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ORIGINAL ARTICLE

ADAMTS9 is a functional tumor suppressor through inhibiting AKT/mTOR pathway and associated with poor survival in gastric cancer

W Du[1,2,7], S Wang[1,7], Q Zhou[3], X Li[1], J Chu[4], Z Chang[5], Q Tao[3], EKW Ng[6], J Fang[2], JJY Sung[1] and J Yu[1]

Using genome-wide promoter methylation analysis, we identified *a disintegrin-like and metalloprotease with thrombospondin type 1 motif 9 (ADAMTS9)* is methylated in cancer. We aim to clarify its epigenetic inactivation, biological function and clinical implication in gastric cancer. ADAMTS9 was silenced in 6 out of 8 gastric cancer cell lines. The loss of *ADAMTS9* expression was regulated by promoter hypermethylation and could be restored by demethylation agent. Ectopic expression of ADAMTS9 in gastric cancer cell lines (AGS, BGC823) inhibited cell growth curve in both the cell lines ($P<0.0001$), suppressed colony formation ($P<0.01$) and induced apoptosis ($P<0.001$ in AGS, $P<0.01$ in BGC823). Moreover, conditioned culture medium from ADAMTS9-transfected cell lines significantly disrupted the human umbilical vein endothelial cell tube formation capacity on Matrigel ($P<0.01$ in AGS, $P<0.001$ in BGC823). The *in vivo* growth of ADAMTS9 cells in nude mice was also markedly diminished after stable expression of ADAMTS9 ($P<0.001$). On the other hand, ADAMTS9 knockdown promoted cell proliferation ($P<0.001$). We further revealed that ADAMTS9 inhibited tumor growth by blocking activation of Akt and its downstream target the mammalian target of rapamycin (mTOR). ADAMTS9 also reduced phosphorylation of mTOR downstream targets p70 ribosomal S6 kinase, eIF4E-binding protein and downregulated hypoxia-inducible factor-1α. Therefore, this is the first demonstration that ADAMTS9 is a critical tumor suppressor of gastric cancer progression at least in part through suppression of oncogenic AKT/mTOR signaling. Moreover, promoter methylation of *ADAMTS9* was detected in 29.2% (21/72) of primary gastric tumors. Multivariate analysis showed that patients with *ADAMTS9* methylation had a poorer overall survival (relative risk (RR) = 2.788; 95% confidence interval, 1.474–5.274; $P=0.002$). Kaplan–Meier survival curves showed that *ADAMTS9* methylation was significantly associated with shortened survival in gastric cancer patients ($P=0.001$, log-rank test). In conclusion, ADAMTS9 acts as a functional tumor suppressor in gastric cancer through inhibiting oncogenic AKT/mTOR signaling pathway. Methylation of *ADAMTS9* is an independent prognostic factor of gastric cancer.

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Keywords: ADAMTS9; tumor suppressor; AKT/mTOR pathway; gastric cancer; prognosis

INTRODUCTION

Gastric cancer is a major cause of cancer-related death worldwide. However, the biological and molecular mechanisms underlying gastric cancer development remain largely unclear. Inactivation of tumor-related genes through hypermethylation of CpG islands (CpG islands are defined as sequence ranges where the Obs/Exp value is greater than 0.6 and the GC content is greater than 50%) in the promoter region has an important role in the development of gastric cancer.[1–3] Using promoter methylation array analysis,[4,5] we have recently identified a novel preferentially methylated gene, *a disintegrin-like and metalloprotease with thrombospondin type 1 motif 9 (ADAMTS9)*, in gastric cancer. The identification of novel gene silenced by promoter methylation may provide a new insight to understand the molecular mechanisms of gastric cancer and to determine potential diagnostic and therapeutic target for gastric cancer.

ADAMTS9 is one of the ADAMTS protease family members and is a secreted mammalian metalloprotease that localizes to the cell-surface and/or extracellular matrix.[6] Genetic and epigenetic analyses have supported that ADAMTS9 acts as antitumor protease in esophageal squamous cell carcinoma and nasopharyngeal carcinoma.[7,8] However, the role and the clinical implication of *ADAMTS9* in gastric cancer remain unclear. In this study, the epigenetic regulation, biological function, molecular basis and clinic application of *ADAMTS9* in gastric cancer were examined.

RESULTS

Transcriptional silence of *ADAMTS9* in gastric cancer cell lines

ADAMTS9 was widely expressed in many normal adult tissues and fetal tissues.[9,10] Stronger expression of ADAMTS9 was observed in

[1]Institute of Digestive Disease and Department of Medicine and Therapeutics, Li Ka Shing Institute of Health Sciences, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong; [2]Division of Gastroenterology, Shanghai Jiao-Tong University School of Medicine Renji Hospital, Shanghai Institution of Digestive Disease; Key Laboratory of Gastroenterology & Hepatology, Ministry of Health; State Key Laboratory of Oncogene and Related Genes, Shanghai, China; [3]Department of Clinical Oncology, The Chinese University of Hong Kong, Shatin, Hong Kong; [4]Center for Cancer and Inflammation Research, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong; [5]School of Medicine, Department of Biological Sciences and Biotechnology, State Key Laboratory of Biomembrane and Membrane Biotechnology, Tsinghua University, Beijing, China and [6]Department of Surgery, The Chinese University of Hong Kong, Shatin, Hong Kong. Correspondence: Professor J Yu, Institute of Digestive Disease and Department of Medicine and Therapeutics, Li Ka Shing Institute of Health Sciences, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, NT, Hong Kong or Professor J Fang, Division of Gastroenterology, Renji Hospital, Shanghai Jiao-Tong University, Shanghai, China.
E-mail: junyu@cuhk.edu.hk or jingyuanfang@yahoo.com
[7]These authors contributed equally to this work.
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stomach during mouse embryo development.[11] Our reverse-transcription–PCR result showed that *ADAMTS9* was expressed in human normal gastric tissue and normal human gastric epithelial cell line (GES1) (Figure 1a). However, *ADAMTS9* was silenced or downregulated in 6 out of 8 (75%) gastric cancer cell lines (Figure 1a). We next explored the role of promoter methylation in silencing *ADAMTS9* by methylation specific PCR. Full or partial methylation was detected in six gastric cancer cell lines (BGC823, MGC803, AGS, SNU719, YCCEL1 and Kato III), which showed silenced or downregulated ADAMTS9 expression, whereas methylation was not detected in the cell lines (MKN28 and MKN45) with ADAMTS9 expression (Figure 1a). To confirm whether promoter methylation mediates ADAMTS9 silencing, four methylated cell lines that showed silencing of ADAMTS9 were treated with demethylation agent 5-Aza-2′-deoxycytidine. This treatment restored ADAMTS9 expression in all of them (Figure 1b). The methylation density within the *ADAMTS9* promoter region was then characterized and validated by high-resolution bisulfite genomic sequencing. The bisulfite genomic sequencing results were consistent with those of methylation specific PCR in which dense methylation was found in methylated cell lines (AGS, BGC823, SNU719 and MGC803), but not in unmethylated MKN28, MKN45 and normal gastric tissues (Figure 1c). These results indicated that promoter methylation was associated with the transcriptional silence of *ADAMTS9* in gastric cancer cells.

ADAMTS9 inhibits gastric cancer cell growth

To elucidate the functional significance of ADAMTS9 in gastric cancer, we examined the growth inhibitory effect through ectopic expression of ADAMTS9 in silenced gastric cancer cell lines AGS and BGC823. Restored expression of ADAMTS9 was evidenced by reverse-transcription–PCR and western blot (Figure 2a), which dramatically suppressed cell growth curve in both the cell lines ($P<0.0001$) (Figure 2b). The inhibitory effect on cell growth was further confirmed by colony formation assay that ADAMTS9 inhibited the number of colonies in AGS and BGC823 ($P<0.0001$) (Figure 2c). Moreover, ADAMTS9 reduced protein expression of proliferation marker proliferating cell nuclear antigen, a marker of cell proliferation (Figure 2d). On the other hand, two independent small interfering (siRNA) mediated knockdown of ADAMTS9 in normal GES1 significantly promoted cell growth ($P<0.001$) (Figure 2e).

ADAMTS9 inhibits tumor growth in nude mice

We further tested whether ADAMTS9 could suppress the growth of gastric cancer cells *in vivo*. The subcutaneous tumor growth of BGC823 transfected with ADAMTS9 or empty vector in nude mice is shown in Figure 2f. The tumor growth was significantly lower in ADAMTS9-transfected nude mice as compared with the vector control mice ($P<0.001$), inferring that ADAMTS9 does function as a tumor suppressor in gastric carcinogenesis.

ADAMTS9 induces apoptosis

To explore the mechanism of tumor suppression by ADAMTS9, we performed an apoptosis assay using Annexin V-APC (BD Biosciences, Bedford, MA, USA) and 7-amino-actinomycin double staining (Figure 3a). Ectopic expression of ADAMTS9 resulted in a significant increase in apoptotic cells as compared with vector control both in AGS ($P<0.001$) and in BGC823 ($P<0.01$) cells (Figure 3b). Induction of apoptosis was further evidenced by the enhanced expression of cleavage poly-(ADP-ribose) polymerase, a key apoptosis-related factor, in ADAMTS9-transfected cells (Figure 3c).

ADAMTS9 reduces angiogenesis *in vitro*

ADAMTS9 was reported to be an antiangiogenic metalloprotease and its activation suppressed esophageal cancer and nasopharyngeal carcinoma formation by inhibiting angiogenesis.[8,12] We thus speculated that ADAMTS9 might inhibit gastric carcinogenesis at least in part through inhibiting angiogenesis. Human umbilical vein endothelial cell (HUVEC) tube formation assay was performed to test the effect of ADAMTS9 on angiogenesis of gastric cancer cells *in vitro*. As shown in Figure 4a conditioned culture medium from ADAMTS9-transfected AGS and BGC823 cells significantly reduced the tube-forming capacity of HUVEC on Matrigel. Reduction of angiogenesis was further confirmed by the analysis of the expression of key angiogenesis-related factor vascular endothelial growth factor A (VEGFA) that ADAMTS9 significantly inhibited the mRNA and protein expression of VEGFA in both the cell lines ($P<0.001$) (Figure 4b).

ADAMTS9 inhibits AKT/the mammalian target of rapamycin (mTOR) signaling pathway

To elucidate the molecular mechanisms modulated by ADAMTS9 in tumor inhibition, we elucidated the downstream signaling

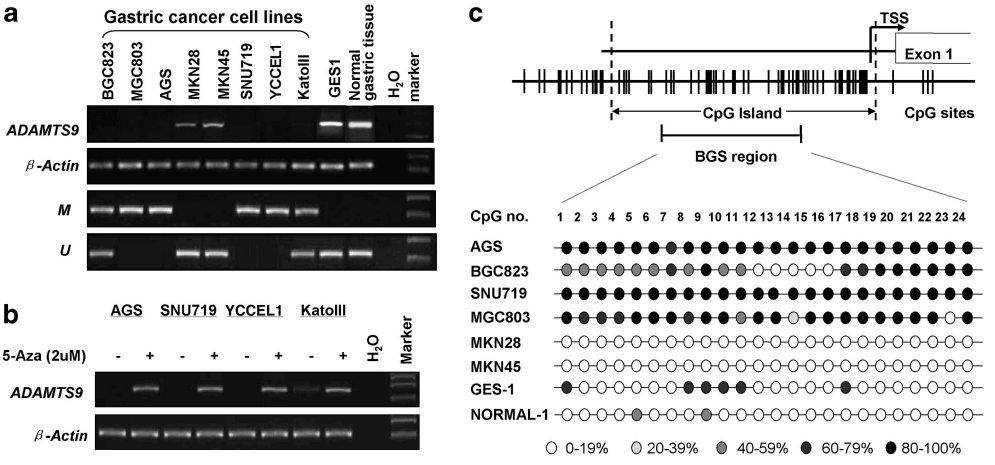

Figure 1. ADAMTS9 was inactivated by promoter methylation in gastric cancer. (**a**) *ADAMTS9* was frequently silenced or reduced in gastric cancer cell lines by promoter methylation. GES1, normal gastric epithelial cell line. (**b**) The mRNA expression of *ADAMTS9* was restored after treatment with demethylation agent 5-Aza-2′-deoxycytidine. (**c**) Methylation status of the *ADAMTS9* promoter was confirmed by bisulfite genomic sequencing.

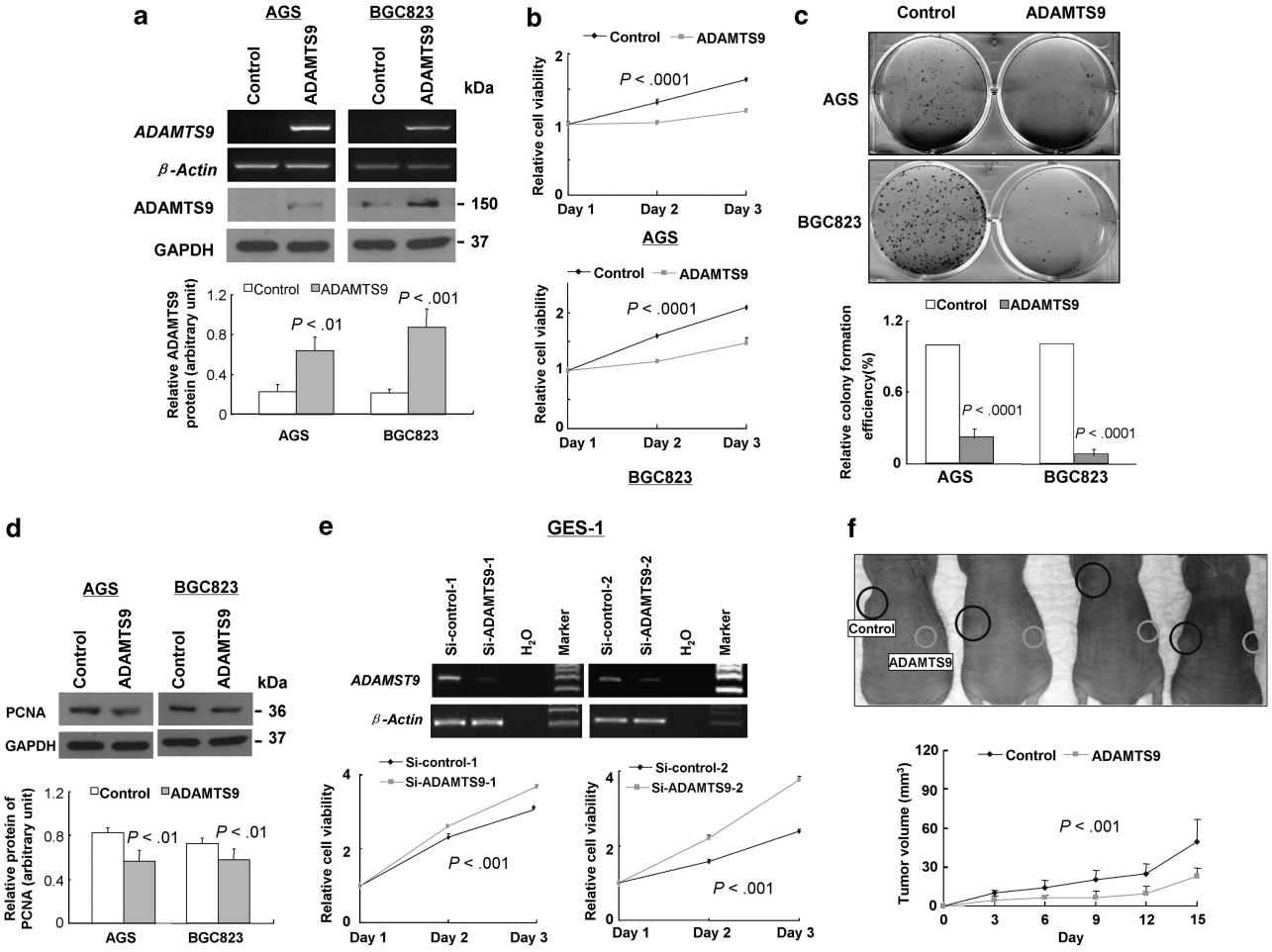

Figure 2. Effect of ectopic ADAMTS9 expression on tumor growth. (**a**) Ectopic expression of ADAMTS9 in gastric cancer cell lines (AGS and BGC823) stably transfected with ADAMTS9 was confirmed by reverse-transcription–PCR and western blot. (**b**) Cell growth curve was inhibited by ADAMTS9 in AGS and BGC823 cells. (**c**) Re-expression of ADAMTS9-suppressed colony formation. (**d**) Re-expression of ADAMTS9 suppresed the expression of proliferating cell nuclear antigen protein in AGS and BGC823 cells. (**e**) ADAMTS9 knockdown by si-ADAMTS9 increased cell growth ability in a normal GES1. (**f**) ADAMTS9 inhibited tumor growth in nude mice subcutaneously inoculated with BGC823/ADAMTS9 compared with BGC823/vector *in vivo*. A full colour version of this figure is available at the *Oncogene* journal online.

pathways modulated by ADAMTS9 through screening three key cancer-related signaling pathways (phosphor-STAT3, phosphor-Erk1/2 and phosphor-AKT/mTOR) by western blot. We found that oncogenic signaling phospho-AKT and its downstream phosphor-mTOR were downregulated after re-expression ADAMTS9 in both AGS and BGC823 cells (Figure 5a), while the other two pathways (phosphor-STAT3 and phosphor-Erk1/2) remained unchanged (Supplementary Figure 1). Accordingly, AKT/mTOR signaling downstream events, phosphor-p70S6K and phosphor-eIF4E-binding protein (4E-BP1) were also inhibited in ADAMTS9 expressed cells (Figure 5a), both of which affect mRNA translation initiation and elongation and control the rate of protein synthesis.[13] As AKT/mTOR signaling promotes the translation of oncogenes through p70S6K and 4E-BP1,[13] the protein expression of the well-established downstream effectors of p70S6K and 4E-BP1 was examined. Our results showed that cell proliferation regulators including cyclin D1, cyclin-dependent kinase 4 and c-Myc, and antiapoptotic B-cell CLL/lymphoma 2 (Bcl-2) were downregulated in ADAMTS9-transfected AGS and BGC823 as compared with the vector-transfected controls (Figure 5a). On the other hand, two independent siRNA-mediated knockdown of ADAMTS9 in normal gastric epithelial GES1 cells significantly enhanced the expression of phospho-AKT, phospho-mTOR, phospho-p70S6K and phospho-4E-BP1 (Figure 5b).

Hypoxia-inducible factor 1α (HIF1α) is another critical effector in mTOR-drived tumorigenesis for stimulating angiogenesis and glycolysis.[13–16] The expression of HIF1α was downregulated after re-expression of ADAMTS9 ($P<0.01$) (Figure 5c). This was further proved by decreased level of HIF1α downstream angiogenic VEGFA by ADAMTS9 transfection (Figure 4b). Our data also showed that HIF1α downstream target glycolysis-related genes glucose transporter 1 ($P<0.001$), phospho-glycerate kinase 1 ($P<0.01$) and hexokinase 2 ($P<0.05$) were significantly reduced in ADAMTS9 expressed BGC823 cells (Figure 5d).

Promoter methylation of *ADAMTS9* is associated with poor survival of gastric cancer patients

The clinical application of *ADAMTS9* methylation was evaluated in 72 primary gastric cancers and in 20 healthy gastric tissue samples. Among 72 gastric cancer cases, partial and dense promoter methylation of *ADAMTS9* was detected in 29.2% (21/72) cases, but none in 20 healthy gastric tissue samples (Figure 6a). There was no correlation between *ADAMTS9* methylation and clinicopathologic features such as age, gender, tumor location, tumor staging, histologic type and pathologic stage, except *H. pylori* infection ($P<0.05$) (Supplementary Table 1).

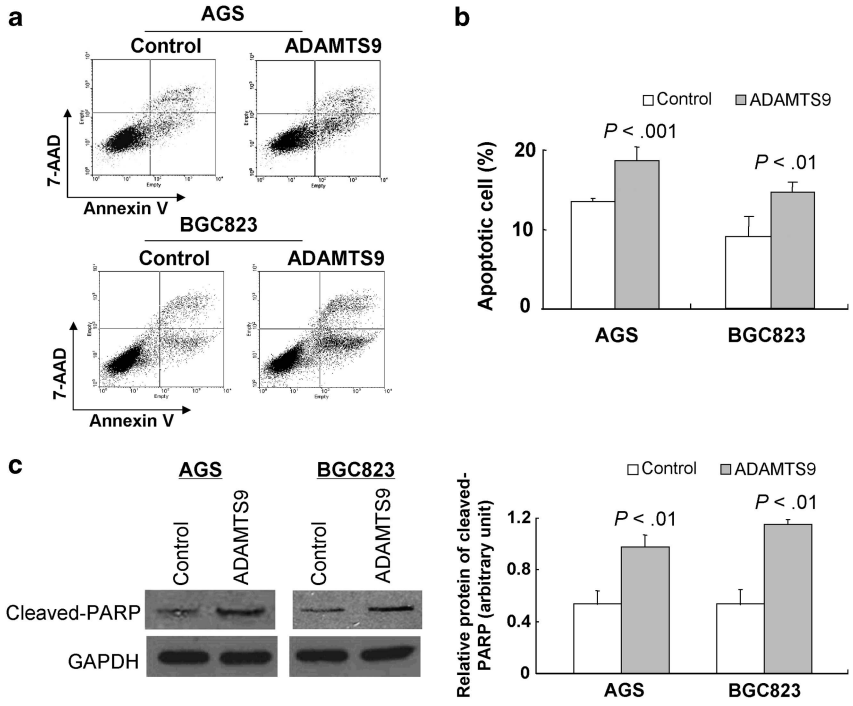

Figure 3. ADAMTS9 induced apoptosis of gastric cancer cells. (**a**) Cell apoptosis was examined by flow cytometry analysis of Annexin V-APC and 7-amino-actinomycin double-staining. (**b**) ADAMTS9 induced cell apoptosis in AGS and BGC823 cells. (**c**) ADAMTS9 induced protein expression of cleaved poly-(ADP-ribose) polymerase in AGS and BGC823 cells by western blot.

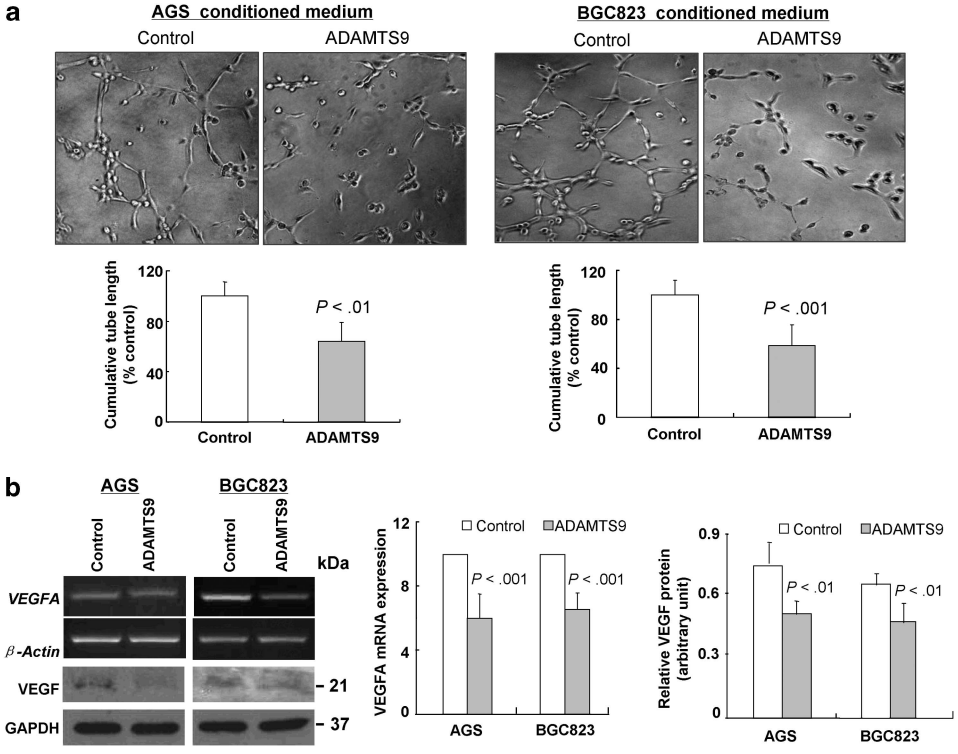

Figure 4. ADAMTS9-suppressed angiogenesis *in vitro*. (**a**) Conditioned media from ADAMTS9-expressing cells suppressed HUVEC tube formation in AGS and BGC823 cells. (**b**) ADAMTS9 inhibited mRNA expression and protein expression of VEGFA by semiquantitative PCR, real-time PCR and western blot. The intensity of bands of western blot were quantified by scanning densitometry.

In univariate Cox regression analysis (Supplementary Table 2), *ADAMTS9* methylation in tumor tissues was associated with an increased risk of cancer-related death (RR = 1.936; 95% confidence interval, 1.123–3.337; $P < 0.017$). In the multivariate model (Table 1), after the adjustment for potential confounding factors, ADAMTS9 methylation in tumor tissues was found to predict

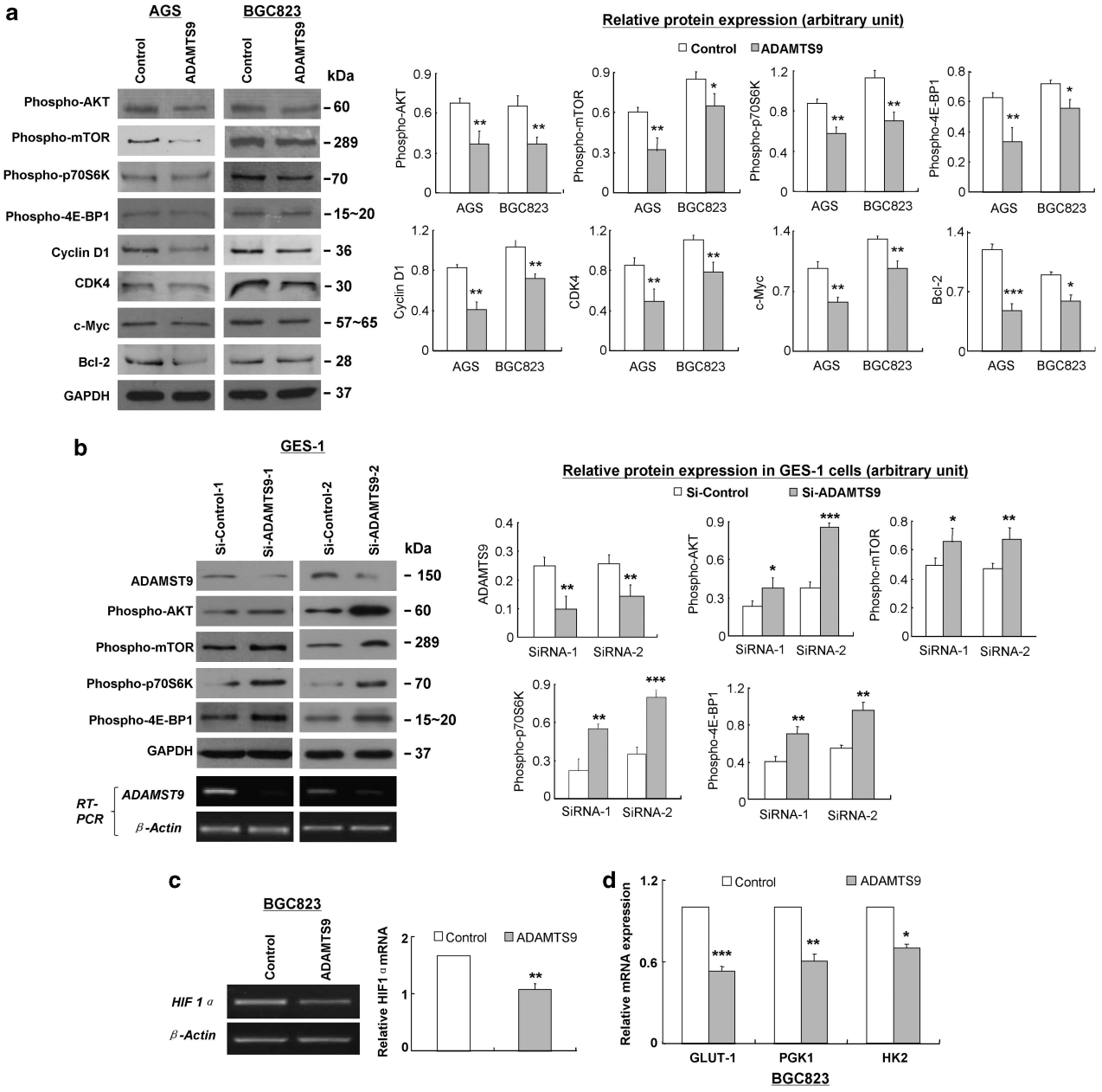

Figure 5. ADAMTS9 regulated its downstream molecular targets. (**a**) The effect of ADAMTS9 overexpression on its downstream targets in AKT/mTOR signaling pathway was assessed by western blot. (**b**) The effect of ADAMTS9 knockdown on its downstream targets in AKT/mTOR signaling pathway was assessed by western blot (upper panel). The knock down efficiency of two independent siRNA to ADAMTS9 was confirmed by western blot and reverse-transcription–PCR (lower panel). The effects of ADAMTS9 overexpression on mRNA expression of HIF1α (**c**) and its downstream glycolysis-related targets (**d**) by reverse-transcription–PCR. The intensity of bands of western blot were quantified by scanning densitometry. Data are mean ± s.d., $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

poorer survival (RR = 2.788; 95% confidence interval, 1.474–5.274; $P=0.002$). Tumor TNM (tumor, node, metastasis) stage was another independent predictor for overall survival. Patients in stages I–III had a significantly better survival when compared with patients with a stage IV tumor (Table 1).

As shown in the Kaplan–Meier survival curves, gastric cancer patients with *ADAMTS9* methylation had significantly shorter survival than those without ADAMTS9 methylation ($P=0.001$, log-rank test) (Figure 6b). The difference in survival remained significant even if patients were further stratified by tumor TNM staging. The overall survival of patients with ADAMTS9 methylation was significantly shorter than that of other gastric cancer patients in all stages, respectively (Figure 6c).

DISCUSSION

In this study we found that ADAMTS9 is widely expressed in normal adult tissues and fetal tissues, whereas absent or down-regulated in 6 out of 8 gastric cancer cell lines. The reduced expression was mostly attributed to the promoter methylation of *ADAMTS9* gene as evaluated by methylation specific PCR. Bisulfite genomic sequencing of the *ADAMTS9* promoter region confirmed dense methylation in gastric cancer cell lines and in gastric cancer

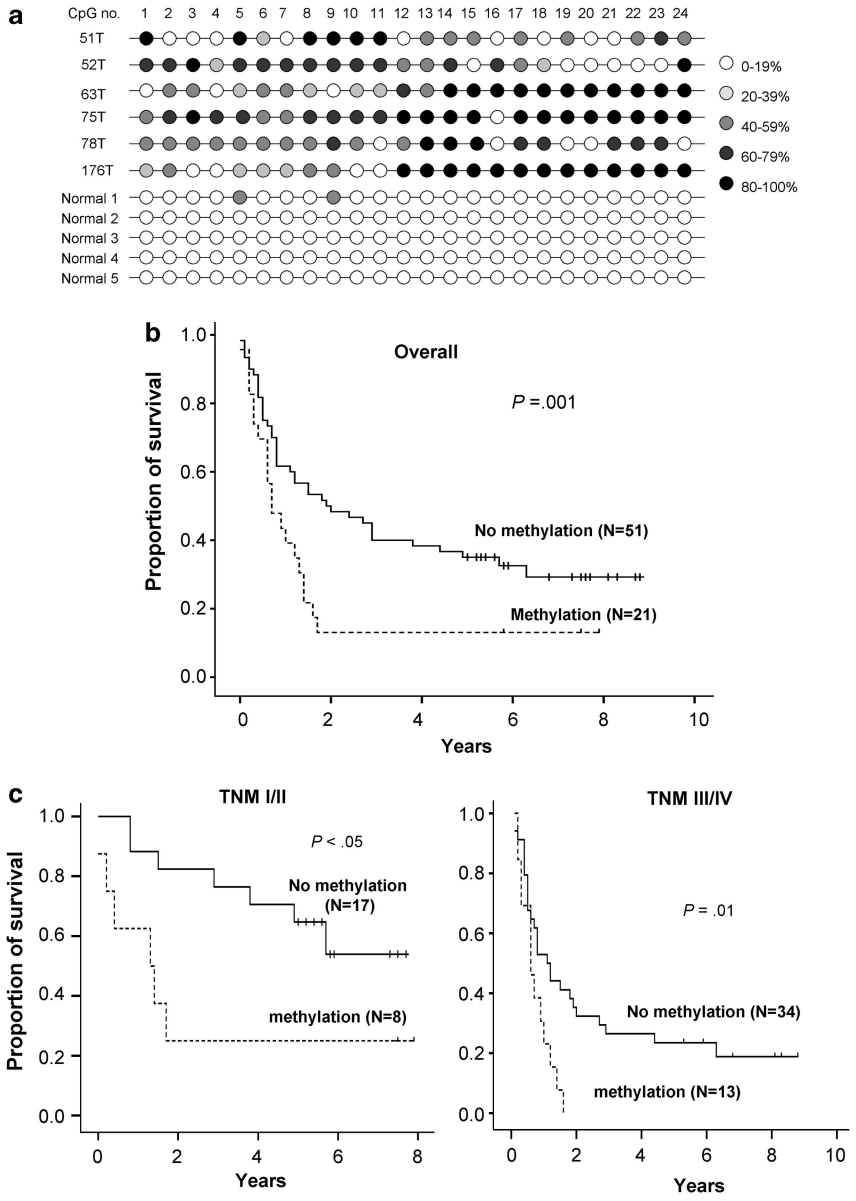

Figure 6. Promoter methylation of *ADAMTS9* is associated with poor survival of gastric cancer patients. (**a**) *ADAMTS9* was frequently methylated in primary gastric cancers. Detailed bisulfite genomic sequencing analysis confirmed dense methylation in tumor tissues, but less in normal tissues. Filled circle, methylated; open circle, unmethylated. (**b**) Kaplan–Meier survival curves show that gastric cancer patients with *ADAMTS9* methylation had poorer survival than those without *ADAMTS9* methylation based on log-rank test ($P<0.001$). (**c**) Kaplan–Meier curves of gastric cancer patients stratified by methylation status of different TNM stages (I/II and III/IV), survival was significantly shorter in the methylation group.

tissues, whereas no methylation in the normal gastric tissues. Furthermore, the silencing of *ADAMTS9* can be reversed by pharmacological demethylation treatment, suggested that promoter methylation was a predominant regulatory mechanism of *ADAMTS9* inactivation in gastric cancer.

ADAMTS9 gene inactivation in the gastric cancer cell lines and tissues, but expression in normal tissues suggested that ADAMTS9 could be a potential tumor suppressor and its downregulation could have functional role in the development of gastric cancer. Therefore, the tumor suppressor role of ADAMTS9 in gastric cancer was investigated both *in vitro* and *in vivo*. Ectopic expression of ADAMTS9 in the silenced AGS and BGC823 cells showed significant growth suppressing effect by inhibition of cell proliferation and colony formation. The diminution of tumor growth by ADAMTS9 was further confirmed by the reduced tumorigenesis in nude mice. On the other hand, siRNA-mediated knockdown of ADAMTS9 in normal gastric epithelial GES1 cell significantly increased cell proliferation. These results indicated that ADAMTS9 functions as a tumor suppressor in gastric carcinogenesis.

Given these data apparently suggesting that ADAMTS9 could be a growth inhibitor in gastric cancer, a better understanding of the mechanism of the action of ADAMTS9 is required. We therefore analyzed the effects of ADAMTS9 re-expression on cell apoptosis and angiogenesis in two human gastric-cancer cell lines. In both AGS and BGC8230 cells, re-expression of ADAMTS9 significantly increased apoptosis. This was confirmed by upregulation of cleaved poly (ADP-ribose) polymerase by ADAMTS9. The

activation of poly (ADP-ribose) polymerase causes loss of DNA repair, cellular disassembly and finally undergoing apoptosis.[17] In addition to inhibition of cell proliferation, the growth inhibitory effect of ADAMTS9 was related to induction of apoptosis. Moreover, on the basis of the angiogenesis analysis, our results clearly showed that ADAMTS9 significantly suppressed HUVEC tube formation in both AGS and BGC823 cells. ADAMTS9 disrupted HUVEC tube formation by downregulating angiogenesis factor-VEGFA. Thus, ADAMTS9-mediated growth inhibition occurs also by modulation of angiogenesis.

We further elucidate the downstream signaling pathways that ADAMTS9 exerts its tumor suppressor function in gastric cancer and found that ADAMTS9 inhibited AKT signaling pathway. AKT pathway has a critical role in cancer development and progression by regulating cell proliferation, apoptosis and angiogenesis.[18,19] In keeping with this, we reveled that ADAMTS9 inhibited the activation of AKT by downregulation of phosphor-AKT as demonstrated by gain- and loss-ADAMTS9 function analyses. It is therefore possible that the inhibitory properties of ADAMTS9 against gastric cancer cell growth observed in this study could be explained, at least in part, by the resultant inhibition of AKT signaling pathway (Figure 7).

Table 1. Multivariate Cox regression analysis of potential prognostic factors for gastric cancer patients

Variable	*HR (95% CI)*	P-*value*
Age	0.980 (1.005–1.031)	0.704
Gender		
Male	0.625 (0.338–1.154)	0.133
Female	1.00	
TNM stage		
I	0.177 (0.072–0.436)	<0.001
II	0.146 (0.052–0.415)	<0.001
III	0.357 (0.175–0.727)	0.005
IV	1.00	
ADAMTS9 methylation		
Methylated	2.788 (1.474–5.274)	0.002
Non-methylated	1.00	

Abbreviations: *ADAMTS9*, a disintegrin-like and metalloprotease with thrombospondin type 1 motif 9; CI, confidence interval; HR, hazard ratio.

It is known that mTOR is a direct target of AKT oncogenic signaling, which promotes cancer progression through regulating cell proliferation, apoptosis, metabolism and angiogenesis.[13,14,19] mTOR drives tumorigenesis by promoting the translation of oncogenes through relieving 4E-BP1-mediated inhibition of eukaryotic translation initiation factor 4E and activating p70S6K, both of which associate mRNA and regulate mRNA translation initiation and elongation.[13,20–22] It is therefore of interest in the present study to investigate the effects of ADAMTS9 on activation of mTOR and its substrate p70S6K and 4E-BP1. Our results showed that ADAMTS9 could inhibit phosphorylation of p70S6K and phosphorylation of 4E-BP1 (Figures 5 and 7). Moreover, a number of well-known downstream targets translationally regulated by p70S6K and 4E-BP1 including the key pro-growth proteins cyclin D1, cyclin-dependent kinase4 and the oncogenic transcription factor c-Myc and antiapoptotic protein Bcl-2 were suppressed concomitantly by ADAMTS9 (Figure 5).[14,23,24] Cyclin D1 and cyclin-dependent kinase4 contribute to tumor growth by promoting cell cycle progression.[25,26] Reduced Bcl-2 leads to the permeabilization of the mitochondrial outer membrane, which causes poly (ADP-ribose) polymerase to cleave, finally resulting in chromosomal DNA fragmentation and cellular apoptosis.[24,27,28] Therefore, ADAMTS9 suppresses gastric cancer cells proliferation and induce apoptosis likely by a mechanism that involves mediating AKT/mTOR signaling pathway (Figure 7).

The AKT/mTOR pathway has been found to promote expression of genes involved in glucose uptake and glycolysis through normoxic upregulation of the HIF1α transcription factor.[13,14] Glucose uptake and consumption by glycolysis provide tumor cells a proliferative advantage over normal cells under conditions

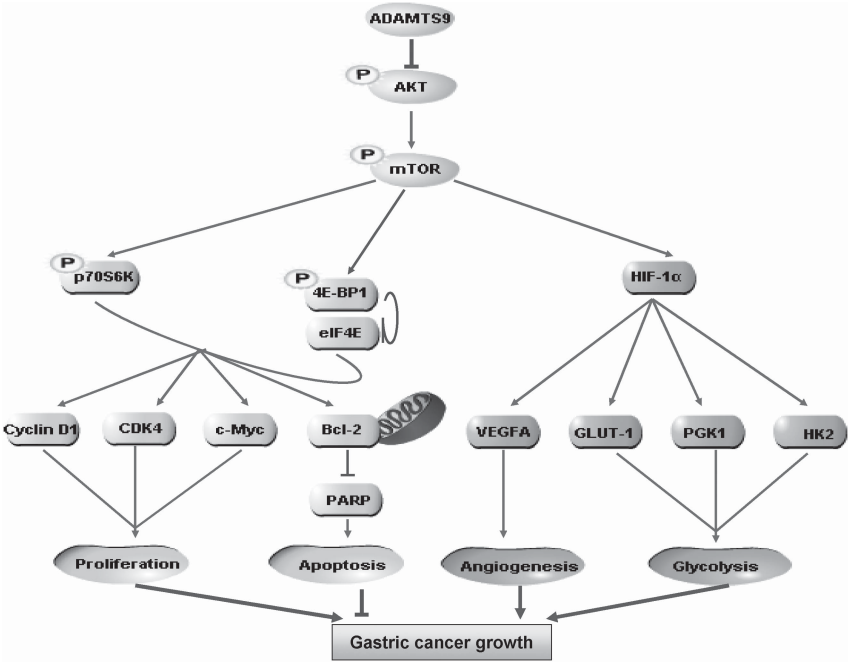

Figure 7. Schematic diagram for the molecular basis of *ADAMTS9* as a tumor suppressor gene in gastric cancer by decreasing cell proliferation, inducing cell apoptosis, inhibiting glycolysis and angiogenesis through regulating AKT/mTOR signaling pathway. A full colour version of this figure is available at the *Oncogene* journal online.

of intermittent hypoxia within the growing tumor. In this connection, we analyzed the effects of ADAMTS9 on glucose regulation and found that ADAMTS9 inhibited expression of glucose transporter -1, a major carrier to regulate glucose into cell, two glycolytic enzymes PGK1 and hexokinase 2 (Figures 5d and 7) and their upstream mediator HIF1α. The findings indicated that ADAMTS9 inhibited glucose uptake and glycolysis through inhibiting AKT/mTOR/HIF1α pathway, which contributes to its tumor suppressive function. Furthermore, reduced AKT/mTOR/HIF1α signaling also provided an explanation for the antiangiogenic function by ADAMTS9. ADAMTS9 reduced the expression of HIF1α downstream target VEGFA, which contributes to the blunted HUVEC tube formation (Figure 4a), adding further weight to the significance of ADAMTS9 inhibiting tumorigenesis by decreasing AKT/mTOR/HIF1α signaling pathway (Figure 7).

As a highly conserved member of the ADAMTS family, ADAMTS9 is an extracellular matrix metalloprotease with proteolytic activity and localizes to the cell-surface and extracellular matrix.[29] It has been reported that ADAMTS1, a member of ADAMTS family, enhances extracellular signal-regulated kinase signaling by activating epidermal growth factor receptor through shedding of the transmembrane precursors of heparin-binding epidermal growth factor.[30] Several ADAMTS9 substrates have been identified including versican and aggrecan, which are important extracellular matrix proteoglycans in cell biology.[29,31] It is thus possible that the active metalloproteinase domain of ADAMTS9 cleaves extracellular matrix components or membrane proteins, leading to alteration of the composition of the extracellular matrix/media, ultimately affecting intracellular signaling and process. On the other hand, the AKT cascade can be mediated by many membrane proteins including receptor tyrosine kinases, integrins, cytokine receptors, G protein-coupled receptors that induces the production of phosphatidylinositol 3,4,5 triphosphates by phosphoinositide 3-kinase.[19] Thus, the inhibited intracellular AKT/mTOR signaling by ADAMTS9 may be due to changes in the concentration or localization of extracellular signaling molecules, which can be released from or withheld in the matrix. However, the direct link between ADAMTS9 with intracellular AKT/mTOR signaling need to be investigated in future study.

Keeping with our findings, previous studies had shown that *ADAMTS9* was aberrantly epigenetically silenced in esophageal cancer and nasopharyngeal carninoma.[7,8] ADAMTS9 inhibited tumorigenicity of esophageal and nasopharyngeal cancer cells in nude mice by suppressing angiogenesis with reduced VEGFA production.[6] Our study demonstrated that ADAMTS9 contributed to tumor inhibition not only by reducing angiogenesis but also by regulating cell proliferation, apoptosis and glycolysis. Furthermore, we for the first time elucidated the molecular mechanism of tumor suppressive function of ADAMTS9, which was through regulating oncogenic AKT/mTOR signaling pathway.

The clinical implication of ADAMTS9 in primary gastric cancers was evaluated. Among our panel of 72 primary gastric tumors, the ADAMTS9 promoter methylation status was not associated with clinicopathologic variables except *H. pylori* infection. This consistent with the reports that DNA methylation is associated with exposure to *H. pylori* as *H. pylori* infection increases DNA methyltransferase activity.[32,33] In addition, the promoter methylation of *ADAMTS9* was significantly associated with poorer survival in gastric cancer patients independent of patient characteristics ($RR = 2.788$, $P = 0.002$) by multivariate cox regression analysis, suggesting that *ADAMTS9* methylation could be regarded as a potential prognostic factor for gastric cancer. As TNM staging is the most important clinical predictor of patient outcome, we evaluated both methylation status and tumor stage by Kaplan–Meier survival analysis. The methylation was associated significantly with shorter survival for stages I/II and stages III/IV gastric cancer patients. The phenomenon that methylation alteration of *ADAMTS9* affects the prognostic of gastric cancer patients may provide an additional evidence for the role of *ADAMTS9* as a tumor suppressor gene in the development of gastric cancer.

In conclusion, we have identified a novel functional tumor suppressor gene *ADAMTS9* inactivated by promoter methylation in gastric cancer cell lines and primary gastric cancers. ADAMTS9 contributes to the suppression of tumorigenesis by decreasing cell proliferation, inducing cell apoptosis and inhibiting angiogenesis through regulating AKT/mTOR signaling pathway. *ADAMTS9* methylation detected in gastric tumor tissues is associated with poor survival of gastric cancer patients and may serve as a potential epigenetic biomarker to predict outcome for patients with gastric cancer.

MATERIALS AND METHODS

Cell lines

Eight gastric cancer cell lines (BGC823, MGC803, AGS, MKN28, MKN45, SNU719, YCCEL1 and Kato III) were obtained from the American Type Culture Collection (Manassas, VA, USA) and human non-tumorigenic GES 1 was obtained from Cancer Research Institute of Beijing, Beijing University, China. They were cultured in Dulbecco's modified Eagle's medium or RPMI 1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C.

Patients and gastric biopsy specimens

Gastric biopsy specimens were obtained from gastric cancer patients during endoscopy before any therapeutic intervention. The biopsy specimens were snap-frozen in liquid nitrogen and stored at −80 °C for molecular analyses. A total of 83 patients with confirmed gastric normal tissues were examined. Tumor was staged according to the TNM staging system. *H. pylori* infection was defined by the presence of the gram-negative curved bacilli on histology. All histological assessments were made by an experienced pathologist (KFTo). Patients were being followed up regularly in our clinic and the median follow-up duration since the time of diagnosis was 16.8 months. Data for *H. pylori* infection status, Lauren classification and TNM stage/survival time were missing in 31, 19 and 11 patients, respectively. All patients were treated according to a standard protocol with surgery being the mainstay of treatment. All patients and controls gave informed consent, and the study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong.

RNA extraction, semiquantitative reverse-transcription–PCR and real-time PCR analyses

Total RNA was extracted from gastric cell lines using Trizol reagent (Invitrogen, Carlsbad, CA, USA). complementary DNA was synthesized from total RNA using Transcriptor Reverse Transcriptase (Roche Applied Sciences, Indianapolis, IN, USA). For semiquantitative reverse-transcription–PCR, *ADAMTS9* gene was amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) as reported previously, with β-actin as internal control. Real-time PCR was performed using SYBR Green master mixture on HT7900 system according to the manufacturer's instructions (Applied Biosystems). Primer sequences are listed in Supplementary Table 3.

Methylation-specific PCR and Bisulfite genomic sequencing

Genomic DNA was extracted from the gastric tissues or cell lines using QIAamp DNA Mini kit (Qiagen, Hilden, Germany). DNA was chemically modified with sodium metabisulphite. The bisulfite-modified DNA was amplified by using primers that specifically amplify either methylated or unmethylated sequences of the *ADAMTS9* genes (Supplementary Table 3).

Bisulfite genomic sequencing was performed to characterize the methylation density in the promoter of *ADAMTS9* using the BigDye Terminator Cycle Sequencing kit version 1.0 (Applied Biosystems) (Supplementary Table 3). Twenty four CpG sites spanning the −601 and −995 bp regions were evaluated. Sequences were analyzed by using SeqScape software (Applied Biosystems) and Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

5-Aza-2′-deoxycytidine treatment

Cell lines with silenced *ADAMTS9* expression were treated with 2 mM DNA demethylating agent 5-Aza-2′-deoxycytidine (Sigma-Aldrich, St Louis, MO, USA) for 5 days. 5-Aza-2′-deoxycytidine was replenished every day. Controlled cells were treated with an equivalent concentration of dimethyl sulfoxide. Cells were then harvested for RNA extractions.

Colony formation assay

AGS and BGC823 cells transfected with pCEP4-ADAMTS9-expressing or pCEP4 empty vector (originally from Dr Suneel S Apte, Department of Biomedical Engineering, Lerner Research Institute, Cleveland, OH, USA)[34] were selected with Hygromycin B (Invitrogen) for 2 weeks. pCEP4-ADAMTS9 plasmid encodes the full-length human ADAMTS9 complementary DNA (NM_182920) and was sequence-verified. Colonies with cell numbers of >50 cells per colony were counted after staining with crystal violet solution. All the experiments were performed in triplicate wells in three independent experiments.

Western blot analysis

AGS and BGC823 cells were harvested and total protein were extracted at 48 h after transfection of pCMV-ADAMTS9 or pCMV empty vector. The pCMV-ADAMTS9 plasmid was constructed by our collaborator Dr Qian Tao, using pCEP4-ADAMTS9 as ADAMTS9 complementary DNA template. pCMV-ADAMTS9 plasmid encodes the full-length human *ADAMTS9* complementary DNA (NM_182920), which was sequence-verified. GES 1 was harvested and total protein was extracted at 48 h after transfection of *ADAMTS9* siRNA-1 (forward 5′-CGACAAAUGUGAUACCUUAdTdT-3′; and reverse 5′-dTdT GCUGUUUACACUAUGGAAU-3′) and control siRNA-1 (Ribobio, Guangzhou, China), *ADAMTS9* siRNA-2 (forward 5′-GAGGG CAUAAACAACGAAA dTdT-3′; and reverse 5′-UUUCGUUGUUUAUGCC CUCdTdT-3′) and control siRNA-2 (GenePharma, Shanghai, China), respectively. Thirty micrograms of protein were separated by sodium dodecyl sulfate–PAGE and transferred onto nitrocellulose membrane. The dilution of primary antibodies was according to the company's recommendation. Proteins were visualized using ECL Plus Western Blotting Detection Reagents (RPN2132, GE Healthcare, Chalfont St Giles, UK). The intensity of bands of western blot were quantified by scanning densitometry. Antibodies are listed in Supplementary Table 4.

Cell viability assay

Cell viability was measured by the MTS assay (Promega, Madison, WI, USA). Briefly, AGS and BGC823 cells (5×10^3 per well) were seeded in 96-well plates and transient transfected with pCMV-ADAMTS9 vector or pCMV empty vector. GES 1 (1×10^3) was seeded in 96-well plates and transient transfected with *ADAMTS9* siRNA or control siRNA. After transfection for 1, 2 or 3 days, 20 ml of reaction solution was added to cultured cells in 100 ml culture medium for 1 h at 37 °C and measured at a wave-length of 490 nm. The experiment was repeated three times independently.

Annexin V apoptosis assay

Apoptosis was assessed using the dual staining with Annexin V:FITC (BD Biosciences) and 7-amino-actinomycin (BD Biosciences). Briefly, pCMV-ADAMTS9 or empty vector-transfected cells were harvested at 48 h post transfection pCMV-ADAMTS9 vector or pCMV empty vector. Annexin V:FITC and 7-amino-actinomycin were added to the cellular suspension according to the manufacturer's instructions and was analyzed using FACS-Calibur System (Becton Dickinson Pharmingen, San Jose, CA, USA). Both early and late apoptotic cells were counted for relative apoptotic changes. All the experiments were performed three times.

Tube formation assay

Conditioned media were collected by incubating pCMV-ADAMTS9 and pCMV vector-alone transfected cells (BGC823 and AGS) without serum for 24 h. After Matrigel (Millipore, Billerica, MA, USA) thawed on ice, the 96-well plate coated with 50 μl Matrigel, each well was then incubated at 37 °C for 30 min to allow the Matrigel to polymerize. A total of 1×10^4 HUVEC cells were seeded into each well and incubated with 100 μl conditioned media from ADAMTS9 and vector-alone transfectants plus 1% fetal bovine serum. Cells were then incubated for 4 h to allow the formation of tube-like structures. Image analysis of tube length was carried out using Image software (NIH website, USA).

In vivo tumorigenicity

BGC823 cells (5×10^5 cells in 0.2 ml phosphate-buffered saline) transfected with pCMV-ADAMTS9 vector or pCMV empty vector were injected subcutaneously into the dorsal flank of 5-week-old male Balb/c nude mice, separately. Tumor diameter was measured every 3 days for 15 days. Tumor volume (mm^3) was estimated by measuring the longest and shortest diameter of the tumor and calculating as follows: volume = (shortest diameter)^2 × (longest diameter) × 0.5.[5] All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Statistical analysis

Data were expressed as mean ± standard deviation (s.d.). The difference in tumor growth rate between the two groups of mice was determined by repeated-measures analysis of variance. The χ^2-test was used for comparison of patient characteristics and distributions of methylation by vital status. Patient age (at entry or follow-up evaluation) by vital status was compared using the *t*-test. RRs of death associated with ADAMTS9 methylation and other predictor variables were estimated from univariate Cox proportional hazards model first. Multivariate Cox models also were constructed to estimate the RR for ADAMTS9 methylation. Overall survival in relation to methylation status was evaluated by the Kaplan–Meier survival curve and the log-rank test. All analyses were performed using SPSS for Windows 11.0.1 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

ABBREVIATIONS

7-AAD, 7-amino-actinomycin; 5-Aza, 5-Aza-2′-deoxycytidine; ADAMTS9, a disintegrin-like and metalloprotease with thrombospondin type 1 motif 9; BCL-2, B-cell CLL/lymphoma 2; BGS, bisulfite genomic sequencing; CDK4, cyclin-dependent kinase 4; eIF4E, eukaryotic translation initiation factor 4E; 4E-BP1, eIF4E-binding protein; EGF, epidermal growth factor; GLUT-1, glucose transporter 1; HIF1α, hypoxia-inducible factor 1α; HK2, hexokinase 2; HUVEC, human umbilical vein endothelial cell; MSP, methylation specific PCR; mTOR, the mammalian target of rapamycin; PARP, nuclear enzyme poly (ADP-ribose) polymerase; PGK1, phosphoglycerate kinase 1; PI3K, phosphoinositide 3-kinase; PCNA, proliferating cell nuclear antigen; VEGFA, vascular endothelial growth factor A.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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