Extracellular Protease ADAMTS9 Suppresses Esophageal and Nasopharyngeal Carcinoma Tumor Formation by Inhibiting Angiogenesis


Abstract

ADAMTS metallocathepsin family member ADAMTS9 maps to 3p14.2 and shows significant associations with the aerodigestive tract cancers esophageal squamous cell carcinoma (ESCC) and nasopharyngeal carcinoma (NPC). However, the functional impact of ADAMTS9 on cancer development has not been explored. In this study, we evaluated the hypothesized antiangiogenic and tumor-suppressive functions of ADAMTS9 in ESCC and NPC, in stringent tumorigenicity and Matrigel plug angiogenesis assays. ADAMTS9 activation suppressed tumor formation in nude mice. Conversely, knockdown of ADAMTS9 resulted in clones reverting to the tumorigenic phenotype of parental cells. In vivo angiogenesis assays revealed a reduction in microvessel numbers in gel plugs injected with tumor-suppressive cell transfectants. Similarly, conditioned medium from cell transfectants dramatically reduced the tube-forming capacity of human umbilical vein endothelial cells. These activities were associated with a reduction in expression levels of the proangiogenic factors MMP9 and VEGFA, which were consistently reduced in ADAMTS9 transfectants derived from both cancers. Taken together, our results indicate that ADAMTS9 contributes an important function in the tumor microenvironment that acts to inhibit angiogenesis and tumor growth in both ESCC and NPC. Cancer Res; 70(13); 5567–76. ©2010 AACR.

Introduction

The a disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motifs (ADAMTS) family consists of 19 secreted enzymes with a well-defined domain structure. These enzymes consist of a prometalloprotease domain and a characteristic ancillary domain containing one or more thrombospondin type 1 motifs (1). Through analysis of mutant mice and human genetic disorders, the roles of ADAMTS in skin pigmentation, organogenesis, limb development, connective tissue assembly, and fertility were shown (2). Moreover, altered expression of some ADAMTS genes has been shown in various cancers and arthritis (1, 2). Three ADAMTS proteases (ADAMTS1, ADAMTS8, and ADAMTS9) were previously shown to have antiangiogenic activity. ADAMTS1 and ADAMTS8 inhibited vascular endothelial growth factor (VEGF)-induced angiogenesis as assayed by the chick chorioallantoic membrane assay, suppressed fibroblast growth factor-induced vascularization in the cornea pocket assay, and inhibited endothelial cell proliferation in vitro (3). ADAMTS9 was recently shown to be a constitutive product of microvascular endothelial cells in both embryonic and adult mice and to act as a cell-autonomous angiogenesis inhibitor (4).

The ability of a tumor to progress from a nonangiogenic to an angiogenic phenotype is critical to cancer progression and is termed the “angiogenic switch” (5). Expansion of a tumor mass beyond its initial microscopic size is dependent on the recruitment of its own vascular supply, by angiogenesis and/or blood vessel cooption (6–8). Failure of a tumor to recruit new microvascular endothelial cells or to reorganize the existing surrounding vasculature results in growth-limited, nonangiogenic tumors (9). Although related matrix metalloproteases (e.g., ADAM and ADAMTS proteases) have been implicated in tumor progression and angiogenesis, the specific role of ADAMTS9 in tumor angiogenesis is less clearly defined. Our previous functional genomic studies show that ADAMTS9 is associated with tumor suppression in two aerodigestive tract cancers, namely esophageal squamous cell
carcinoma (ESCC) and nasopharyngeal carcinoma (NPC). Downregulation of ADAMTS9 expression was commonly observed in tumor tissues and cell lines of both cancers. Promoter hypermethylation contributes to ADAMTS9 gene silencing in both ESCC and NPC (10, 11). Importantly, previous studies indicate that ADAMTS9 protein expression in NPC is significantly associated with lymph node metastases (11). The role of this protein in cancer development remains unclear. In the present study, we investigated the in vivo and in vitro functional roles of ADAMTS9 in angiogenesis and ESCC and NPC tumorigenesis. Antiangiogenic and tumorsuppressive activities of ADAMTS9 were studied by stringent in vivo tumorigenicity and Matrigel plug angiogenesis assays. The effects of conditioned medium from ADAMTS9 stable transfectants were assessed in in vitro tube formation ability assays using human umbilical vein endothelial cells (HUVEC) to better understand its role in this important process.

Materials and Methods

Cell lines and culture conditions

The ESCC cell line KYSE30, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (12), and immortalized esophageal epithelial cell line NE1 were cultured as previously described (10). Stable ESCC ADAMTS9 transfectants (EC-AD clones) and pCR3.1 vector-alone control (EC-V clone) were cultured in medium containing 400 μg/mL neomycin. The recipient NPC HONE1 cell line and the previously established HONE1/chromosome 3 microcell hybrid (MCH) cell line MCH8.12 were used for the ADAMTS9 knockdown analysis. MCH8.12 contains an extra-truncated chromosome 3 (deleted at 3p24) transferred by microcell-mediated chromosome transfer to the recipient HONE1 cell; it exhibits a prolonged latency period before tumour formation. HONE1 and MCH8.12 were maintained as previously described (13). Stable ADAMTS9 knockdown clones were maintained in culture medium containing 500 μg/mL neomycin and 5 μg/mL blasticidin. The immortalized nasopharyngeal epithelial cell line NP460 was described as previously described (14). Construction of a pETE-Bsd–responsive vector and a HONE1 cell line, HONE1-2, producing the tetracycline transactivator tTA, was described by Protopopov and colleagues (15). Construction of a pETE-Bsd–responsive vector and a HONE1/chromosome 3 microcell hybrid (MCH) cell line MCH8.12 were used for the ADAMTS9 knockdown analysis. MCH8.12 contains an extra-truncated chromosome 3 (deleted at 3p24) transferred by microcell-mediated chromosome transfer to the recipient HONE1 cell; it exhibits a prolonged latency period before tumour formation. HONE1 and MCH8.12 were maintained as previously described (13). Stable ADAMTS9 knockdown clones were maintained in culture medium containing 500 μg/mL neomycin and 5 μg/mL blasticidin. The immortalized nasopharyngeal epithelial cell line NP460 was described as previously described (14). Construction of a pETE-Bsd–responsive vector and a HONE1 cell line, HONE1-2, producing the tetracycline transactivator tTA, was described by Protopopov and colleagues (15). Stable NPC transfectants with ADAMTS9 transgene (NPC-AD clones) or with pETE-Bsd vector-alone (NPC-V clone) were maintained in culture medium containing 500 μg/mL neomycin and 5 μg/mL blasticidin. HUVECs (Lonza) were cultured as previously described (16). All cultures were regularly monitored for Mycoplasma contamination and were uniformly negative.

Reverse transcription-PCR and real-time quantitative reverse transcription-PCR analyses

Semiquantitative and quantitative PCR were performed as previously reported (10, 11). Real-time quantitative PCRs were performed using ADAMTS9 and GAPDH Taqman probes or the SYBR Green PCR master mix in a StepOnePlus Real-Time PCR System (Applied Biosystems). The primers used for semiquantitative PCR are listed in Supplementary Table S1. All PCR assays were performed in triplicate in two independent experiments. For the analysis of mRNA stability of MMP9 and VEGFA, the transcription inhibitor, actinomycin D (Sigma-Aldrich, 5 μg/mL; ref. 17), was added to ADAMTS9 stable transfectants.

Western blot analysis

Western blot analysis of ADAMTS9 was performed as previously reported (18). The ADAMTS9 propeptide domain targeting antibody (Abcam) and Ab-1 (Calbiochem) were used as primary antibodies for the detection of ADAMTS9 and α-tubulin, respectively.

Stable transfection of ADAMTS9

To generate stable clones, which express wild-type ADAMTS9 in ESCC and NPC cell lines, KYSE30 and HONE1-2 cells were transfected with pCR3.1–ADAMTS9 and pETE-Bsd–ADAMTS9, respectively, as previously described (11, 18).

Knockdown of ADAMTS9 in MCH8.12 cells

ADAMTS9 knockdown was achieved by using BLOCK-iT Pol II miRNAi Expression Vector Kit (Invitrogen), and the sequences of the pair of the shRNA oligonucleotides are 5′-TGCTGTCACCAAGCCAGTTAATCGTTGTTTGCGC-3′ and 5′-CCTGTCACCAAGCCAGTTAATCGTTGTTTGCGC-3′, which target at nucleotide positions 770 to 780 of the human ADAMTS9 cDNA (NM_182920). In brief, pcDNA6.2GM-shRNA770 plasmid with the shRNA oligonucleotide or the vector-alone pcDNA6.2-GW/EmGFPMiR (pcDNA6.2GM) plasmid was stably transfected into the recipient cell line, MCH8.12, which strongly expresses ADAMTS9 (11).

Tumorigenicity assay and tumor segregant analysis

The cell lines were injected subcutaneously into three 6- to 8-week-old female athymic BALB/c Nu/Nu mice. Subcutaneous injection and preparation of tumor segregants were performed as previously described (10, 19). In brief, 5 × 106 and 1 × 107 cells were injected into both flanks of three nude mice (six sites) for each ESCC and NPC cell line, respectively. The tumor sizes were measured weekly. Tumors arising from nonsuppressing ADAMTS9 transfectants were subsequently excised and reconstituted into tissue culture. These are the tumor segregant cell lines used for further analysis. For inhibition of the tetracycline-inducible expression of ADAMTS9 in NPC transfectant cell lines in vivo, 200 μg/mL doxycycline was added to the drinking water of mice 1 week before injection; water containing doxycycline was changed twice a week.

HUVEC tube formation assay

Conditioned media were collected by incubating ADAMTS9 and vector-alone ESCC and NPC transfectant cells with Dulbecco’s modified Eagle’s medium (DMEM) without serum for 24 hours. For the NPC ADAMTS9 and vector-alone transfectants, conditioned media ± 0.2 μg/mL doxycycline...
were obtained. A total of $4 \times 10^4$ HUVEC cells were seeded into each well coated with 50 μL Matrigel (BD Biosciences) and incubated with 100 μL conditioned media from vector-alone and ADAMTS9 transfectants plus 1% fetal bovine serum. Cells were then incubated for 5 hours to allow formation of tube-like structures (16). The images at 100× magnification were captured using an inverted microscope (Nikon Instruments, Inc.). Total tube length was measured and compared for three different viewing fields by the SPOT software (Diagnostic Instruments). The primary ADAMTS9 targeting antibody (Abcam) was used as a neutralizing antibody for blocking the effects of the extracellular ADAMTS9 protein in the conditioned media. Another irrelevant rabbit polyclonal antibody was used as a negative control immunoglobulin.

**In vivo Matrigel plug angiogenesis assay**

A total of $5 \times 10^6$ ESCC cells or $1 \times 10^6$ NPC cells in 50 μL DMEM mixed with 250 μL ice-cold Matrigel (BD Biosciences) were subcutaneously injected into nude mice. Each cell line was injected into one site for five nude mice. Matrigel containing the cell suspension polymerized after injection and formed a plug impregnated with tumor cells. The gel plugs were removed after 7 days, fixed with formalin, and embedded in paraffin. Histologic sections were stained with hematoxylin and eosin and endothelial cell marker anti-CD34 monoclonal antibody (Santa Cruz Biotechnology). The slides were incubated with the anti-CD34 antibody (1:40 dilution) for immunohistochemistry as previously described (18). CD34-positive staining of vascular endothelial cells was analyzed by ImageScope v10 software (Aperio).

**Human angiogenesis antibody array**

Conditioned media were obtained as previously described. Proteins in conditioned media were hybridized with a human angiogenesis antibody array dotted with 43 human angiogenesis–related antibodies (RayBiotech). The assay was performed as described in the manufacturer’s manual.

**Gelatin zymography**

Matrix metalloproteinase 9 (MMP9) protein expression was measured by gelatin zymography and was performed as previously described (20). In brief, conditioned medium was mixed with loading buffer without β-mercaptoethanol, and loaded onto a 10% SDS-PAGE gel with 0.1% gelatin. After the samples were fractionated, the gel was washed twice with 2.5% Triton X-100 and then incubated at 37°C with reaction buffer [50 mmol/L Tris-Cl (pH 7.5), 5 mmol/L CaCl2, and 0.02% NaN3] overnight. The gel was stained with 0.1% Coomassie brilliant blue R-250 (Sigma-Aldrich). MMP9 activity was visualized as a clear band on a blue background with a size of 92 kDa. The assay was performed in three independent experiments. Quantitation of the MMP band was performed by using the Quantity One Gel Documentation System (Bio-Rad).

**Human VEGFA immunoassay**

VEGFA in conditioned media secreted by various ESCC and NPC cell lines was detected by the Quantikine Human VEGF Immunoassay system (R&D Systems). The assay was performed according to the manufacturer’s instructions. Absorbance was detected by Labsystems Multiskan MS Plate Reader (Thermo Fisher Scientific, Inc.). The assay was performed in three independent experiments.

**Statistical analysis**

Statistical analysis was performed using SPSS11.0 statistics calculation software (SPSS, Inc.). Comparisons between ADAMTS9 and vector-alone transfectants in all experiments were performed by Student’s t test. A P value of <0.05 was considered as significant.

**Results**

**Activation of ADAMTS9 expression suppresses tumor formation in vivo**

ADAMTS9 expression is downregulated in the cell lines KYSE30 and HONE1 derived from ESCC and NPC, respectively (10, 11); therefore, they were chosen for the stable ADAMTS9 transfection and the subsequent functional analyses. As shown in Fig. 1A and B, gene and protein expression of ADAMTS9 was induced in both ESCC ADAMTS9 transfectants, EC-AD7 and EC-AD9, which were analyzed by quantitative real-time PCR and Western blot analyses, respectively. ADAMTS9 expression in four NPC transfectants, NPC-AD1, NPC-AD20, NPC-AD23, and NPC-AD25, was induced in the absence of doxycycline (Fig. 1A and B). Doxycycline treatment inactivates the tTA transcriptional activator, resulting in reduction of transgene and protein expression. Reduction of ADAMTS9 transcript and protein levels was observed following doxycycline treatment in the HONE1-2 stable clones (Fig. 1A and B). High gene and protein expression levels were detected in NPC-AD1, NPC-AD20, NPC-AD23, and NPC-AD25 cell lines [without doxycycline treatment (−dox)]; ADAMTS9 levels only dropped to those observed in the vector-alone control with NPC-AD25 [with doxycycline treatment (+dox); Fig. 1A and B].

Recipient KYSE30 and HONE1 cells are highly tumorigenic and formed palpable tumors of 150 mm³ 1 week after injection in all six injection sites. When ADAMTS9 was expressed in EC-AD9 and NPC-AD25 (+dox), prolonged latency periods for tumor formation of up to 4 to 6 weeks were observed, which was significantly longer than the 1- to 2-week latency period observed with vector-alone clones (Fig. 1C; Table 1). When ADAMTS9 expression was reduced in NPC-AD25 (+dox), the average tumor size was 883 mm³, whereas when transcription was switched on, the average size was reduced to 149 mm³ 4 weeks after injection. The difference was statistically significant (P = 0.02). These results suggest that ADAMTS9 expression by tumor potently suppresses tumor formation in both ESCC and NPC in vivo.

**Loss of expression of ADAMTS9 in tumors and a tumor segregant derived from tumorigenic transfectants is associated with tumorigenicity**

In vivo tumorigenicity results showed that there is no significant tumor suppression for the other ADAMTS9 stable transfectants, EC-AD7 and NPC-AD1, NPC-AD20, and NPC-AD23.
(−dox), when compared with the vector-alone transfectants, EC-V1 and NPC-V1 (−dox; Fig. 1C; Table 1). To check the status of ADAMTS9 following tumor formation in these tumorigenic ADAMTS9 clones, total RNA was isolated from excised tumors (EC-AD7-T1-T3, NPC-AD1-T1-T6, NPC-AD20-T1-T3, NPC-AD23-T1-T6), and cells from one tumor (NPC-AD1-T6-TS) were reconstituted in culture medium. Eight primer pairs spanning the ADAMTS9 transcript were used to analyze the regional loss of ADAMTS9 gene expression in those tumors and the tumor segregants by RT-PCR of the 7,335-bp ADAMTS9 transcript. In general, the NPC-AD1 series of tumors and its tumor segregants showed an extensive deletion in the ADAMTS9 mRNA. The other tumors derived from the EC-AD7 and NPC-AD20 and NPC-AD23 showed smaller discrete deletions. RT-PCR analysis using the primers p4 and p5 consistently showed the loss of ADAMTS9 transcript in all tumors and the tumor segregant; a common minimum region of deletion was observed in the third and fourth thrombospondin type 1 repeats of ADAMTS9 (Fig. 2A). Representative RT-PCR results with and without loss of ADAMTS9 transcript regions in the selected tumors are shown in Fig. 2B. We conclude that functional inactivation of ADAMTS9 during tumorigenesis in nude mice contributes to the lack of tumor suppression of EC-AD7, NPC-AD1, NPC-AD20, and NPC-AD23.

**Tumorigenicity restored by ADAMTS9 knockdown in a microcell hybrid cell line**

To further confirm the tumor-suppressive effect of ADAMTS9, ADAMTS9 knockdown in the nontumorigenic HONE1/chromosome 3 MCH8.12, which expresses high levels of ADAMTS9 mRNA (11), was performed. Stable ADAMTS9 knockdown transfectants were obtained by transfecting pcDNA6.2GM-shRNA770 into MCH8.12. Reduction of ADAMTS9 protein expression was observed in the stable clones, 8.12-A9-shRNA770-N59 and 8.12-A9-shRNA770-N60 (Fig. 2C), when compared with the vector-alone 8.12-pcDNA-V1. The specificity of the shRNA oligonucleotide was tested; RT-PCR results show that gene expression of ADAMTS1 and ADAMTS8 was not affected in stable ADAMTS9 knockdown transfectants 8.12-A9-shRNA770-N59 and 8.12-A9-shRNA770-N60 (data not shown). Tumorigenicity was suppressed in the vector-alone clone 8.12-pcDNA-V1.
and tumors were observed at only two sites of injection for up to 8 weeks after inoculation (Fig. 2D; Table 1). Tumors appeared with both ADAMTS9 knockdown clones in at least four injection sites, which showed similar growth kinetics. The differences were statistically significant when compared with the vector-alone controls (P = 0.01 and 0.003, respectively). When compared with the tumor growth rate of HONE1 cells, the two ADAMTS9 knockdown MCHs were significantly slower (P = 0.02 and 0.016, respectively; Fig. 2D; Table 1).

ADAMTS9 reduces tube formation by HUVEC in vitro

The “angiogenic switch” is critical for solid tumor formation (5), and ADAMTS9 was shown to suppress tumor formation in ESCC and NPC in the current study. This study examines the antiangiogenic property of ADAMTS9 and how it can contribute to its tumor-suppressive function. HUVEC tube formation assay was used to test the effect of ADAMTS9 on angiogenesis in vitro. ADAMTS9 is a secreted protein associated with the cell surface, but has also been shown to be present in the conditioned medium of expressing cells (4). Concentrated conditioned media collected from both ESCC and NPC ADAMTS9 transfectants were analyzed by Western blotting to show the presence of secreted ADAMTS9 proteins compared with vector-alone transfectants, EC-V1 and NPC-V1 (Fig. 3A). Secreted ADAMTS9 protein expression was significantly reduced in NPC-AD25 (+dox; Fig. 3A). Conditioned media from EC-AD7 and EC-AD9 and NPC-AD25 (−dox) inhibited formation of tube-like structures of HUVEC cells, as compared with their respective vector-alone controls (Fig. 3B). The tube-forming ability of HUVEC cells was significantly decreased to 66.4%, 61.1%, and 61.5% after incubation with EC-AD7 and EC-AD9 and NPC-AD25 (−dox) cell conditioned media, respectively, as compared with the vector-alone controls (Fig. 3C). The inhibitory effect on tube formation was restored to control levels in NPC-AD25 (+dox) when transgene expression was repressed (Fig. 3B and C). To further validate the specific inhibitory effect of secreted ADAMTS9 protein in the conditioned media, ADAMTS9-neutralizing antibody was added together with the conditioned medium to block its activities; reduction in the activities of secreted ADAMTS9 subsequently decreased the tube-forming ability of the three ESCC/NPC ADAMTS9 transfectants (Fig. 3B and C). The ADAMTS9-neutralizing antibody had no significant effect on tube formation in the vector-alone clones (Fig. 3B and C). An irrelevant negative control antibody had no significant effect on the tube formation in the ADAMTS9 transfectants (data not shown).

ADAMTS9 inhibits in vivo angiogenesis

The antiangiogenic activity of ADAMTS9 in vivo was assessed in mice with the Matrigel plug assay. The tumor-suppressive ESCC and NPC transfectants, EC-AD9 and NPC-AD25, were used, as the ADAMTS9 transgenes of these clones are stable in vivo conditions. The anti-CD34 antibody microvesSEL–stained gel plugs were analyzed; representative results are shown in Fig. 4A and B (top panels). The numbers of microvessels of both ESCC and NPC clones, EC-AD9 and NPC-AD25 (−dox), were substantially reduced

### Table 1. Tumorigenicity assays of ADAMTS9 transfectants and ADAMTS9 knockdown MCHs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Identification</th>
<th>Dox</th>
<th>Tumor formation (no. tumors/no. sites)</th>
<th>Latency period (wk) for tumor volume of 150 mm³</th>
<th>P*115</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC-V1</td>
<td>KYSE30× pCR3.1</td>
<td>−</td>
<td>6/6</td>
<td>1–2</td>
<td>−</td>
</tr>
<tr>
<td>EC-AD7</td>
<td>KYSE30× pCR3.1-ADAMTS9</td>
<td>−</td>
<td>6/6</td>
<td>1</td>
<td>0.71*</td>
</tr>
<tr>
<td>EC-AD9</td>
<td>KYSE30× pCR3.1-ADAMTS9</td>
<td>−</td>
<td>3/6</td>
<td>4–6</td>
<td>0.002*</td>
</tr>
<tr>
<td>NPC-V1</td>
<td>HONE1-2× pETE-Bsd</td>
<td>−</td>
<td>6/6</td>
<td>1–2</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6/6</td>
<td>1–2</td>
<td>0.4†</td>
<td></td>
</tr>
<tr>
<td>NPC-AD1</td>
<td>HONE1-2× pETE-Bsd-ADAMTS9</td>
<td>−</td>
<td>6/6</td>
<td>1–2</td>
<td>0.14*</td>
</tr>
<tr>
<td>NPC-AD20</td>
<td>HONE1-2× pETE-Bsd-ADAMTS9</td>
<td>−</td>
<td>4/6</td>
<td>1–4</td>
<td>0.35*</td>
</tr>
<tr>
<td>NPC-AD23</td>
<td>HONE1-2× pETE-Bsd-ADAMTS9</td>
<td>−</td>
<td>5/6</td>
<td>1–4</td>
<td>0.23*</td>
</tr>
<tr>
<td>NPC-AD25</td>
<td>HONE1-2× pETE-Bsd-ADAMTS9</td>
<td>−</td>
<td>5/6</td>
<td>4–6</td>
<td>0.01*</td>
</tr>
<tr>
<td>HONE1</td>
<td>Parental NPC cell line</td>
<td>−</td>
<td>6/6</td>
<td>1–3</td>
<td>−</td>
</tr>
<tr>
<td>8.12-pcDNA-V1</td>
<td>8.12 × pcDNA6.2GM</td>
<td>−</td>
<td>2/6</td>
<td>NA</td>
<td>0.004‡</td>
</tr>
<tr>
<td>8.12-A9-shRNA770-N59</td>
<td>8.12 × ADAMTS9-shRNA770</td>
<td>−</td>
<td>4/6</td>
<td>4–7</td>
<td>0.01†, 0.02§</td>
</tr>
<tr>
<td>8.12-A9-shRNA770-N60</td>
<td>8.12 × ADAMTS9-shRNA770</td>
<td>−</td>
<td>4/6</td>
<td>4–6</td>
<td>0.003‡, 0.016‡</td>
</tr>
</tbody>
</table>

Abbreviations: Dox, doxycycline; NA, not applicable.

*P value obtained by comparison with EC-V1/NPC-V1 (−dox).
†P value obtained by comparison between tumor sizes ± dox.
‡P value obtained by comparison with HONE1.
§P value obtained by comparison with pcDNA6.2GM.
to 58.8% and 46.9%, respectively, of the vector-alone controls (Fig. 4A and B, bottom panels). Repression of the transgene in NPC-AD25 (+dox) restored the angiogenesis levels observed in the gel plug to that of the vector-alone control (Fig. 4B). In addition, cell necrosis was frequently observed in the central regions of tumor nodules in the gel plug of NPC-AD25 (-dox; Fig. 4C). This is possibly connected with poorer vasculature of the tumor cells in these plugs.

**Downregulation of MMP9 and VEGFA gene and protein expression in media derived from ADAMTS9 stable transfectants**

We used an antibody array containing several angiogenesis-related antibody probes to study the proteins associated with the antiangiogenic activities of ADAMTS9. Conditioned media from the ESCC ADAMTS9 and vector-alone transfectants were collected and hybridized to this antibody array to determine differential expression levels of the angiogenesis-related proteins. Downregulated expression of VEGFA, MMP9, interleukin 8 (IL-8), granulocyte macrophage colony-stimulating factor (GM-CSF), and transforming growth factor-β1 (TGF-β1) was observed in ADAMTS9 transfectants (data not shown). Real-time quantitative PCR was used to confirm their expression in both ESCC and NPC transfectants, EC-AD7 and EC-AD9 and NPC-AD1, NPC-AD20, NPC-AD23, and NPC-AD25. MMP9 gene expression was consistently decreased in all ADAMTS9-expressing ESCC and NPC clones (Fig. 5A). After transgene repression in NPC clones, MMP9 gene expression was higher in all four NPC transfectants (+dox), as compared with the clones expressing ADAMTS9 (-dox). We further used gelatin zymography to detect and quantify the MMP9 protein in conditioned media. Strong MMP9 signals were observed in the vector-alone EC-V1 and NPC-V1 (Fig. 5B). When ADAMTS9 was overexpressed in EC-AD9 and NPC-AD25 (-dox), MMP9 signals were significantly reduced. MMP9 activity was restored when ADAMTS9 expression was suppressed in NPC-AD25 (+dox).

On the other hand, real-time PCR results show that VEGFA-189 gene expression decreased in the two ESCC...
clones (EC-AD7 and EC-AD9) and three of four NPC clones (NPC-AD1, NPC-AD23, and NPC-AD25; Fig. 5A). The other angiogenesis-related genes, IL-8, TGF-\(\beta_1\), and GM-CSF, were less consistently downregulated in the ESCC and NPC clones (data not shown). To quantitate VEGFA protein expression, we used enzyme-linked immunosorbent assay (ELISA) to detect VEGFA secreted by ADAMTS9-overexpressing transfectants in the conditioned media. Figure 5C shows that VEGFA protein expression was reduced to 55% and 17% in EC-AD9 and NPC-AD25 (−dox), as compared with their vector-alone controls, respectively. VEGFA protein expression was restored in NPC-AD25 (+dox) when the ADAMTS9 gene expression was switched off.

To investigate the regulatory mechanism of MMP9 and VEGF by ADAMTS9, NPC-AD25 was treated with actinomycin D for 24 hours to determine whether transcription or mRNA stability of MMP9 and VEGF-189 would be affected. Figure 5D shows that the MMP9 and VEGF-189 transcripts were still stable after treatment with actinomycin D. The increased expression of MMP9 and VEGF-189 after the addition of doxycycline was reduced to less than basal levels when the cell line was treated with actinomycin D. Hence, it is likely that transcription of both MMP9 and VEGFA-189, and not their stability, was regulated by ADAMTS9.

**Discussion**

Functional analyses of ADAMTS9 strongly support its important role in vivo and in vitro. Using inducible and constitutive gene expression systems, in vivo and in vitro angiogenesis and tumorigenicity assays clearly show that the overexpression of ADAMTS9 is sufficient to induce potent suppression of tumor formation and angiogenesis in both ESCC and NPC. Importantly, by knockdown of ADAMTS9 expression in a nontumorigenic MCH, we show that ADAMTS9 knockdown clones revert to the tumorigenic phenotype of parental cells. Gene and protein expression analyses of ADAMTS9 transfectants revealed that its reduced expression is associated with the transcriptional regulation of proangiogenic factors, MMP9 and VEGFA, in both ESCC and NPC. Hence, the data strongly suggest that ADAMTS9 plays a critical role in the angiogenic switch and transforms both ESCC and NPC cell lines from a proangiogenic to a nonangiogenic phenotype. Inhibition of tumor angiogenesis is a common mechanism for tumor suppression in both tumor types. Based on the deletion patterns of the ADAMTS9 transcript in tumors and a tumor segregant derived from the tumorigenic transfectants, it is tempting to speculate that the tumor-suppressive activity of ADAMTS9 in ESCC and NPC...
is associated with the thrombospondin (TSP) domains in the COOH-terminal region of the gene.

Our findings strongly suggest that the antiangiogenic activities of ADAMTS9 play the critical role of tumor suppression in both ESCC and NPC. These findings are consistent with recent findings in which the ADAMTS9 protein expressed by the microvascular endothelial cells was shown to be antiangiogenic in both the \( \text{Adamts}^{9+/-} \) mice and the siRNA knockdown of cultured human microvascular endothelial cells. In contrast, unlike ADAMTS1, which exhibits its antiangiogenic effects by cleavage of TSPs and sequestration of VEGFA-165, ADAMTS9 neither cleaves TSP-1 and TSP-2, nor binds VEGFA-165 (4). A key conclusion of those studies was the cell-autonomous effect of ADAMTS9 in endothelial cells. The present studies highlight a non-cell autonomous mechanism by which ADAMTS9 produced by tumor cells has an effect on angiogenesis; thus, ADAMTS9 may act on endothelial cells through a dual mechanism.

For the tumorigenic ADAMTS9 transfectants, EC-AD7, NPC-AD1, NPC-AD20, and AD-23 (−dox), tumors formed 1 week after injection. After inoculation of these clones in nude mice, both reduction and loss of the region encoding the third and the fourth thrombospondin type 1 repeats were observed by RT-PCR analysis of mRNA in all tumors and the representative tumor segregant derived from tumorigenic clones. This kind of functional inactivation due to elimination of transgene overexpression was observed in our previous studies of the tumor-suppressor gene cell adhesion molecule 1 (CADM1, formerly called TSLC1) and THY1 in NPC (19, 21). The in vitro angiogenesis assay results show that EC-AD7 could significantly suppress tube formation of HUVEC cells, as the ADAMTS9 transgene is stable and remains activated in vitro conditions.

The present functional studies were also performed using the stable NPC MCH8.12 ADAMTS9 knockdown clones. Both clones showing reduced ADAMTS9 expression clearly reverted back to their tumorigenic phenotype. However, because tumor growth kinetics was still lower than that of the original recipient HONE1 cells, it is possible that other growth-inhibitory gene(s) besides ADAMTS9 might still be present on chromosome 3. Previous studies indicate that PTPRG and RASSFIA and BLU, at the nearby 3p14-21 and 3p21.3 regions, respectively, are identified as tumor-suppressor genes in NPC (22–25).

Except for the initial association of microvascular endothelial cells expressing ADAMTS9 with angiogenesis (4), the antiangiogenic activities of ADAMTS9 in human cancer cells have yet to be reported. The results of these studies thus provide clear evidence for the importance of ADAMTS9 in tumor suppression.

**Figure 4.** Representative results from the Matrigel plug assay in ESCC (A) and NPC (B). Endothelial cells were stained with anti-CD34 antibody, as indicated by arrows. CD34 staining index of ADAMTS9 transfectants as compared with their corresponding vector-alone controls. *, \( P < 0.05 \), statistically significant difference from the vector-alone clone. C, representative image of cell necrosis (acellular areas indicated by *) observed in the center region of tumor nodules formed by the NPC ADAMTS9 transfectant NPC-AD25 clone (−dox). H&E, hematoxylin and eosin.
of ADAMTS9 in angiogenesis and tumor development in two important malignancies, ESCC and NPC. Our results suggest that ADAMTS9 inhibition of angiogenesis in both cancers is associated with reduction of gene expression levels of the proangiogenic factors MMP9 and VEGFA. In future studies, it will be important to define the precise relationship between ADAMTS9 and these two key regulators of angiogenesis.

Figure 5. A, real-time quantitative PCR analysis of MMP9 and VEGFA-189 in ESCC and NPC ADAMTS9 transfectants. Fold change of the relative gene expression of MMP9 and VEGFA-189 of each clone was compared with their corresponding vector-alone transfectants. Relative ddCt of the ADAMTS9 gene expression of each clone was compared with that of the corresponding vector-alone transfectants. B, zymography of conditioned media from ESCC and NPC ADAMTS9 stable transfectants. Average MMP9 activities were calculated from three independent experiments. Fold change of MMP9 activities of each clone was compared with that of the vector-alone controls. Representative zymography results are shown on the right. MMP9 and MMP2 are indicated. C, quantitative analysis of human VEGFA in conditioned media by ELISA. Relative VEGFA expression in conditioned medium from ADAMTS9 stable transfectants was compared with their vector-alone clone. *, P < 0.05, statistically significant difference from the vector-alone clone. D, real-time quantitative PCR analysis of MMP9 and VEGFA-189 in NPC-AD25 (±dox) after actinomycin D treatment. Fold change of the relative gene expression of MMP9 and VEGFA-189 in each of the treated cells was compared with that of the untreated cells.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support


The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 12/11/2009; revised 04/14/2010; accepted 04/28/2010; published OnlineFirst 06/15/2010.

References

Extracellular Protease ADAMTS9 Suppresses Esophageal and Nasopharyngeal Carcinoma Tumor Formation by Inhibiting Angiogenesis


Cancer Res 2010;70:5567-5576. Published OnlineFirst June 15, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-4510

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/06/22/0008-5472.CAN-09-4510.DC1

Cited articles
This article cites 25 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/13/5567.full.html#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
/content/70/13/5567.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.