Research Article

Genetic Inactivation of ADAMTS15 Metalloprotease in Human Colorectal Cancer

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Abstract

Matrix metalloproteinases have been traditionally linked to cancer dissemination through their ability to degrade most extracellular matrix components, thus facilitating invasion and metastasis of tumor cells. However, recent functional studies have revealed that some metalloproteases, including several members of the ADAMTS family, also exhibit tumor suppressor properties. In particular, *ADAMTS1, ADAMTS9,* and *ADAMTS18* have been found to be epigenetically silenced in malignant tumors of different sources, suggesting that they may function as tumor suppressor genes. Herein, we show that *ADAMTS15* is genetically inactivated in colon cancer. We have performed a mutational analysis of the *ADAMTS15* gene in human colorectal carcinomas, with the finding of four mutations in 50 primary tumors and 6 colorectal cancer cell lines. Moreover, functional *in vitro* and *in vivo* studies using HCT-116 and SW-620 colorectal cancer cells and severe combined immunodeficient mice have revealed that ADAMTS15 restrains tumor growth and invasion. Furthermore, the presence of ADAMTS15 in human colorectal cancer samples showed a negative correlation with the histopathologic differentiation grade of the corresponding tumors. Collectively, these results provide evidence that extracellular proteases, including ADAMTS15, may be targets of inactivating mutations in human cancer and further validate the concept that secreted metalloproteases may show tumor suppressor properties. [Cancer Res 2009;69(11):4926–34]

Introduction

The establishment of causal relationships between proteolytic activities and tumor progression has prompted to consider proteases as suitable targets for anticancer therapy (1–5). However, an increasing body of evidence indicates that some proteases can confer antitumorigenic properties (6, 7). Among them, there are several members of the a disintegrin and metalloproteinase with thrombospondin domains (ADAMTS) family including ADAMTS1, ADAMTS8, ADAMTS9, ADAMTS12, ADAMTS15, and ADAMTS18 (7). All of them share a complex structural design characterized by the presence of a metalloproteinase domain linked to a variety of specialized ancillary domains including a series of thrombospondin-1 repeats (8–11). ADAMTS are secreted enzymes, which, on secretion, remain located at the pericellular space through interactions of their thrombospondin-1 motifs and spacer regions with extracellular matrix components (11, 12). Several reports have shown that the tumor suppressor activities of ADAMTS1 and ADAMTS8 derive from the angio-inhibitory capacities of their thrombospondin-1 domains (13, 14), whereas ADAMTS12 inhibits tumor growth through its ability to modulate the Ras-dependent extracellular signal-regulated kinase (ERK) signaling pathway (15). In addition to these biochemical studies, genetic and epigenetic analyses have supported the proposal that ADAMTS family members may act as antitumor proteases. Thus, *ADAMTS1, ADAMTS9*, and *ADAMTS18* have been identified as epigenetically silenced genes in several carcinomas (16–18). The putative role of ADAMTS15 as a potential tumor-protective protease has recently emerged after the finding that its overexpression is a sign of favorable outcome in breast cancer patients (19). Furthermore, mutational analysis of most protein coding genes in a small set of human breast and colorectal carcinomas has revealed the occurrence of some mutations in *ADAMTS15* (20, 21). However, no functional studies have been done to evaluate the potential consequences of these mutations in tumor development and progression.

In this work, we have investigated the potential role of ADAMTS15 as a candidate tumor suppressor protease through an exhaustive analysis of both *ADAMTS15* genetic and epigenetic alterations in a large collection of human colorectal carcinomas. We have also performed a series of functional analyses to assess the protective nature of ADAMTS15, with the finding that depletion of this protease markedly promotes *in vitro* and *in vivo* tumor growth and invasion, whereas overexpression of the enzyme reverses the phenotype. Finally, data obtained from human tissue microarrays show a negative correlation between *ADAMTS15* expression and differentiation grade of colon carcinomas. Overall, our data indicate that ADAMTS15 is a metalloprotease with tumor suppressor properties.

Materials and Methods

Mutational analysis. Genomic DNA from a set of 50 normal/tumor paired colorectal tissue samples were obtained from patients subjected to surgery at Institut Català d'Oncologia. PCR amplification of *ADAMTS15* coding exons was done as described by Sjoblom et al. (20). Sequence data were analyzed using Mutation Surveyor software (20). Both tumor and normal paired tissue samples were resequenced to validate potential mutations and discard germ-line variants. Potential effect of an amino acid change in the ADAMTS15 protein function was predicted *in silico* using the Sorting Intolerant From Tolerant algorithm (22). This computational Web-based approach calculates a score, ranging from 0 to 1, which corresponds to the probability that a particular change in an amino acid is tolerated.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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This software uses alignments among the members of one family to predict if an amino acid is essential for the function of the protein.

cDNA constructs. *ADAMTS15* full-length human cDNA (23) was subcloned in pcDNA3.1 expression vector. The same cDNA was employed to delete a guanine at position 2547 (pcDNA-*ADAMTS15*ΔG2547 vector; ADAMTS15_G849fs protein). To this end, cassette mutagenesis was carried out by overlap extension (24) using the following primers: TS15bamFOR 5′-CCAAATGGGATCCCTATGGCC-3′, TS15REV 5′-CGCGGAGCACGGCCC-CAGCTGCCAGCCAC-3′, TS15FOR 5′-GCAGCTGGGGCCGTGCTCCGC-GAGCTGCGG-3′, and TS15hindREV 5′-AAGCTTGCACGGCCTCAGGAC-3′. An additional construct was generated using a modified pCEP4 expression vector and a FLAG epitope at the COOH-terminal region of the protein.

Cell culture and transfection. Colorectal tumor cell lines HCT-116, SW-480, SW-620, LoVo, DLD-1, and Caco-2 were purchased from the American Type Culture Collection. Cells were routinely maintained in DMEM, with the exception of SW-620 cells that were grown in L-15 medium. Cells were transfected with the Lipofectamine reagent, and stable clones were selected in the presence of 2 μg/mL puromycin.

DNA methylation analysis and reverse transcription-PCR analysis. Methyl Primer Express version 1.0 software (Applied Biosystems) was used to identify CpG islands in an interval of 2.0 kb upstream and 1.5 kb downstream from the first ATG codon. The same program was used to design primers to amplify methylated or unmethylated DNA in both promoter and first exon regions. To evaluate *ADAMTS15* expression, cDNA was synthesized using 1 μg purified RNA and the ThermoScript Reverse Transcription-PCR System (Invitrogen). To quantitatively assess *ADAMTS15* expression levels in colorectal cancer cells, quantitative reverse transcription-PCR was done using the Taqman probe Hs01054400_g1 from Applied Biosystems. All reactions were done in quadruplicates.

RNA interference. A panel of four *ADAMTS15* specific short hairpin RNA (shRNA; 5′-TCTTCAGACCTGCGACGCTGCTTCTATTC-3′, 5′-AGA-GAAGCTGCTCTGTCATTGAGGACGAT-3′, 5′-GCTACAACCACAGCAC-CAACCGGCTCACT-3′, and 5′-TCTGTGTCCAAGGCAAGTGCATCAAGGCT-3′) introduced into the pRetroSuper (pRS) vector was purchased from OriGene. SW-620 or 293-EBNA cells were transfected with these vectors or with pRS control vector and selected with 1 μg/mL puromycin.

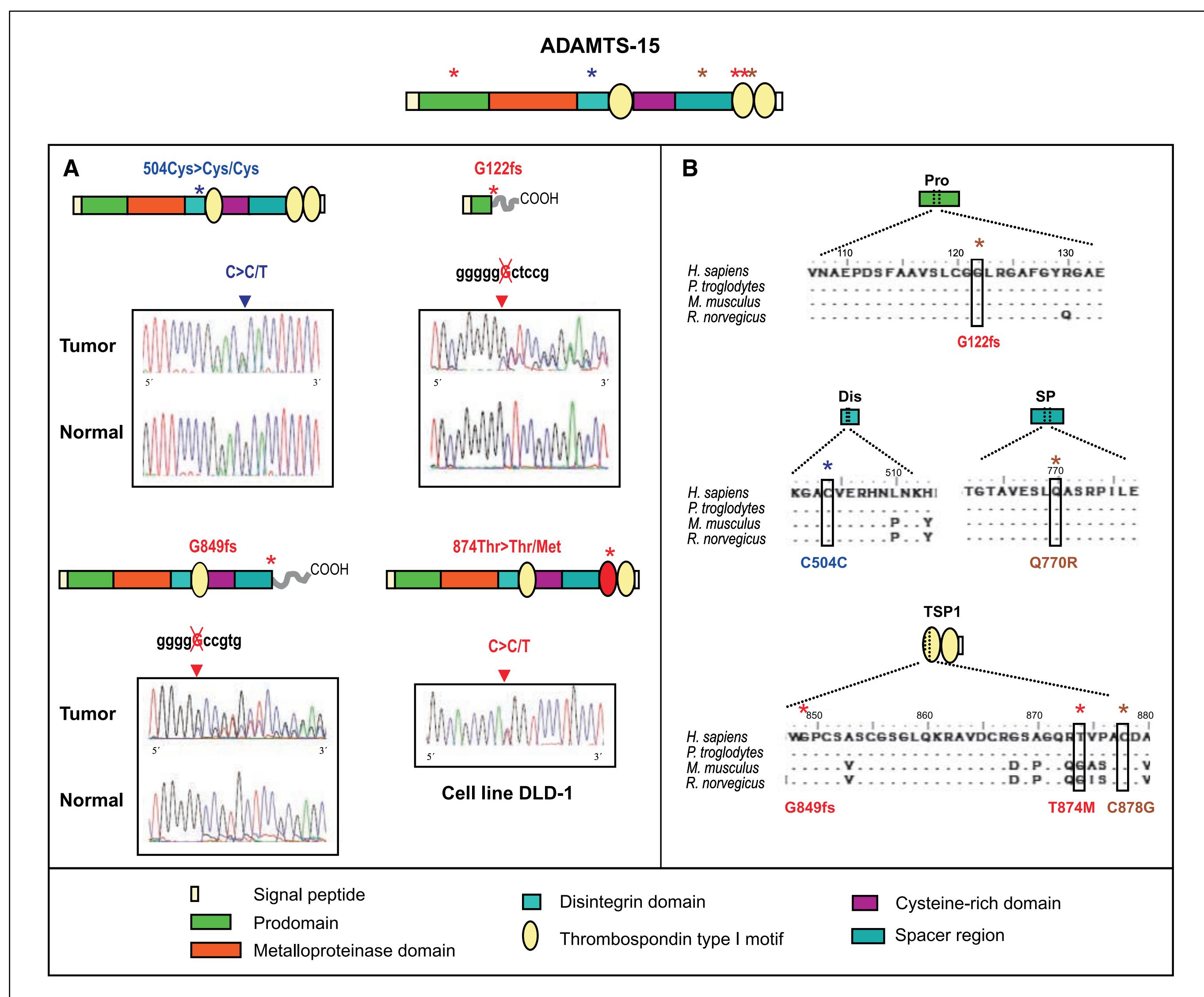

Figure 1. Somatic mutations in *ADAMTS15* gene. *A*, domain organization of ADAMTS15 and sequencing electropherograms obtained from paired normal/tumor colorectal samples and cell lines. *Arrowheads,* positions of somatic mutations; *asterisks,* nonsynonymous mutations (*red*), synonymous mutation (*blue*), and previously reported mutations in colorectal cancer (*brown*). *B*, alignment of the identified mutations with ADAMTS15 orthologues from the indicated species. Sequences are fully conserved with the exception of the indicated amino acids. Altered amino acids by somatic mutations are boxed.

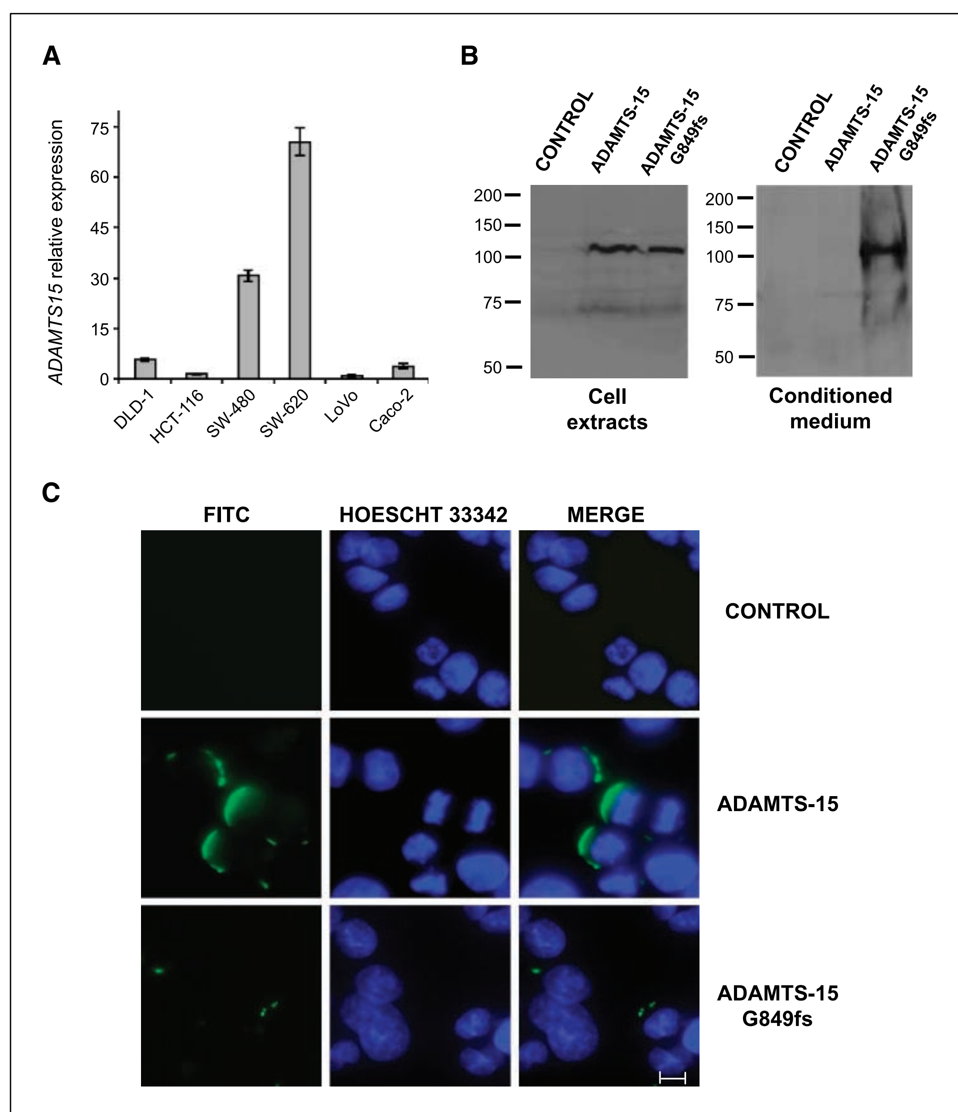

Figure 2. ADAMTS15 expression and localization in HCT-116 cells. *A,* relative expression of *ADAMTS15* determined by quantitative reverse transcription-PCR in a panel of six human colorectal cancer cell lines. *B,* detection of recombinant forms of wild-type ADAMTS15 and ADAMTS15_G848fs mutant was determined in HCT-116 lysates and in conditioned medium using the H-135 antibody. *C,* immunofluorescence analysis. The same antibody was employed to detect both wild-type and mutant ADAMTS15 in HCT-116. A secondary FITC-conjugated goat anti-rabbit antibody was used. Cell nuclei were stained with Hoechst 33342. *Bar*, 10 μm.

Colony formation assays. HCT-116 cells plated in 60 mm dishes were transfected with the wild-type or mutant *ADAMTS15*ΔG2547 expression vectors along with the corresponding control vector. Then, cells were trypsinized, plated in 6-well dishes, and cultured in medium containing 0.4 mg/mL geneticin for 15 days. SW-620 cells were also transfected with the interfering constructs along with the control vector as described above. Colonies were stained with 0.5% crystal violet in 20% methanol and counted in eight randomly selected microscopic fields.

Invasion assays. In vitro invasion potential was evaluated using 24-well Matrigel-coated invasion chambers with an 8 μm pore size (BD Biosciences). For HCT-116, 5×10^4 cells were allowed to migrate for 48 h using 5% fetal bovine serum as a chemoattractant. In the case of SW-620 cells, invasion of 1×10^5 cells was evaluated after 96 h using 2% fetal bovine serum. Cells that reached the lower surface were stained with crystal violet and counted in 10 randomly selected microscopic fields.

Western blot. Western blot analysis was done using the following primary antibodies: H-135 anti-ADAMTS15 (Santa Cruz Biotechnology), anti-p44/42 mitogen-activated protein kinase, anti-phospho-p44/42 mitogen-activated protein kinase (Cell Signaling Technology), anti-FLAG-M2 (Sigma-Aldrich), and anti-actin (Abcam). Immunoreactive bands were visualized using horseradish peroxidase-labeled secondary antibodies and chemiluminescent horseradish peroxidase substrate (Millipore). To detect recombinant proteins, the indicated cells were transfected with pCEP-*ADAMTS15*-FLAG (15), pcDNA-*ADAMTS15*ΔG2547, or the corresponding empty vector. Cell extracts along with the conditioned medium were used for Western blot analysis.

Immunostaining. Two human tissue arrays with normal and tumor colorectal samples were obtained from the Hospital Universitario Central de Asturias and used to evaluate *ADAMTS15* expression. After dewaxing and rehydrating, samples were incubated overnight with H-135 antibody at 1:200 dilution. Sections were incubated with EnVision System-labeled polymer horseradish peroxidase anti-rabbit (DAKO) followed by incubation with diaminobenzidine colorimetric reagent (DAKO) and counterstaining with hematoxylin. To detect ADAMTS15 in HCT-116 cells, immunofluorescence was carried out as described (15).

***In vivo* tumorigenicity.** Experimentation with severe combined immunodeficient mice (C.B-17/IcrCrl-scid-BR; Charles River Laboratories) followed institutional guidelines approved by the local animal review board. To induce subcutaneous tumors, two groups of six severe combined immunodeficient mice were injected at one flank with 7×10^6 cells of two different SW-620-ADAMTS15–interfered clones (sh clone2 and sh clone12). As control, the opposite flank in each animal was injected with a stable clone transfected with the pRS empty vector. Tumor growth was monitored as described (15). Histopathologic characteristics of the tumors were evaluated in H&E-stained sections.

Results

Genetic and epigenetic analysis of *ADAMTS15* in human colorectal cancer. Genomic DNA from a collection of 50 human colorectal cancer cases and 6 colon cancer cell lines was screened for mutations through the eight coding exons and intron-exon junctions of the *ADAMTS15* gene. Combining colorectal cancer samples and cell line data, four heterozygous mutations were identified, three mutations in patients and one mutation in cell lines (Fig. 1). All these changes were shown to be somatic by analysis of DNA from normal tissues of the same individuals. No correlation between the appearance of mutations and histopathologic features was observed (Supplementary Table S1). In tumors, one of these changes was a single-base substitution and the other two were single-base deletions (Fig. 1*A*). The observed nucleotide substitution consisted of a cytosine-to-thymine transition at nucleotide 13777 of the gene (exon 4), resulting in a silent mutation at codon 504. Hence, the cysteine residue encoded by this codon remains unaltered in heterozygosis (504Cys>Cys/Cys; Fig. 1*A* and *B*). The remaining changes were nonsynonymous mutations and consequently may directly affect the ADAMTS15 protein function. Thus, the coding changes corresponding to deletion 366ΔG in exon 1 (G122fs; Fig. 1*A*) would cause a frameshift generating a truncated protein of 244 amino acids (compared with 950 in the wild-type protein), only containing the signal peptide and part of the prodomain. In the case of deletion 24544ΔG (G849fs; Fig. 1*A*), it would also produce a frameshift finally leading to the production of an altered protein of 947 amino acids and lacking the last two thrombospondin-1 motifs. These deletions were heterozygously detected in the same tumor sample and may cause the loss of ADAMTS15 normal function.

Mutational analysis of colorectal cancer cell lines revealed the presence of one point mutation in DLD-1 cells, consisting of a single-base substitution 24616C>C/T (874Thr>Thr/Met) in exon 8 (Fig. 1*A* and *B*). This change would affect the first of the two contiguous thrombospondin-1 domains present at the COOH-terminal region. Interestingly, we also detected a heterozygous polymorphism in exon 4 (13589T>T/C; 442Tyr>Tyr/His) that had not been described previously. This single nucleotide polymorphism was also detected in two tumor/normal colorectal samples and in LoVo cells and affects a tyrosine residue that is conserved in the disintegrin domain of nine different analyzed species (not shown). We next evaluated the potential effect of the Thr^{874}Met mutation in metalloprotease function using the Sorting Intolerant From Tolerant algorithm (22). The normalized probability for a Thr^{874}Met substitution at the second thrombospondin-1 domain of ADAMTS15 was <0.01, thus predicting a deleterious effect. Similarly, the germ-line variant Tyr^{442}His was also predicted to be deleterious. Overall, these results indicate that, with the exception of the silent mutation C13777T (Cys^{504}Cys), the functional relevance of which is uncertain, all remaining mutations found in this mutational analysis of *ADAMTS15* in colon cancer could affect the normal function of the protein.

Additionally, we examined whether *ADAMTS15* could be epigenetically silenced through methylation. To do this, we first determined the occurrence of CpG islands in its 5′ region and performed a methylation-specific PCR amplification analysis in a variety of randomly selected colorectal tissue samples. Moreover, the *ADAMTS15* expression pattern in the same samples was analyzed. No relation between expression levels, normal or tumor status of the tissues, and promoter methylation was observed (Supplementary Table S1; Supplementary Fig. S1). Consistent with these results, *ADAMTS15* did not show the expression pattern characteristic of epigenetically silenced genes (Supplementary Fig. S1).

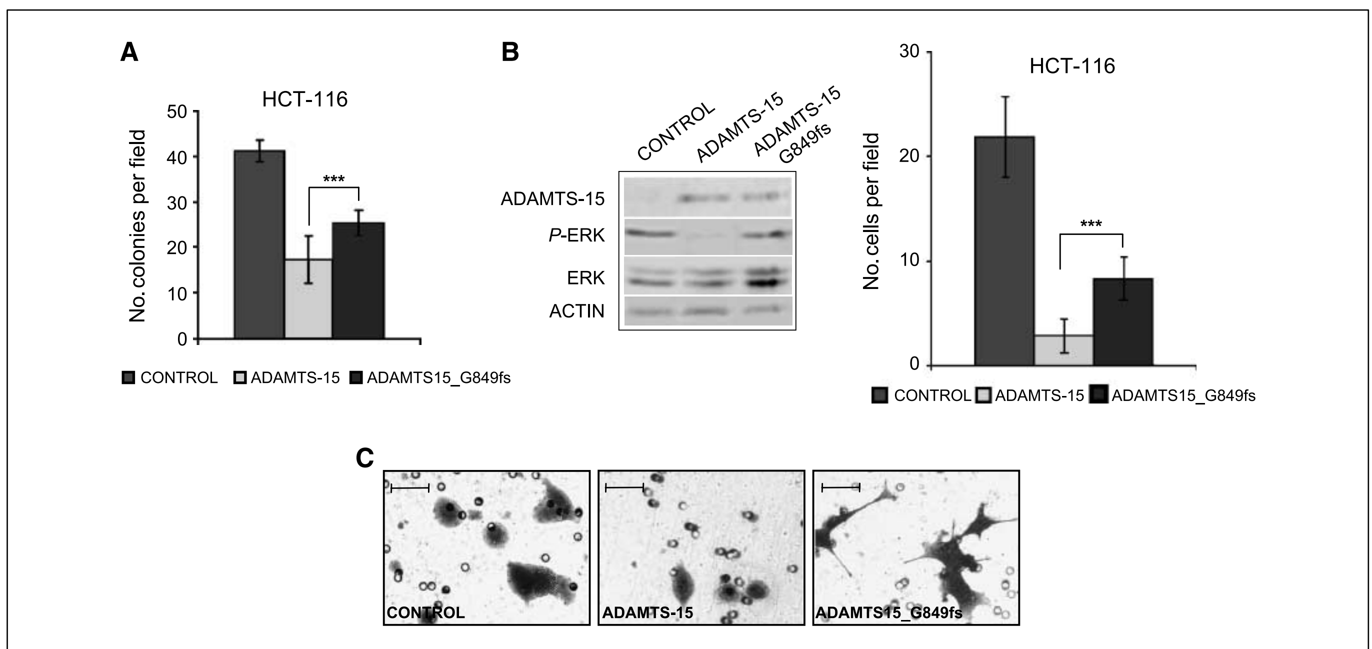

Figure 3. *ADAMTS15* expression modifies colony-forming ability and invasion properties of HCT-116 colorectal cancer cells. *A,* average colony formation after 15 days of selected HCT-116 cells expressing *ADAMTS15* and *ADAMTS15_G849fs. Bars,* SD. ***, $P < 0.005$, Student's *t* test. *B, left,* expression of *ADAMTS15* and *ADAMTS15_G849fs* in HCT-116 clones was determined by Western blot analysis; *right,* HCT-116 cells expressing *ADAMTS15* and *ADAMTS15_G849fs* were plated in Matrigel invasion chambers and the average number of invasive cells was evaluated. *Bars,* SD. ***, $P < 0.005$. Levels of ERK and phospho-ERK (*P-ERK*; *left*) were evaluated by Western blot of HCT-116 cells expressing *ADAMTS15* or *ADAMTS15_G849fs. C,* morphologic changes induced in HCT-116 *ADAMTS15* and *ADAMTS15_G849fs* cells. *Bar,* 40 μm.

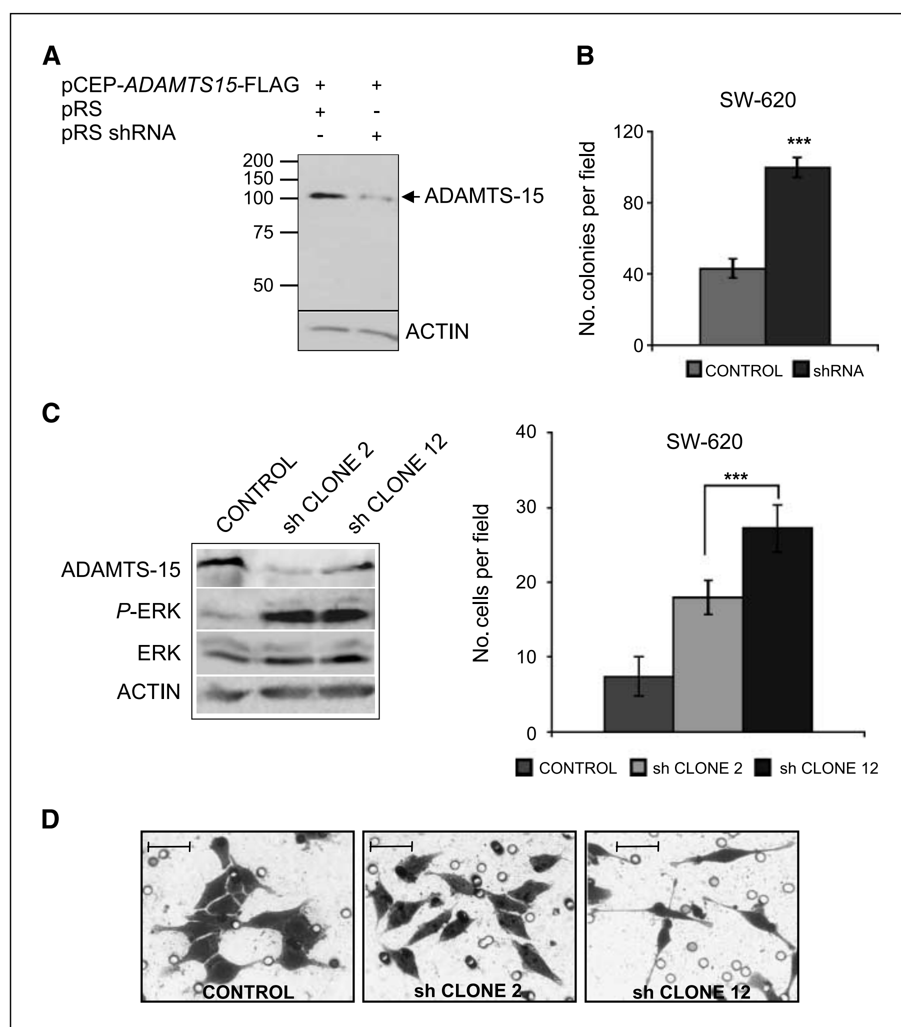

Figure 4. *ADAMTS15* down-regulation modifies colony-forming ability and invasion properties of SW-620 colorectal cancer cells. *A,* 293-EBNA cells were cotransfected with the *ADAMTS15* epitope-tagged cDNA construct (pCEP-*ADAMTS15*-FLAG) and a set of four shRNA interfering plasmids (pRS-shRNA) or with the control vector (pRS). *ADAMTS15* expression levels were evaluated by Western blot analysis. *B,* average colony formation selected ADAMTS15-interfered SW-620 cells. *Bars,* SD. ***, $P < 0.005$, Student's *t* test. *C, left, ADAMTS15* expression in two interfered SW-620 clones was determined by Western blot; *right,* ADAMTS15-interfered SW-620 cells along with control cells were plated in Matrigel invasion chambers, and the average number of invasive cells was evaluated. *Bars,* SD. ***, $P < 0.005$. Levels of ERK and phospho-ERK (*left*) were also evaluated. *D,* morphologic changes induced in ADAMTS15-interfered SW-620 cells versus control. *Bar,* 40 μm.

G849fs mutation alters the localization of ADAMTS15. To examine the functional relevance of the G849fs alteration, we constructed a vector containing the *ADAMTS15* cDNA harboring this mutation. HCT-116 cells were used for this analysis, as they do not express *ADAMTS15* (Fig. 2*A*). Western blot analysis using the H-135 antibody on whole-cell lysates identified specific bands on extracts from both ADAMTS15 and ADAMTS15_G849fs HCT-116 transfected cells (Fig. 2*B*). These bands were absent in cells transfected with an empty vector, indicating that they likely correspond to the wild-type and mutant forms of ADAMTS15, as their molecular masses agree well with those predicted for these proteins, which are very similar in size but distinct at their COOH-terminal ends (ADAMTS15, ∼103 kDa; ADAMTS15_G849fs, ∼102 kDa). However, only the mutant form can be detected in conditioned medium (Fig. 2*B*). Moreover, immunostaining of HCT-116 cells expressing intact ADAMTS15 indicated that the protease remained attached to the extracellular matrix following secretion (Fig. 2*C*). These results suggest that the thrombospondin-1 units are important to retain the full-length ADAMTS15 protein attached to the cell surface, because the absence of thrombospondin-1 domains as occurs in ADAMTS15_G849fs drives the protein to the cell medium. Similar results were obtained in 293-EBNA cells transfected with vectors expressing wild-type and mutant forms of ADAMTS15 tagged with a FLAG epitope (data not shown), thus ruling out the possibility that these changes in protease localization are cell line specific. These data indicate that different forms of ADAMTS15 may show different cellular localizations, which could result in the abrogation of the normal function of the protease or in the acquisition of novel functional properties.

***ADAMTS15* expression in colorectal cancer cell lines reduces colony formation and cell invasion.** HCT-116 cells expressing exogenous ADAMTS15 were selected to examine the ability of the enzyme to induce antitumor properties. As shown in Fig. 3*A*, ectopic expression of wild-type *ADAMTS15* strongly inhibited colony formation (58.1% colony reduction) compared with control cells, whereas expression of the ADAMTS15_G849fs form exhibited a substantially diminished inhibitory effect (38.4% colony reduction). Similar results were obtained in SW-480 colorectal cells (data not shown). Following transfection in HCT-116 cells, individual clones were isolated and screened for *ADAMTS15* expression by quantitative reverse transcription-PCR and Western blot (Fig. 3*B, left*; data not shown). Clones expressing >10-fold *ADAMTS15* and *ADAMTS15_G489fs* mRNA levels compared with control cells were selected for further analysis. As shown in Fig. 3*B* (*right*), ectopic expression of *ADAMTS15* considerably inhibited invasion of HCT-116 cells (86.8% reduction). Likewise, the mutant form of the protein conferred a diminished invasive phenotype to cells (61.6% reduction; Fig. 3*B, right*). Altogether, these findings indicate that

overexpression of ADAMTS15 protein in HCT-116 colon cancer cells results in reduction of both plating efficiency and *in vitro* invasive properties, thus reinforcing the proposed role for ADAMTS15 as a tumor-protective metalloprotease.

Then, and considering that both *Xenopus* ADAMTS1 (25) and human ADAMTS12 (15) modulate the Ras-dependent ERK pathway, we examined the levels of the phosphorylated form of ERK in HCT-116 cells expressing ectopic ADAMTS15 and ADAMTS15_G849fs. As shown in Fig. 3*B* (*left*), phospho-ERK levels were very low in cells expressing wild-type ADAMTS15. By contrast, cells expressing the mutant protein retained the ability to phosphorylate this kinase, indicating that the COOH-terminal thrombospondin-1 domains of ADAMTS15 are required for an effective inhibition of ERK activity. Moreover, a catalytically inactive ADAMTS15 also confers antitumor properties to HCT-116 (Supplementary Fig. S2), suggesting that these domains are responsible for its antitumor role. Furthermore, ADAMTS15_G849fs overexpression induced clear morphologic changes in the invasive HCT-116 cells as illustrated by the fact that ADAMTS15_G849fs-expressing cells were larger, flatter, and spindle-shaped in contrast to the smaller and round-shaped morphology of both control and *ADAMTS15*-transfected cells (Fig. 3*C*). Additionally, we observed that neither wild-type ADAMTS15 nor the mutant-expressing cells showed altered motility in wound-healing assays (not shown).

Down-regulation of *ADAMTS15* by shRNA increases clonogenicity and invasive properties of colon cancer cells. According to their significant *ADAMTS15* expression levels (Fig. 2*A*), SW-620 cells were selected to perform RNA interference experiments. To do that, four shRNA constructs were employed to target different regions of *ADAMTS15*. First, and to evaluate the effectiveness of the shRNAs, 293-EBNA cells were transfected either with the pCEP-*ADAMTS15*-FLAG and the set of four shRNA plasmids or with pCEP-*ADAMTS15*-FLAG and the pRS empty vector. Western blot analysis revealed that the immunoreactive signal corresponding to ADAMTS15 was greatly reduced in the interfered cells when compared with control cells (Fig. 4*A*). Then, we examined the functional consequences of knocking down this protease. To this end, polyclonal populations of SW-620 cells transfected with the shRNA plasmids were plated along with the control cells, and their colony-forming ability was tested 15 days later. Figure 4*B* shows that *ADAMTS15* down-regulation confers an increased clonogenicity to SW-620 colon cancer cells.

Following puromycin selection, individual clones were expanded and *ADAMTS15* expression levels analyzed by quantitative reverse transcription-PCR and Western blot (Fig. 4*C, left*; data not shown). Clones 2 and 12 showed a similar reduction in *ADAMTS15* mRNA levels (51.9% and 53.5%, respectively) and were selected to evaluate their invasive potential using Matrigel-coated invasion chambers. As shown in Fig. 4*C* (*right*), these clones exhibited an enhancement of their invasive properties compared with cells transfected with the empty vector (1.4- and 2.6-fold, respectively). Moreover, and consistent with the above findings in the HCT-116 clones expressing *ADAMTS15*, levels of phospho-ERK were significantly augmented in both clones (Fig. 4*C, left*). Furthermore, *ADAMTS15* down-regulation also induced morphologic changes, which are reminiscent of the spindle-shaped phenotype observed previously in HCT-116 cells expressing mutant ADAMTS15_G849fs (Fig. 4*D*). Collectively, these data are also consistent with the proposal that ADAMTS15 is an antitumor protease.

We next evaluated whether the observed *in vitro* functional alterations elicited by ADAMTS15 expression could also be extended to *in vivo* models. To this purpose, we examined the tumorigenic potential of ADAMTS15-depleted cells in subcutaneously injected severe combined immunodeficient mice. Consistent with the above *in vitro* data, the volume of tumors generated by ADAMTS15-interfered clones 2 and 12 versus control cells was significantly augmented over time (Fig. 5*A*). Histopathologic analysis revealed a higher percentage of necrotic regions in tumors derived from both interfered clones when compared with control tumors (Fig. 5*B*). Other histologic markers such as angiogenesis or

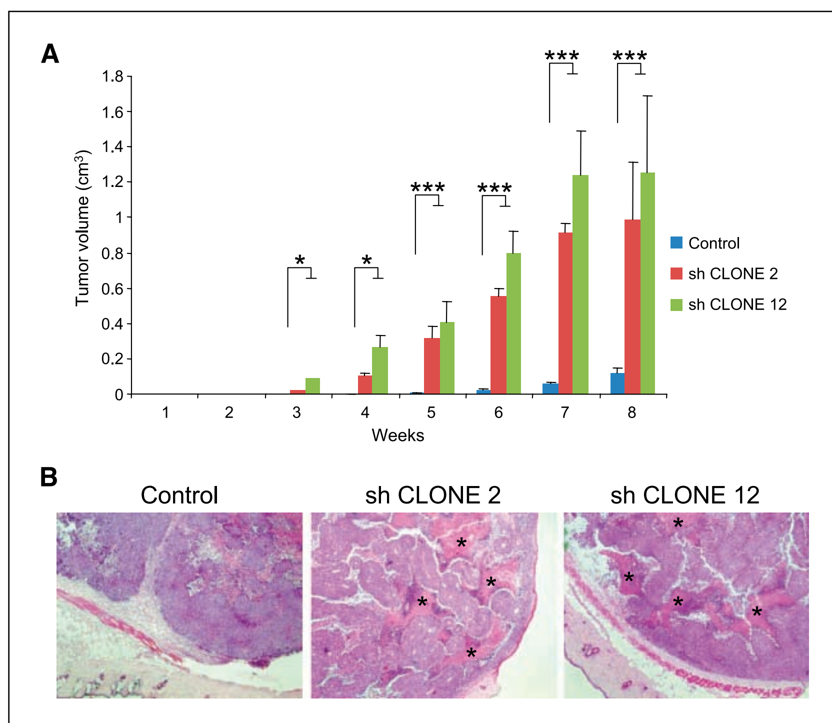

Figure 5. *In vivo* subcutaneous tumor growth of SW-620 ADAMTS15-interfered clones. *A,* severe combined immunodeficient mice were injected with control SW-620 cells or with the ADAMTS15-interfered clones (2 or 12). Tumor growth was followed during 8 wk postinjection. *Bars,* SD. *, $P < 0.05$; ***, $P < 0.005$, Student's t test. *B,* representative H&E staining of formalin/paraffin-embedded tumors obtained after 8 wk of subcutaneous growth (×20). *Asterisk,* necrotic tumor region.

local invasion showed no remarkable differences among the different tumors. In all cases, tumors were classified as poorly differentiated adenocarcinomas (Fig. 5*B*; data not shown). Collectively, these results support the conclusion that ADAMTS15 exhibits an *in vivo* antitumorigenic effect.

***ADAMTS15* expression negatively correlates with the histopathologic differentiation grade in colon adenocarcinoma.** To further evaluate the putative clinical relevance of *ADAMTS15* expression in colon cancer, we performed an immunohistochemical analysis of two tissue arrays containing normal and tumor colorectal specimens (Fig. 6). ADAMTS15 was detected in normal samples, preferentially in the apical side of the colon mucosa. However, in most analyzed samples, ADAMTS15 expression was reduced in the tumor tissue in comparison with the corresponding normal epithelia. Moreover, ADAMTS15 immunostaining inversely correlated with the histopathologic grade of colorectal tumors (Fig. 6). Thus, we evaluated *ADAMTS15* expression levels in 77 areas ranging from adenomatous polyps to different grades of colon adenocarcinoma. Immunoreactivity was scored as shown in Supplementary Table S2. The intensity of immunostaining in adenomatous polyps varied among samples: 25% of areas with an intense staining, 37.5% with a slight staining, and the remaining 37.5% areas with negative staining. A direct correlation between ADAMTS15 down-regulation and tumor development was assessed by the observation that positive ADAMTS15 staining was found in 58.3% of well-differentiated tumors, 21% of moderately differentiated tumors, and 0% of poorly differentiated colorectal carcinomas. These data suggest that ADAMTS15 could represent an effective differentiation marker for the histopathologic grading of colonic adenocarcinoma.

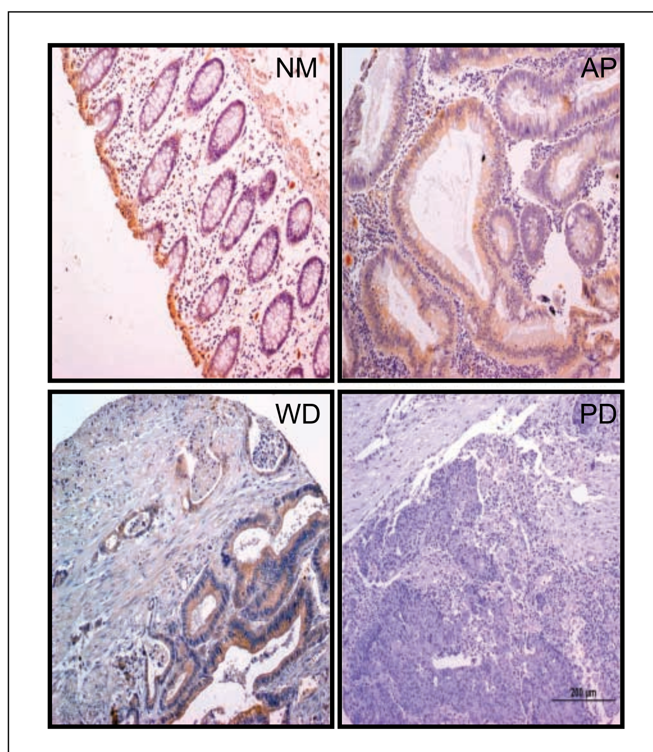

Figure 6. *ADAMTS15* expression inversely correlates with histopathologic differentiation grade in human colorectal carcinomas. Human colorectal tissue arrays were immunostained for ADAMTS15 and four representative tissue sections are shown. ADAMTS15 shows positive immunostaining in luminal epithelia of normal mucosa (*NM*). Adenomatous polyp (*AP*) also shows strong immunoreactivity to ADAMTS15. Well-differentiated adenocarcinoma (*WD*) still presents significant expression. Poorly differentiated high-grade adenocarcinoma (*PD*) does not show staining for the metalloprotease.

Discussion

In this work, we provide functional evidence that ADAMTS15 is a new enzyme in the growing category of tumor-defying proteases (7). The gene encoding this metalloprotease was originally cloned in our laboratory as part of our long-term studies aimed at identification of proteases associated with tumor progression (23). However, its precise physiologic and pathologic relevance is currently unknown. The first indication regarding a potential protective role for ADAMTS15 derived from the observation that low *ADAMTS15* expression levels coupled to high *ADAMTS8* levels conferred poor prognosis to breast cancer patients (19). Moreover, *ADAMTS15* was identified as one of the so-called CAN genes found to be mutated in a small set of colorectal cancers (20). To provide functional support to the putative relevance of ADAMTS15 as a tumor suppressor protease, we extended the mutational analysis of this metalloprotease gene to a panel of 50 colorectal cancers and 6 colon cancer cell lines. This work allowed us to identify four new mutations, validating the proposal that *ADAMTS15* is mutated in human colon carcinomas. Next, we performed different functional approaches aimed at evaluating the antitumorigenic properties of ADAMTS15 using cellular models. As a first step to this purpose, we proceeded to exogenously express *ADAMTS15* in HCT-116 cells, which led to a considerable reduction in both colony formation and invasive capacities of this cell line. Moreover, functional analysis of selected *ADAMTS15*-knockdown SW-620 clones showed an enhancement of their invasive capacities and growing ability when compared with control clones. These data provide the first functional evidence on the tumor suppressor properties of ADAMTS15.

Furthermore, we could observe in these colon cancer cell lines that defective or tumor-derived mutant ADAMTS15 invasive clones exhibited morphologic alterations characteristic of cells that have lost their epithelial features and acquired a mesenchymal phenotype. Epithelial-to-mesenchymal transition is a characteristic feature of cells undergoing invasion, which is an essential step for transformed cells to progress and metastasize in distant organs (26). In agreement with these data, we also found that the Ras/mitogen-activated protein kinase oncogenic pathway, known to induce epithelial-to-mesenchymal transition events, is modulated by ADAMTS15. Thus, we observed a marked inhibition of ERK phosphorylation in *ADAMTS15*-expressing HCT-116 clones and an activation in the *ADAMTS15* knockdown SW-620 clones. Previous studies have shown that sustained activation of Ras/mitogen-activated protein kinase pathway is involved in promoting cellular processes associated with tumor progression (27). The presence of thrombospondin-1 domains in ADAMTS15 could be responsible for switching off this pathway. In fact, ectopic expression of thrombospondin-1 markedly inhibits tumor formation (28). Moreover, expression of thrombospondin-1 is up-regulated by the tumor suppressor gene p53 and down-regulated by oncogenes such as Myc and Ras (29). Furthermore, it has been reported that both *Xenopus* ADAMTS1 and human ADAMTS12 can also inhibit this pathway (15, 25). These two ADAMTS undergo proteolytic processing events to generate fragments with prometastatic or antimetastatic activities depending on the presence of thrombospondin-1 domains in them (15, 30, 31). In fact, it has been proven

that these domains confer angio-inhibitory properties to different ADAMTS (13) and may be involved in the modulation of the ERK signaling pathway (15, 25). Interestingly, the 24544ΔG mutation identified in our *ADAMTS15* mutational analysis would result in an enzyme lacking the last two thrombospondin-1 domains. We have engineered an ADAMTS15 isoform mimicking this situation, which shows reduced invasive properties compared with the full-length protein. Moreover, cells expressing this form displayed higher levels of phosphorylated ERK than those detected in cells expressing intact ADAMTS15. These data would indicate that the thrombospondin motifs are essential for ADAMTS15 to act as a tumor suppressor protease. Furthermore, we have also found that the presence of these domains is important to immobilize the protease in the pericellular space following its secretion, which would facilitate a putative interaction of the thrombospondin-1 domains with their receptors as a mechanism to inhibit ERK activation (25). In contrast, the form lacking these domains is secreted to the cell medium, thus hindering this putative ERK-inhibitory function.

Consistent with the above data, *in vivo* experiments based on subcutaneous injection of the interfered clones in severe combined immunodeficient mice revealed that ADAMTS15 knockdown markedly promotes *in vivo* tumor growth. Additionally, we carried out an immunohistochemical analysis of ADAMTS15 in colorectal tissue samples with the finding that this metalloprotease is preferentially detected at the apical side of the colonic normal mucosa, whereas its presence shows a clear decay within the tumor tissue. Interestingly, the negative correlation between *ADAMTS15* expression and the differentiation status of the colorectal tumors agrees with the suggested protective properties of ADAMTS15 in tumors of colorectal origin.

We have also examined whether *ADAMTS15* can be an epigenetically silenced gene in colon cancer, a quite common mechanism to inactivate several tumor suppressor genes in colorectal carcinomas (32). Moreover, *ADAMTS1* and *ADAMTS18* have been found to be hypermethylated in these malignancies (16, 18). However, our results revealed that this is not the case for *ADAMTS15* because an analysis of paired normal/tumor samples and cDNAs from colon carcinomas did not show the expected expression pattern for epigenetically silenced genes. Thus, genetic mutations rather than epigenetic silencing seem to be the mechanism that contributes to *ADAMTS15* inactivation in some cases of human colorectal carcinomas. The total number of identified mutations in the *ADAMTS15* gene is relatively low but similar to that found in other genes reported as novel targets of genetic inactivation in cancer (20, 21, 33). Nevertheless, it is very interesting that mutation events may result in the inactivation of this protease gene in other human malignant tumors as assessed by the recent findings showing that *ADAMTS15* is mutated in pancreatic (33) and lung cancers,[5] although no functional analysis of these mutations has been reported yet. Additionally, it is also noteworthy that *ADAMTS15* gene is located in chromosome 11q24.3 between markers *D11S912* and *D11S969*, a region of frequent loss of heterozygosity in different tumors including colorectal cancer (34). This fact would provide a new clue on the potential relationship between loss-of-function of *ADAMTS15* and development of colorectal cancer.

In summary, the results presented in this work provide strong support to the proposal that ADAMTS15 is a metalloprotease with tumor-protective functions. This finding, together with previous observations on the antitumorigenic properties of different proteases (7), emphasizes the need to more precisely characterize the cancer degradome (35). These approaches will contribute to identify those proteolytic enzymes that constitute appropriate therapeutic targets and to distinguish them from those that confer tumor protection. Ultimately, the structural and functional discrimination between protease targets and antitargets in cancer will be very helpful to design new inhibitors aimed to specifically block the unwanted proteolytic activities associated with tumor progression.

[5] http://www.sanger.ac.uk/genetics/CGP/cosmic/

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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