmol/L; Calbiochem, San Diego, CA) and U0126 (20 μmol/L; Promega); phospholipase C-γ inhibitor U73122 (2 μmol/L; Calbiochem) and atypical protein kinase inhibitor GF109203X (1 μmol/L; Calbiochem). Serum-free media was supplemented with these inhibitors and SKRC39 cells were incubated for 24 hours then scratched as above and incubated for a further 24 hours.

**Anchorage-independent growth assay.** SKRC39, UMR2, or SKRC45 clones (three of each) stably expressing wtSPINT2 or the empty vector control were suspended in DMEM 10% FCS agar. Cells were maintained by

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addition of 200 μL of DMEM 10% FCS, supplemented with 1 mg/mL G418 weekly. After 8 weeks of growth, a final count of colonies was done.

Statistical analysis. Paired t tests and χ² tests were carried out using SPSS 12.0 and statistical significance was taken at the 0.05 level.

Results

Identification of Epigenetically Down-regulated Genes in Renal Cell Carcinoma Cell Lines

The RCC-derived cell lines KTCL26, SKRC39, SKRC45, and SKRC47 were treated with the demethylating agent 5-azacytidine (5 μmol/L) for 5 days to reactivate epigenetically silenced/down-regulated genes in these cell lines. Changes in gene expression were measured by microarray analysis of chips containing 11,000 transcripts (32). Fifty-seven genes showed significant up-regulation (>5-fold in at least one cell line or a minimum of 2-fold in multiple cell lines) following demethylation including genes known to be epigenetically silenced in RCC (e.g., TFPI2 and CDH1; refs. 34, 35; Supplementary Table S1).

We noted that expression of HAI-2/SPINT2, a Kunitz-type protease inhibitor, was increased following 5-azacytidine in two of four cell lines (56-fold in SKRC39 and 3-fold in KTCL26). We then analyzed the expression of HAI-2/SPINT2 by RT-PCR pretreatment and posttreatment with 5-azacytidine in the original four RCC cell lines and in a further seven RCC cell lines. HAI-2/SPINT2 expression was up-regulated following treatment in 5 of 11 (SKRC39, UMRC2, 786-0, Caki-1, and KTCL26) lines. Before treatment, expression was completely absent in two of these (SKRC39 and UMRC2; Fig. 1A).

Expression and Methylation Status of HAI-2/SPINT2 in Sporadic Renal Cell Carcinoma

Expression of HAI-2/SPINT2 protein in sporadic renal cell carcinoma. The expression of HAI-2/SPINT2 protein in RCC and matched normal renal tissue was analyzed by Western blotting in 31 sporadic RCC, 29 clear cell RCC, and 2 nonclear cell RCC (Fig. 1B). Compared to adjacent tumor-free material, HAI-2/SPINT2 protein expression was down-regulated in 29% (9 of 31) of RCC (9 of 29 clear cell RCC and 0 of 2 nonclear cell RCC).

HAI-2/SPINT2 promoter region methylation status and mutation analysis in sporadic renal cell carcinoma. To determine if HAI-2/SPINT2 promoter region methylation was linked to down-regulation of HAI-2/SPINT2 protein expression in RCC cell lines and tumors, we analyzed the methylation status of a CpG island 5′ of the transcriptional start of HAI-2/SPINT2 (Fig. 2A) by direct sequencing and combined restriction analysis (Fig. 2B). Promoter region methylation was detected in four cell lines with silencing or down-regulation of HAI-2/SPINT2 mRNA expression and not (or minimally) in RCC cell lines without silencing or down-regulation. Of the four methylated cell lines, dense methylation was observed in two (UMRC2 and SKRC39), which showed total silencing of HAI-2/SPINT2 by RT-PCR (Figs. 1A and 2B). To investigate the frequency of HAI-2/SPINT2 promoter region methylation in primary RCC tumors, we analyzed 102 sporadic RCC (64 clear cell RCC and 38 papillary RCC), by methylation-specific PCR (Fig. 2C). Promoter region methylation was detected in 33% (34 of 102) of RCC, 30% (19 of 64) of clear cell RCC, and 40% (15 of 38) of papillary RCC (frequency of methylation in clear cell RCC verses papillary RCC: χ² = 1.028, P = 0.31). Unmethylated
specific PCR (USP) products were amplified in most of the tumors that were methylated. This was not unexpected as the tumor samples were not microdissected and, thus, there is some normal tissue contamination.

HAI-2/SPINT2 promoter region methylation was infrequent in normal kidney tissue (2 of 38 samples).

To determine whether somatic HAI-2/SPINT2 mutations were a feature of sporadic RCC, we did direct sequencing of the HAI-2/SPINT2 coding exons in 9 RCC cell lines and 30 sporadic RCC (15 clear cell RCC and 15 papillary RCC). An exon 3 c.331C > T transition resulting in a nonconservative missense substitution (P111S) was detected in a RCC cell line (SKRC47) that did not show methylation (Fig. 3). This sequence variant was not detected in 180 control chromosomes. Sequencing of cDNA clones from SKRC47 indicated that both the mutant and wild-type alleles were expressed. We also analyzed germ line DNA from six probands with familial non-VHL RCC, but no mutations were found. A single G > A polymorphism was identified in exon 7 (G689A, R230H) of two clear cell RCC, two papillary RCC, and all six familial RCC tumors. This was also present in 70% of control samples ($n = 90$).

Analysis of the Tumor-suppressing Activity of HAI-2/SPINT2 in Renal Cell Carcinoma

To determine the possible consequences of HAI-2/SPINT2 down-regulation in RCC, we undertook a series of investigations to determine the effect of HAI-2/SPINT2 expression on RCC cell line growth and migration.

Restoration of HAI-2/SPINT2 expression reduces colony formation. The tumor suppressor activity of HAI-2/SPINT2 was assessed by in vitro colony formation assays. Following transfection of a wild-type HAI-2/SPINT2 expression plasmid (or empty vector) into SKRC39 (Fig. 4A), there was a significantly reduced number of G418-resistant colonies (mean 73%, $t = 5.56, P = 0.031$) compared with SKRC39 cells transfected with an empty vector control in three independent experiments (Fig. 4B). The reexpression HAI-2/SPINT2 promoter/CpG island region. The predicted promoter region is underlined and transcriptional start is indicated by an arrow. Primers used for nested combined restriction analysis (COB) and methylation-specific PCR are highlighted. Two sets of methylation-specific PCR primers were designed to amplify either methylated or unmethylated promoter fragments. CpG dinucleotides analyzed are numbered 1 to 57. B, direct sequencing indicated methylation in RCC cell lines correlated with HAI-2/SPINT2 down-regulation: fully shaded box, complete methylation; half-shaded box, partial methylation; empty box, unmethylated. In particular, the cell lines in which HAI-2/SPINT2 was silenced, UMRC2 and SKRC39 (see Fig. 1), had densely methylated CpG islands. C, methylation-specific PCR analysis of 102 sporadic RCC identified HAI-2/SPINT2 promoter methylation in 30% of clear cell RCC and 40% of papillary RCC. Shown are representative samples; tumors 20, 27, 47, 28, 9, and 29 were methylated. The cell lines SKRC 18 and SKRC 39 are shown as unmethylated and methylated controls, respectively. M, methylation-specific primers; U, unmethylated specific primers.
missense substitution was identified in the cell line SKRC47 at exon 3; C331T

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**Figure 3.** HAI-2/SPINT2 mutation analysis. A single, nonconservative, missense substitution was identified in the cell line SKRC47 at exon 3; C331T (P111S).

Reexpression of HAI-2/SPINT2 inhibits anchorage-independent growth. The effect HAI-2/SPINT2 on anchorage-independent growth in a soft agar colony formation assay was assessed in SKRC39 cells transfected with empty pcDNA3.1 vector or pcDNA 3.1-wt-HAI-2/SPINT2. Following selection, clones expressing high levels of HAI-2/SPINT2 were isolated and cells were seeded and incubated in soft agar for 8 weeks, each experiment was done in triplicate with three independent clones. Cells transfected with empty vector showed robust colony growth, whereas colony growth was greatly reduced when HAI-2/SPINT2 was reexpressed; both the number and size of colonies was reduced [the number of colonies ≥100 μm was reduced by 66% (t = 14.2, P = 0.005) in clones expressing HAI-2/SPINT2] when compared with the control clones (Fig. 5A and B). In a duplicate experiment using UMRC2 clones, the number of large (≥100 μm) colonies was reduced by 53% (t = 23.8, P = 0.02; Fig. 5C). In contrast, the formation of SKRC45 (which expresses endogenous wild-type HAI-2/SPINT2) anchorage-independent colonies was not significantly reduced (mean 11%, t = 1.4 P = 0.31; Fig 5D) despite the overexpression of wtHAI-2/SPINT2.

Reexpression of HAI-2/SPINT2 reduces cell motility. Following growth in serum-free media, confluent dishes of stable SKRC39-empty vector and SKRC39-wt-HAI-2/SPINT2 clones were scratched with a 200 μL pipette tip. Twenty-four hours later, SKRC39-pCDNA 3.1 cells had fully invaded the resulting "wound," whereas SKRC39 cells reexpressing HAI-2/SPINT2 had not noticeably moved into the scratch region (Fig. 6A). This result was consistent for three experiments conducted on different HAI-2/SPINT2–expressing clones.

This observation was confirmed by repeating the scratch test on untransfected SKRC39 cells following the addition of serum-free, conditioned, media obtained from COS-7 cells transiently transfected with empty pCDNA 3.1 or pCDNA 3.1-HAI-2/SPINT2 vector. As with the stably transfected clones, 24 hours following introduction of a scratch, SKRC39 cells maintained in HAI-2/SPINT2-conditioned media did not reenter the wound. On the other hand, cells maintained in empty vector conditioned media reentered the wound (UMRC2 was not studied as it does not grow to confluence in vitro; Fig. 6C).

To further assess the functional significance of the P111S substitution, the wound healing assay was repeated with SKRC39 clones stably expressing mtP111S-HAI-2/SPINT2. Twenty-four hours after the scratch (and in contrast to cells expressing wtHAI-2/SPINT2), the wound had been filled (Fig. 6B). Moreover, untransfected SKRC39 cells incubated in mtP111S-HAI-2/SPINT2–conditioned media also filled the wound within 24 hours (Fig. 6C). These results suggest that the P111S missense substitution is pathogenic and are consistent with the lack of colony formation inhibition by mutant P111S HAI-2/SPINT2.

Modulation of MET-downstream signal transduction and response to HAI-2/SPINT2 inactivation. A major function of HAI-2/SPINT2 is to inhibit HGF, an activator of MET (36, 37). To investigate the relationship between HAI-2/SPINT2 inactivation, activation of MET downstream signal transduction pathways, and HAI-2/SPINT2 tumor suppressor functions, we evaluated the effect of inhibiting extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), phospholipase C-γ, and atypical protein kinase C (PKC) on HAI-2/SPINT2 inhibition of wound healing (see above). Addition of ERK/MAPK inhibitors PD98059 or U0126 to serum-free media, at a concentration of 30 and 20 μmol/L, respectively, resulted in the partial inhibition of wound healing by HAI-2/SPINT2-null SKRC39 cells over a period of 24 hours (Fig. 6D). Phospholipase C-γ recruitment to Gαi is essential for HGF-MET–induced cell motility (38, 39) and wound healing was also partially inhibited by the addition of the phospholipase C-γ inhibitor U73122 at 2 μmol/L (Fig. 6D). In contrast, the inhibition of atypical protein kinase C by GF109203X at 1 μmol/L did not inhibit the wound healing activity of HAI-2/SPINT2-null SKRC39 cells (Fig. 6D). These results indicate that, at least in part, the increased cell motility associated with loss of HAI-2/SPINT2 expression can be inhibited by downstream antagonists of MET signaling.

**Discussion**

We have shown (a) tumor suppressor effects of HAI-2/SPINT2 in RCC cell lines, (b) frequent loss of expression and promoter...
Figure 4. Reexpression of HAI-2/SPINT2 in RCC cells results in growth suppression. A, Western blot analysis of HAI-2/SPINT2. Representative blots of isolated clones (Cl. –) transfected with empty pCDNA3.1 vector, pCDNA3.1-wtHAI-2/SPINT2, or pCDNA3.1mtHAI-2/SPINT2 (C331T). The antibody used binds to two distinct bands, perhaps as a consequence of posttranslational processing of HAI-2/SPINT2. It is interesting that the lower band is not present in mtHAI-2/SPINT2 blots, but a higher molecular weight band is present. Clones expressing wtHAI-2/SPINT2 were isolated from transfections of SKRC39, UMRC2 (both of which do not express endogenous HAI-2/SPINT2), and SKRC45 cell lines. (The SKRC45 panel has been overexposed to allow a clear representation of the endogenous levels of wtHAI-2/SPINT2 present in pCDNA3.1 clones. Equal or further overexposure of SKRC39 and UMRC2 blots did not reveal HAI-2/SPINT2 in pCDNA3.1 transfected clones.) Clones expressing mtHAI-2/SPINT2 were isolated from transfections of the SKRC39 line. 

B, equal amounts of empty vector (3.1) and pCDNA3.1-wtHAI-2/SPINT2 (wtSPINT2) were transfected into SKRC39 cells. Each experiment was done in triplicate; columns, means. The mean number of colonies counted in the 3.1 plates was taken as 100%. There was a statistically significant reduction of colonies in each of the wtSPINT2 transfectants (P = 0.027). C, a replicate experiment using UMRC2 cells revealed a similar reduction of colony formation (P = 0.01). D, transfecting wtHAI-2/SPINT2 into SKRC45 cells, which express endogenous HAI-2/SPINT2, did not result in a significant reduction of colony formation (P = 0.32). E, the introduction of mtHAI-2/SPINT2 into SKRC39 cells did not have the same growth-suppressing effect as the wild-type. Colony formation was not significantly reduced (P = 0.35) compared with those plates transfected with empty vector.

Figure 5. Reexpression of wtHAI-2/SPINT2 inhibits anchorage-independent growth. A, clones (Cl.) of SKRC39-pCDNA3.1 and SKRC39-wtHAI-2/SPINT2 were seeded at the same density into soft agar and incubated for 8 weeks. Clones not expressing wtHAI-2/SPINT2 (pCDNA3.1) produced many large (>100 μm) colonies. In contrast, SKRC39 clones expressing exogenous wtHAI-2/SPINT2 did not grow as robustly resulting in fewer large colonies after 8 weeks of incubation (magnification, ×100). B, a graphical representation of three independent experiments; the number of colonies >100 μm expressing wtHAI-2/SPINT2 was significantly reduced (P = 0.02). C, in an identical experiment, the number of large colonies after 8 weeks following initial seeding of UMRC2-wtHAI-2/SPINT2 was reduced by 53% compared with UMRC2-pCDNA3.1 clones (P = 0.01). D, in contrast, exogenous expression of wtHAI-2/SPINT2 in SKRC45 clones did not significantly reduce the number of large colonies formed in soft agar (P = 0.13).
region methylation of HAI-2/SPINT2 in RCC cell lines and primary tumors, and (c) a HAI-2/SPINT2 mutation with reduced tumor suppressor function in a RCC cell line. These findings implicate HAI-2/SPINT2 in the pathogenesis of clear cell and nonclear cell RCC, extend the role of disordered HGF/MET signaling in the pathogenesis of RCC, and provide a basis for developing novel therapeutic strategies.

Promoter methylation and transcriptional silencing of HAI-2/SPINT2 has been described previously in hepatocellular carcinoma cell lines and tumors (31). In addition, Yamauchi et al. (40) found that HAI-2/SPINT2 mRNA was abundantly expressed in normal kidney but was down-regulated in advanced stage RCC. In methylation profiles of RCC by us and others, only four (CASP8, RASSF1A, SLIT2, and TIMP3) of 17 tumor suppressor genes tested showed promoter methylation in >20% of RCC (24, 25). Thus, tumor suppressor gene methylation is not a generalized feature of RCC, and, relative to many other known tumor suppressors, methylation of HAI-2/SPINT2 in primary RCC tumors is frequent. We also investigated whether somatic mutations in HAI-2/SPINT2 were a common cause of HAI-2/SPINT2 inactivation in RCC. We did not detect mutations in 30 primary RCC tumors, but a single missense mutation (that inhibited HAI-2/SPINT2 tumor suppressor function) was detected in a RCC cell line. Thus, HAI-2/SPINT2 resembles other candidate RCC tumor suppressor genes, such as RASSF1A and NORE1A, for which epigenetic inactivation is the major mechanism of inactivation (22, 41, 42).

HAI-2/SPINT2 is a Kunitz-type serine protease inhibitor that has a broad inhibitory spectrum against serine proteases, such as plasmin, trypsin, tissue, and plasma kallikreins and factor Xia (43, 44). HAI/SPINT2 was independently identified as placental bikunin. In studies of ovarian cancer, HAI-2/SPINT2/bikunin has been implicated as an inhibitor of tumor cell invasion and metastasis. In addition to inhibiting serine proteases, HAI-2/SPINT2 can also bind to high-affinity cell surface receptors (45) and down-regulate urokinase plasminogen activator (uPA) and its receptor (uPAR), probably by the MAP kinase pathway (46). Generation of plasmin from plasminogen by uPA can induce extracellular matrix degradation and promote tumor cell migration and metastasis. Hence, inactivation of HAI-2/SPINT2 might promote tumorigenesis by multiple mechanisms. Nevertheless, it seems likely that loss of HAI-2/SPINT2 inhibition of HGF/MET signaling would be implicated in promoting renal tumorigenesis.

Figure 6. Reexpression of wtHAI-2/SPINT2 reduces cell motility. A, SKRC39 clones reexpressing wtHAI-2/SPINT2 (wtSPINT2) or transfected with empty vector (pCDNA3.1) were grown to confluence. Following incubation in serum-free media, an artificial wound was scratched through them (0 hour). Nonexpressing SKRC39 (pCDNA3.1) cells had invaded the wound within 24 hours, whereas wtHAI-2/SPINT2–expressing cells did not reenter the wound (24 hours). The cells were maintained in serum-free media prior and during the experiment to exclude the possibility of cell growth masking the effects of cell migration. B, in contrast, SKRC39 clones expressing mtHAI-2/SPINT2 (mtSPINT2) invaded the wound in a similar manner to clones transfected with empty vector. C, similar repression of motility was observed when untransfected SKRC39 cells were maintained in serum-free conditioned media obtained from COS-7 cells transiently transfected with pCDNA3.1-wtHAI-2/SPINT2. Whereas the addition of serum-free–conditioned media obtained from COS-7 cells transiently transfected with mtHAI-2/SPINT2 did not inhibit the invasion of the wound. D, increased cell motility associated with loss of HAI-2/SPINT2 can be partially inhibited by downstream inhibitors of MET signaling. SKRC39 cells were maintained in serum-free media supplemented with inhibitory compounds to MAPK/ERK, phospholipase C-γ, or atypical protein kinase C. Following the introduction of a scratch wound, the confluent cells were incubated for a further 24 hours. As with previous experiments, the control cells had fully invaded the wound, as had those cells that had atypical PKC inhibited. However, the inhibition of MAPK/ERK or phospholipase C-γ had also partially inhibited the motile phenotype of these cells.
HGF activation of the MET signaling pathway induces profound effects on epithelial-cell motility, growth, and formation of branched tubules (47). During development, HGF is a potent growth factor for epithelial cells and has a critical role in regulating cellular motility (48–50). Disregulation of the HGF/MET signaling pathway is frequent in human cancer and may result from a variety of mechanisms (47). Thus, up-regulation of HGF and MET expression can activate MET signaling by a paracrine effect. However, the most direct evidence for MET involvement in human cancer was provided by the finding of germ line activating MET mutations in patients with HPBRC1. Nevertheless, somatic MET mutations are infrequent in renal cancer (3), so the finding of frequent HAI-2/SPINT2 inactivation in clear cell and papillary RCC provides an alternative mechanism by which disregulated HGF/MET signaling can be implicated in the pathogenesis of RCC.

We found that restoration of HAI-2/SPINT2 expression had a variety of effects on RCC cell lines including suppression of in vitro colony formation, inhibition of anchorage-independent growth, and reduced cell motility. Tumor formation and metastasis is a complex multistep process that requires the acquisition of a variety of properties (e.g., proliferation, invasion, angiogenesis, and antiapoptosis) that are associated with MET activation (51–54). We note that the biological effects of transfecting RCC cells with HAI-2/SPINT2 were most pronounced when endogenous HAI-2/SPINT2 was silenced, suggesting that the observed effects were specific consequences of HAI-2/SPINT2 overexpression. We have initiated preliminary investigations of the mechanisms of HAI-2/SPINT2 tumor suppression. Interestingly, we found that increased cell motility, associated with HAI-2/SPINT2 silencing, was abrogated by treatment with ERK/MAPK and phospholipase C-γ inhibitors, but not by inhibition of atypical PKC. The apparent partial inhibition of cell motility may be an indicator that HAI-2/SPINT2 inhibits a number of different signaling pathways (although we cannot exclude the possibility that the partial inhibition is an experimental artifact resulting from the relatively short half lives of the inhibitory compounds used). These findings provide a basis for further investigations into HAI-2/SPINT2 function and its dependence on specific downstream signaling pathways.

RCC is curable if detected at an early stage. However, up to 40% of patients with RCC present with locally advanced or metastatic disease that is difficult to treat with chemotherapy or radiotherapy. A major aim of human cancer genetics is to develop novel therapeutic agents based on a detailed knowledge of cancer molecular biology. Such agents could then be administered in individualized treatment regimens. Thus, response to treatment with an epidermal growth factor receptor (EGFR) kinase inhibitor (gefitinib) in lung cancer patients was associated with the presence of somatic EGFR mutations in the lung cancer (55, 56). Intriguingly, bikunin has been investigated as a treatment for ovarian carcinoma. Thus, overexpression of HAI-2/SPINT2 in an ovarian cancer cell line suppressed invasion and peritoneal carcinomatosis (30), and once-daily oral HAI-2/SPINT2 therapy reduced tumor load in a nude mouse model and in human ovarian cancer (57). In addition, a combination of HAI-2/SPINT2 and paclitaxel produced more profound effects on tumor growth (58). We note that conditioned media from HAI-2/SPINT2–expressing cells inhibited cell motility. Thus, our findings suggest that administration of HAI-2/SPINT2, and/or inhibitors of MET signaling (59), might provide novel therapeutic approaches to treating advanced RCC with HAI-2/SPINT2 inactivation.

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