Original Paper

Promotion of Tumor Growth by ADAMTS4 in Colorectal Cancer: Focused on Macrophages

Jianjun Chen^a Yang Luo^a Yong Zhou^b Shaolan Qin^a Yier Qiu^a
Ran Cui^a Minhao Yu^a Jun Qin^a Ming Zhong^a

^aDepartment of Gastrointestinal Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, ^bDepartment of General Surgery, Shanghai Jiading District Hospital of Chinese medicine, Shanghai, P.R. China

Key Words

Adamts • Tumor microenvironment • Colorectal cancer • Macrophages

Abstract

Background/Aims: ADAMTSs (A disintegrin and metalloprotease domains with thrombospondins motifs) are a family of extracellular proteases that have been related to both oncogenic and tumor-suppressive functions. The aim of the present study was to investigate: 1) the mutation, copy-number alterations, and expression profile of ADAMTSs in colorectal cancer and 2) whether ADAMTSs participate in colorectal cancer (CRC) progression and invasion.

Methods: The mutation, copy-number alterations, and expression profile of ADAMTSs in CRC were analyzed in the TCGA cohort using cBioportal. ADAMTS4 expression in tumor tissues and cell lines were determined by immunostaining and real-time quantitative PCR. The role of ADAMTS-4 in CRC progression and the underlying mechanisms were studied by using short hairpin RNA-mediated knockdown of ADAMTS4. The effects of ADAMTS4 in cell proliferation and invasion were determined by clone formation assay and transwell migration assay, respectively. Macrophages were depleted by liposomal clodronate in immune-competent BALB/c mice and tumor growth was analyzed. **Results:** ADAMTS4 was differentially expressed in CRC and predicted a poor prognosis. Elevated ADAMTS4 expression was closely associated with larger tumor size, enhanced TNM stage, and a poor clinical outcome in patients with CRC. ADAMTS4 knockdown had no inhibitory implications on cell proliferation and invasion *in vitro*, but significantly attenuated tumor growth *in vivo*. Mechanistically, we revealed that ADAMTS4 was associated macrophages infiltration and polarization in the tumor microenvironment of CRC. Macrophage depletion largely abolished the promotive effect of ADAMTS4 on tumor growth in the immune competent BALB/c mice. **Conclusion:** ADAMTS4 seemed to be a promising prognostic indicator in CRC. The novel link between ADAMTS4 and macrophages mirrors the potential regulatory roles of ADAMTSs in the inflammatory microenvironment of cancers.

© 2018 The Author(s)
Published by S. Karger AG, Basel

J. Chen, Y. Luo and Y. Zhou contributed equally to this work.

Ming Zhong

Department of Gastrointestinal Surgery, Ren Ji Hospital, School of Medicine,
Shanghai Jiao Tong University, 160th Pujian Road, Shanghai (P.R. China)
Tel. +86-21-68383985, E-Mail drzhongming1966@163.com

KARGER

Introduction

Colorectal cancer (CRC) is the most common cause of cancer-related deaths worldwide. More than 1.2 million new cases of CRC occur every year globally, accounting for 10% of all cancer cases [1]. Due to early screening and improved treatment, the mortality rates of CRC are decreasing [2]. Despite the incidence of CRC, in developed countries, is stabilized and even declined, there is still the necessity for achieving better understanding tumor biology with a view to develop novel therapeutic strategies.

Extracellular matrix (ECM) components are critically involved in the malignant phenotypes of cancers, especially metastatic events [3]. Specifically, protease-induced breakdown of the ECM components is essential for tumor cells to across tissue barriers [4, 5]. ADAMTS (a disintegrin-like and metalloproteinase with thrombospondin motifs) is a family of extracellular metalloproteinases which was first identified in 1997. To date, 19 ADAMTS family members have been identified. Most of ADAMTSs include the following domain structure from the N-terminus to C-terminal: 1) a signal peptide directs ADAMTS to the secretory pathway; 2) a prodomain maintains enzyme latency; 3) a zinc binding metalloproteinase domain; 4) a disintegrin-like domain; 5) a central Thrombospondin Type 1 repeat (TSR); 6) a cysteine rich domain; 7) a spacer domain; 8) variable number of TSRs [6, 7]. ADAMTSs are involved in diverse functions including matrix degradation, collagen maturation, blood coagulation, organogenesis, and inflammation [8, 9]. However, the functions, mechanisms of activation, and substrates of most ADAMTS members remain largely unexplored [10]. In cancers, ADAMTSs can play both oncogenic and tumor-protective roles through regulating matrix-degradation, angiogenesis and metastasis [8, 11-16]. Moreover, ADAMTSs mutation is significantly associated with improved chemotherapy sensitivity and progression-free survival in ovarian cancer [17]. Therefore, exploring the roles of ADAMTSs in cancers might provide promising oncotherapeutic options.

In this study, we firstly studied on the mutation, copy-number alterations, and expression profile of ADAMTSs in CRC. The differentially expressed ADAMTS4 was selected for further study including correlation with clinicopathological factors, prognostic value, *in vitro* and *in vivo* functions, and related mechanisms. As a result, we demonstrated that overexpressed ADAMTS4 predicted a poor prognosis and contributed to tumor growth by modulating macrophages in CRC.

Materials and Methods

Cell Culture

Five human CRC cell lines (LOVO, SW620, SW480, Caco-2, and HCT15) and a mouse tumor cell line CT26 were all obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences in Shanghai, China. The normal colonic epithelial cell line NCM460 was purchased from American Type Culture Collection (Manassas, VA, ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or Roswell Park Memorial Institute (RPMI)-1640 containing 10% fetal bovine serum (FBS) containing 10% fetal bovine serum (FBS). All cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

Data mining

The analyzed TCGA data of colorectal adenocarcinoma (COAD) was downloaded from Broad Institute TCGA Genome Data Analysis Center (<https://cancergenome.nih.gov>). The expression level of ADAMTS family members was quantified as RSEM (RNA-Seq by expectation-maximization) logarithmically transformed (base 2) in order to follow a normal distribution. The R2 database (<http://hgserver1.amc.nl>) was used to analyze ADAMTS4-associated genes. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were further used to explore the function of ADAMTS4-associated genes in this database.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described [18]. Information for tissue microarray used in this study was reported previously [19]. Briefly, tissue sections were deparaffinized in

xylene and rehydrated with graded ethanol. Then the sections were incubated with 3% hydrogen peroxide and boiled in a microwave for 15 min to unmask antigen epitopes and then blocked with 10% BSA (Sangon, Shanghai, China). After washing three times with phosphate-buffered saline (PBS), sections were incubated with primary antibody for ADAMTS4 (Abcam, ab84792, diluted at 1:200), Ki67 (Cell signaling technology, #9449, diluted at 1:400) and CD68 (Abcam, ab955, diluted at 1:200) at 4°C overnight with optimal dilution. In the next day, sections were incubated with second antibody labeled by HRP (rabbit) (Proteintech, Shanghai, China) at room temperature for 1 h. Finally, the bound antibodies were visualized with diaminobenzidine (DAB) and counterstained with haematoxylin.

RNA isolation and quantitative real-time PCR

Total RNA were extracted from the CRC cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and 1 µg RNA was reverse-transcribed into cDNA using PrimerScript RT-PCR kit (Takara, Tokyo, Japan) according to the manufacturer's protocol. Quantitative real time PCR was performed using SYBR green (Applied Biosystems, CA, USA) with a Step One Real-Time PCR system (Applied Biosystems, CA, USA). Gene expression was normalized to GAPDH. Primer sequences are listed as follows: ADAMTS4 forward, 5'-GAGGGAGGCACCCCTAACT-3'; ADAMTS4 reverse, 5'- CCTTGACGTTGCACATGGGA-3'; GAPDH forward, 5'-ACAACCTTTGGTATCGTGAAGG-3'; GAPDH reverse, 5'-GCCATCACGCCACAGTTTC-3'.

Generation of stable cell lines

The short hairpin RNAs targeting ADAMTS4 were designed and purchased from Genepharma (Shanghai, China). The coding sequences of ADAMTS4 were cloned into pCDH-CMV-MCS-EF1-Puro vector (System Biosciences, CA, USA). Virus packaging was performed in 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Viruses were harvested at 48 h after transfection, and virus titers were determined. Indicated cells (1×10^5) were infected with 1×10^6 recombinant lentivirus-transducing units in the presence of 6 µg/ml polybrene (Sigma-Aldrich, Shanghai, China). The infected cells were screened by 2 µg/ml puromycin (Sangon, Shanghai, China). Puromycin resistant single-cell clones stably expressing ADAMTS4 were established and verified by western blotting analysis.

Western blotting

Total protein was extracted using RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0 mM EDTA, 0.1% SDS, and 1% Triton X-100). Protein lysates were separated by a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. The blotted membranes were incubated in blocking solution containing 5 % (w/v) non-fat milk powder and 0.1 % (v/v) Tween-20 for 1 h at room temperature. Then the membranes were incubated with the primary antibody of ADAMTS-4 (Abcam, ab84792, diluted at 1:1000) and GAPDH (Abcam, ab8245, diluted at 2:1000) at 4°C overnight, followed by incubation with secondary horseradish peroxidase (HRP)-conjugate anti-rabbit secondary antibody for 1 h at room temperature. Bound antibodies were visualized with the ECL kit (Beyotime, Shanghai, China).

Enzyme linked immunosorbent assay (ELISA)

The ADAMTS4 level was measured from cell culture supernatants by A Human ADAMTS4 DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Clone formation assay

The indicated cells (1000 per well) were seeded into six-well plates and cultured for 14 days. The medium was changed every 3 days. Subsequently, formed colonies were fixed with 4% paraformaldehyde (PFA) and then stained with 0.5% crystal violet for 30 min. The number of colonies formed in each well was counted under a microscope.

Cell invasion assay

The invasive potential of Caco-2 and HCT15 cells was monitored by transwell assay (Corning, NY, USA) according to the manufacturer's instructions. Briefly, 2×10^4 cells in 100 µl medium were seeded into the upper chamber of matrigel-coated filters (BD Bioscience, San Diego, CA, USA). A total of 700 µl culture medium containing 10 % (v/v) FBS was added to the bottom chamber. After incubation for 48 h, the non-

migrated cells that remained on the upper chamber were removed. The migrated cells were fixed with 4% PFA and stained with 0.5% crystal violet. Finally, the number of invaded cells on the lower surface was counted under a light microscope in six random fields.

Animal experiments

For subcutaneous xenograft assay, athymic nude mice (5-week-old) were injected subcutaneously with 2×10^6 sh-Ctrl and sh-ADAMTS4 HCT15 cells at the right flank. Tumor size was measured by a slide caliper every 3 days and tumor volume was determined by the formula $0.44 \times A \times B^2$ (A indicates tumor base diameter one direction and B the corresponding perpendicular value). For macrophage depletion assay, BALB/c mice were injected intraperitoneally twice per week with clodronate liposomes (1.4 mg/20 g body weight) or with an equivalent volume of PBS liposomes until week 4. At week 2, BALB/c mice were injected subcutaneously with 2×10^6 OV-vector and OV-ADAMTS4 cells at the right flank. Tumor size was monitored every 3 days for 3 weeks. Clodronate liposomes and PBS liposomes were purchased from Dr N Van Rooijen (Vrije Universiteit, Amsterdam, Netherlands). All mice were housed and maintained under specific pathogen-free conditions and used in accordance with institutional guidelines. The animal studies and the process were approved by Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, China.

Flow Cytometry

For detection of tissue macrophages, mice were sacrificed and transcardially perfused with D-Hanks buffer followed by 1mg/ml collagenase type IV (Sigma-Aldrich, Shanghai, China). Cellular suspensions were prepared as follows: isolated tissues were washed in D-Hanks buffer, injected with 5 ml collagenase solution, minced, and digested at 37°C for 30 min. Cells were collected by passing through a 70 μ m strainer. Subsequently, single-cell suspensions were blocked with CD16/CD32 antibody (BD Biosciences, CA, USA) and subsequently labeled with antibodies against CD11b (Miltenyi Biotec, Shanghai, China) and Gr1 (AbD Serotec, Oxford, UK) for 30 minutes at 4°C. Finally, stained cells were analyzed by flow cytometry.

Statistical analysis

Data were presented as the means $\pm$ SD. Statistical analyses were performed using the SPSS 16.0 software (SPSS, Chicago, IL, USA) and graphical representations were performed with GraphPad Prism 5 (San Diego, CA, USA) software. Correlation of ADAMTS4 expression with clinicopathologic parameters was analyzed by Pearson chi-square test. Overall survival rate was calculated according to the Kaplan-Meier method and the difference in survival curves was evaluated by the log-rank test. Independent prognostic factors were analyzed by the Cox proportional hazards regression model. Paired data were analyzed by Student's t test, and group data were analyzed by one-way analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant.

Results

Differentially expressed ADAMTS4 predicts a poor prognosis in CRC

Emerging evidence has demonstrated that ADAMTS are deregulated in human cancer and implicated in tumor progression [8, 13]. Meanwhile, somatic mutations of ADAMTS genes are associated with chemotherapy sensitivity and patients' survival [20, 21]. To gain further insight into the mutation, copy-number alterations, and expression profile of ADAMTS, we examined the TCGA (provisional) cohort by cBioPortal (<http://www.cbioportal.org/>). As a result, we noticed that ADAMTS family members were rarely mutated in CRC with a highest mutation frequency for ADAMTS12 at only 2.9% (Fig. 1). The copy-number alterations, including amplification and deep deletion, were also not frequently observed in CRC (Fig. 2). Next, by data mining the ADAMTS expression in healthy and cancerous colorectal tissues, we found that 1) ADAMTS-1, -2, -4, and -9 were the most abundant ADAMTS in CRC tissues; 2) ADAMTS-1, -8, and -17 were down-regulated in CRC tissues compared with normal tissues; 3) ADAMTS-2, -3, -4, -6, -7, -9, -12, -14, and -18 were significantly over-expressed in CRC tissues (Fig. 3A). By Kaplan-Meier curve analysis of the differentially expressed ADAMTS, we

Fig. 1. The mutation profile of ADAMTS family member in TCGA (Provisional) cohort.

found that only ADAMTS-4 and ADAMTS-6 predicted a poor prognosis in CRC patients in TCGA cohort (Fig. 3B).

From the therapeutic point of view, we focused on the study of ADAMTS4 in CRC due to its expression profile and prognostic value in CRC. To further confirm ADAMTS4 expression at protein level, we carried out immunohistochemical analysis in a tissue microarray containing 345 cases of CRC specimens. Representative ADAMTS4 staining was shown in Fig. 3C. ADAMTS4 protein was highly expressed in 59.1% (204/345) CRC tissues (Table 1). Furthermore, the correlation between the ADAMTS4 expression and corresponding clinicopathological parameters of the CRC patients was analyzed. As shown in Table 1, ADAMTS4 protein expression was significantly correlated with tumor size and TNM stage, whereas no significant difference was found in age, gender, tumor location, serum CEA level and histology. To further determine the prognostic effect of ADAMTS4, the overall survival rate of patients with CRC in Ren Ji cohort was analyzed using Kaplan-Meier survival curves and the log-rank test. As a result, CRC patients with high ADAMTS4 level had a poor overall survival (OS) compared with those with a lower ADAMTS4 protein level (Fig. 3D). By univariate Cox regression analysis, we found that ADAMTS4 expression level, serum CEA level and TNM stage all are significant risk factors for OS (Table 2). And multivariate Cox regression analysis revealed that only TNM stage is an independent factor in the prediction of OS rate of CRC patients (Table 2). Collectively, these results indicated that over-expressed ADAMTS4 seemed to be a prognostic indicator in CRC and might contribute to the development and progression of CRC.

Fig. 2. The putative copy-number alterations of ADAMTS family member in TCGA (Provisional) cohort.

Table 1. Relationship between ADAMTS4 expression and clinicopathological features in 345 CRC patients. Values in parentheses indicate percentage values. The bold number represents the P-values with significant differences

Variable		ADAMTS4 (n)		P
		Low n = 141	High n = 204	
Age	≤ 65 years	77	110	0.913
	> 65 years	64	94	
Gender	Male	74	125	0.121
	Female	67	79	
Tumor size	≤ 5 cm	83	91	0.012
	> 5 cm	58	113	
Tumor location	Rectum	78	124	0.319
	Colon	63	80	
Serum CEA	≤ 5 ng/ml	76	105	0.663
	> 5 ng/ml	65	99	
Clinical stage	0-I	34	51	0.039
	II	53	56	
	III	41	86	
	IV	13	11	
Histology	Mucinous	21	35	0.657
	Non-mucinous	120 (20.8)	169	

Genetic silencing of ADAMTS4 inhibits tumor growth in vivo but not in vitro

Since ADAMTS4 is associated with malignant activity in clinical specimens, we investigated the oncogenic roles of ADAMTS4 *in vitro* and *in vivo* by loss-of-function assay. By real-time qPCR, we found that ADAMTS4 was commonly elevated in CRC cell lines compared

Fig. 3. Differentially expressed ADAMTS4 predicts a poor prognosis in CRC. (A) The expression level of ADAMTS family members in CRC; data were derived from TCGA cohort and were shown as RSEM (log2) value. (B) Kaplan-Meier analysis of the prognostic value of ADAMTS family members in TCGA cohort. (C) Representative immunohistochemical photos of ADAMTS4 in normal bowel tissues and CRC; scale bar: 100 μ m. (D) Kaplan-Meier analysis of survival curve for patients grouped based on ADAMTS4 expression in Ren Ji cohort. * p <0.05; ** p <0.01; *** p <0.001.

to the normal NCM460 cells (Fig. 4A). Two cell lines, Caco-2 and HCT15, which showed higher ADAMTS4 expression, were selected for next investigation. Two specific shRNAs targeting ADAMTS4 resulted in marked decrease in ADAMTS4 protein expression and secreted level (Fig. 4B). Plate clone formation assay showed that knockdown of ADAMTS4 failed to influence cell proliferation *in vitro* (Fig. 4C). Meanwhile, ADAMTS4 knockdown had no implication on the invasive potential of CRC cells as demonstrated by transwell assay (Fig. 4D). As the tumor promoter or suppressor functions of ADAMTS4 are largely dependent on the extracellular matrix, we further tested the effect of ADAMTS4 *in vivo*. Expectedly, silencing of ADAMTS4 resulted in a significant retardation in tumor growth (Fig. 4E). Consistently, the immunoreactivity of the proliferation index, Ki67, was also remarkably reduced in sh-ADAMTS4-1 group compared to the sh-Ctrl group (Fig. 4F). Taken together, these data above suggested that ADAMTS4 acted as an oncogene in CRC and its oncogenic roles might be dependent on the alteration of tumor microenvironment.

Table 2. Univariate and multivariate analysis of prognostic factors for survival in patients with CRC. HR: Hazard ratio; CI: Confidence interval. The bold number represents the P-values with significant differences

Parameters	HR	Univariate analysis		HR	Multivariate analysis	
		95% CI	P value		95% CI	P value
ADAMTS4 expression	2.254	1.305-3.421	0.004	1.412	0.976-2.365	0.074
Age	1.435	0.922-2.232	0.110	-	-	-
Gender	1.336	0.875-2.039	0.180	-	-	-
Tumor location	0.773	0.491-1.218	0.268	-	-	-
Tumor size	1.160	0.761-1.768	0.491	-	-	-
TNM stage	2.172	1.651-2.857	0.000	2.223	1.467-2.796	0.002
Serum CEA level	1.577	1.019-2.439	0.041	1.376	0.903-2.201	0.146

Macrophages mediate the oncogenic role of ADAMTS4 in the tumor growth of CRC

To pursue the mechanism by which ADAMTS4 promoted tumor growth *in vivo*, we annotated 1358 ADAMTS4-associated genes (selected by R2 software, $R > 0.5$) by Gene ontology analysis. The result showed that ADAMTS4 was profoundly involved in the cell adhesion, ECM receptor interaction, PI3K/AKT pathway, and etc (Fig. 5A). Notably, ADAMTS4 also associated with molecules involved in cytokine and chemokine signaling pathway, suggesting that ADAMTS4 might be implicated in inflammatory response (Fig. 6). Given

Fig. 4. Genetic silencing of ADAMTS4 inhibits tumor growth *in vivo* but not *in vitro*. (A) The mRNA expression of ADAMTS4 in five CRC cell lines and the normal NCM460 cells was detected by real-time quantitative PCR. (B) The interference efficiency of ADAMTS4 in HCT15 and Caco-2 cells was measured by western blotting and ELISA, respectively. (C) The sh-Ctrl and sh-ADAMTS4 cells were subjected to clonogenic assay and visualized colonies were then photographed and calculated. (D) The effect of ADAMTS4 knockdown on the invasive potential of CRC cells was analyzed by Transwell assay. (E) Tumor growth curves for tumors derived from sh-Ctrl and sh-ADAMTS4-1 HCT15 cells in athymic nude mice. $n = 6$ in each group. The results were expressed as the means $\pm$ SD. (F) The proliferation index Ki67 expression and sh-ADAMTS4-1 groups was analyzed by immunohistochemistry. Scale bar: 100 μ m. ** $p < 0.01$.

that macrophages are the most abundant immune cells in the tumor microenvironment, we hypothesized that whether ADAMTS4 contributes macrophages infiltration in the tumor microenvironment of CRC [22]. To test this hypothesis, we correlated ADAMTS4 expression with the expression of specific macrophages markers. We found that there was a close correlation between ADAMTS4 and CD68, a classical macrophages marker, indicating that ADAMTS4 may be associated macrophages infiltration in CRC (Fig. 5B). Furthermore, we noticed that ADAMTS4 had a much higher correlation coefficient with the marker of M2 macrophages CD163 than the M1 macrophages marker CD86 (Fig. 5C), indicating an anti-inflammatory response induced by ADAMTS4. To further test that whether macrophages mediates the oncogenic role of ADAMTS4 in CRC, we generated a subcutaneous xenograft derived from ADAMTS4-overexpressing CT26 cells or the control cells in the immune-competent BALB/c mice (Fig. 5D). Liposomal clodronate has been widely used as an effective tool to delete macrophages in various *in vivo* systems. The treatment schedule was shown in Fig. 5E. Treatment of clodronate liposomes was sufficient to deplete the myeloid cells in the spleen and bone marrow as revealed by flow cytometry (Fig. 5F). By IHC analysis, we found that tumors of ADAMTS4-overexpressing CT26 cells had more macrophages infiltration than that in OV-vector cells and the infiltrated macrophages in the tumor tissues were largely depleted by clodronate liposomes (Fig. 5G). Indeed, over-expression of ADAMTS4 promoted tumor growth. And macrophages inhibition had no significant inhibitory role in the OV-vector tumors, but largely abolished the growth advantage induced by ADAMTS4 (Fig. 5H). Therefore, these results demonstrated that macrophages were critically involved in the oncogenic functions of ADAMTS4 in CRC.

Discussion

Emerging data have demonstrated the significant role of ADAMTSs in tumor progression. In this study, we reported the overexpression pattern of ADAMTS4 and its prognostic value

Fig. 5. Macrophages mediate the oncogenic role of ADAMTS4 in the tumor growth of CRC. (A) Gene ontology analysis of ADAMTS4-associated genes ($R > 0.5$) derived from TCGA cohort. A total of 1358 genes was analyzed. (B) The correlation coefficient between CD68 and ADAMTS4. Data were acquired from TCGA cohort. (C) The correlation coefficient between CD86, CD163 and ADAMTS4 was analyzed via Spearman order correlation. (D) The overexpression efficiency of ADAMTS4 in the mouse CT26 cells was detected by western blotting. (E) BALB/c mice were treated with clodronate liposomes or PBS liposomes from week 1 until week 4. At week 2, CT26 cells were administered and tumor growth was monitored for 3 weeks. (F) The percentage of CD68+ cells in the spleen and bone marrow ($n = 5$) after treatment with clodronate liposomes or PBS liposomes was analyzed by flow cytometry. (G) The macrophages in the tumors after treatment with clodronate liposomes or PBS liposomes were analyzed by immunohistochemistry. Scale bar: 100 μm . * $p < 0.05$. (H) Tumor growth curves for OV-vector and OV-ADAMTS4 CT26 cells-derived xenografts in clodronate-liposome or PBS-liposome-treated mice. ** $p < 0.01$.

and oncogenic roles in CRC. Based on bioinformatics analysis, we revealed no significant mutation and copy-number alterations of ADAMTSs in CRC. Meanwhile, we for the first time uncovered a novel link between ADAMTS4 and macrophages, which ultimately facilitate tumor progression.

Previously, it has been reported that ADAMTS mutations contribute to outcomes in ovarian cancer cases without BRCA1 or BRCA2 mutations and may have important clinical implications. However, by a large-scale data mining, we failed to observe significant mutation frequency of ADAMTSs in CRC. The dysregulated ADAMTSs in cancers can be induced by many factors. For example, IL-6 upregulates ADAMTS-2 in human osteosarcoma cells mediated by JNK pathway [23]. ADAMTS-1, -9, and -18 have been found to be epigenetically silenced in malignant tumors [24]. In this study, no significant copy-number alterations of ADAMTSs were found in CRC. However, multiple ADAMTSs were found to be differentially expressed in CRC. Combined with the clinical follow-ups, we identified the overexpressed ADAMTS4 as a crucial factor in CRC progression. Consistently, the overexpressed prolife of ADAMTS4 has been reported in several cancers including glioblastoma [25], head and neck cancer [26], melanoma [27], and epithelial ovarian cancer [12]. By immunochemical analysis of a CRC tissue microarray, we confirmed that ADAMTS4 expression was elevated in CRC tissues compared to normal tissues and predicted a poor prognosis in CRC. ADAMTS4 and its proteolytic fragments can differentially affect tumor growth and angiogenesis in melanoma [27]. In this study, by loss-of-function study, genetic silencing of ADAMTS4 showed no obvious implications on cell proliferation and invasive capacity of CRC cells with high ADAMTS4 expression, but remarkably suppressed tumor *in vivo*, indicating the

Fig. 6. The ADAMTS4-associated genes in TCGA cohort annotated in the chemokine signaling pathway (A) and cytokine-cytokine receptor interaction category (B).

regulatory roles of ADAMTS4 are largely dependent on ECM. Extensive remodeling of the normal ECM in cancers can progress via the degradation of preexisting ECM molecules, such as aggrecan, brevican, and versican [12]. Alterations in the extracellular environment are critical for tumor initiation and progression. Indeed, based on bioinformatic analysis, we found the implication of ADAMTS4 on ECM. Specifically, we also noticed the potential role of ADAMTS4 in the inflammatory network of CRC tumor microenvironment. And we subsequently confirmed a link between ADAMTS4 and macrophages. To the best of our knowledge, our study is the first time to demonstrate this correlation. Macrophages can

promote tumor progression by enhancing chronic inflammation, matrix remodeling and angiogenesis. Deletion of macrophages exhibits antitumor effects in several xenograft tumor models [28]. In this study, clodronate liposomes mediated macrophage depletion did not influence tumor growth remarkably in control group, but largely compromised the promotive effect of ADAMTS4 on tumor growth of CRC in an immunocompetent mouse model, suggesting that the oncogenic roles of ADAMTS4 are at least partially dependent on the macrophages infiltrated in the tumor microenvironment. Notably, the clear mechanism by which ADAMTS4 modulates macrophages polarization and infiltration remains further investigation.

Conclusion

In summary, the results presented in the current study provide both bioinformatic and clinicopathological evidence to show the observation that overexpressed ADAMTS4 correlates tumor progression and predicts poor clinical outcome in CRC. Our findings also revealed a regulatory role of ADAMTS4 on macrophages in the CRC tumor microenvironment.

Disclosure Statement

The authors confirm that there are no conflicts of interest.

References

- Center MM, Jemal A, Ward E: International trends in colorectal cancer incidence rates. *Cancer Res* 2009;18:1688-1694.
- Essink-Bot ML, Dekker E: Equal access to colorectal cancer screening. *Lancet* 2016;387:724-726.
- Crotti S, Piccoli M, Rizzolio F, Giordano A, Nitti D, Agostini M: Extracellular matrix and colorectal cancer: How surrounding microenvironment affects cancer cell behavior? *J Cell Physiol* 2017;232:967-975.
- Freitas-Rodriguez S, Folgueras AR, Lopez-Otin C: The role of matrix metalloproteinases in aging: Tissue remodeling and beyond. *Biochim Biophys Acta* 2017;1864:2015-2025.
- Sakamoto T, Seiki M: Integrated functions of membrane-type 1 matrix metalloproteinase in regulating cancer malignancy: Beyond a proteinase. *Cancer Sci* 2017;108:1095-1100.
- Kumar S, Rao N, Ge R: Emerging roles of adamts in angiogenesis and cancer. *Cancers* 2012;4:1252-1299.
- Stanton H, Melrose J, Little CB, Fosang AJ: Proteoglycan degradation by the adamts family of proteinases. *Biochim Biophys Acta* 2011;1812:1616-1629.
- Filou S, Korpetinou A, Kyriakopoulou D, Bounias D, Stavropoulos M, Ravazoula P, Papachristou DJ, Theocharis AD, Vynios DH: Adamts expression in colorectal cancer. *PloS One* 2015;10:e0121209.
- Kelwick R, Desanlis I, Wheeler GN, Edwards DR: The adamts (a disintegrin and metalloproteinase with thrombospondin motifs) family. *Genome Biol* 2015;16:113.
- Apte SS: A disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif (adamts) superfamily: Functions and mechanisms. *J Biol Chem* 2009;284:31493-31497.
- Wagstaff L, Kelwick R, Decock J, Edwards DR: The roles of adamts metalloproteinases in tumorigenesis and metastasis. *Front Biosci* 2011;16:1861-1872.
- Lima MA, Dos Santos L, Turri JA, Nonogaki S, Buim M, Lima JF, de Jesus Viana Pinheiro J, Bueno de Toledo Osorio CA, Soares FA, Freitas VM: Prognostic value of adamts proteases and their substrates in epithelial ovarian cancer. *Pathobiology* 2016;83:316-326.
- Cal S, Lopez-Otin C: Adamts proteases and cancer. *Matrix Biol* 2015;44-46:77-85.
- Fontanil T, Rua S, Llamazares M, Moncada-Pazos A, Quiros PM, Garcia-Suarez O, Vega JA, Sasaki T, Mohamedi Y, Esteban MM, Obaya AJ, Cal S: Interaction between the adamts-12 metalloprotease and fibulin-2 induces tumor-suppressive effects in breast cancer cells. *Oncotarget* 2014;5:1253-1264.

- 15 Rocks N, Paulissen G, El Hour M, Quesada F, Crahay C, Gueders M, Foidart JM, Noel A, Cataldo D: Emerging roles of adam and adamts metalloproteinases in cancer. *Biochimie* 2008;90:369-379.
- 16 Chen L, Tang J, Feng Y, Li S, Xiang Q, He X, Ren G, Peng W, Xiang T: Adamts9 is silenced by epigenetic disruption in colorectal cancer and inhibits cell growth and metastasis by regulating akt/p53 signaling. *Cell Physiol Biochem* 2017;44:1370-1380.
- 17 Errico A: Gynaecological cancer: Adamts mutations predict sensitivity to chemotherapy in ovarian cancer. *Nat Rev Clin Oncol* 2015;12:504.
- 18 Yu MH, Luo Y, Qin SL, Wang ZS, Mu YF, Zhong M: Up-regulated cks2 promotes tumor progression and predicts a poor prognosis in human colorectal cancer. *Am J Cancer Res* 2015;5:2708-2718.
- 19 Wang ZS, Cong ZJ, Luo Y, Mu YF, Qin SL, Zhong M, Chen JJ: Decreased expression of interleukin-36alpha predicts poor prognosis in colorectal cancer patients. *Int J Clin Exp Pathol* 2014;7:8077-8081.
- 20 Liu Y, Yasukawa M, Chen K, Hu L, Broaddus RR, Ding L, Mardis ER, Spellman P, Levine DA, Mills GB, Shmulevich I, Sood AK, Zhang W: Association of somatic mutations of adamts genes with chemotherapy sensitivity and survival in high-grade serous ovarian carcinoma. *JAMA Oncol* 2015;1:486-494.
- 21 Yasukawa M, Liu Y, Hu L, Cogdell D, Gharpure KM, Pradeep S, Nagaraja AS, Sood AK, Zhang W: Adamts16 mutations sensitize ovarian cancer cells to platinum-based chemotherapy. *Oncotarget* 2016;8:88410-88420.
- 22 Catalano V, Turdo A, Di Franco S, Dieli F, Todaro M, Stassi G: Tumor and its microenvironment: A synergistic interplay. *Semin Cancer Biol* 2013;23:522-532.
- 23 Alper M, Kockar F: Il-6 upregulates a disintegrin and metalloproteinase with thrombospondin motifs 2 (adamts-2) in human osteosarcoma cells mediated by jnk pathway. *Mol Cell Biochem* 2014;393:165-175.
- 24 Vilorio CG, Obaya AJ, Moncada-Pazos A, Llamazares M, Astudillo A, Capella G, Cal S, Lopez-Otin C: Genetic inactivation of adamts15 metalloprotease in human colorectal cancer. *Cancer Res* 2009;69:4926-4934.
- 25 Held-Feindt J, Paredes EB, Blomer U, Seidenbecher C, Stark AM, Mehdorn HM, Mentlein R: Matrix-degrading proteases adamts4 and adamts5 (disintegrins and metalloproteinases with thrombospondin motifs 4 and 5) are expressed in human glioblastomas. *Int J Cancer* 2006;118:55-61.
- 26 Demircan K, Gunduz E, Gunduz M, Beder LB, Hirohata S, Nagatsuka H, Cengiz B, Cilek MZ, Yamanaka N, Shimizu K, Ninomiya Y: Increased mrna expression of adamts metalloproteinases in metastatic foci of head and neck cancer. *Head Neck* 2009;31:793-801.
- 27 Rao N, Ke Z, Liu H, Ho CJ, Kumar S, Xiang W, Zhu Y, Ge R: Adamts4 and its proteolytic fragments differentially affect melanoma growth and angiogenesis in mice. *Int J Cancer* 2013;133:294-306.
- 28 Griesmann H, Drexel C, Milosevic N, Sipos B, Rosendahl J, Gress TM, Michl P: Pharmacological macrophage inhibition decreases metastasis formation in a genetic model of pancreatic cancer. *Gut* 2017;66:1278-1285.