Original Article

ADAMTS12 acts as a cancer promoter in colorectal cancer via activating the Wnt/β-catenin signaling pathway *in vitro*

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Background: ADAMTS12, a member of the ADAMTS family, is reported to be associated with the clinic outcome of colorectal cancer (CRC) patients. However, the functions and precise mechanism in CRC progression have yet to be fully understood.

Methods: By analyzing The Cancer Genome Atlas (TCGA) database, we examined the mRNA level of ADAMTS12 and assessed the prognostic value of ADAMTS12 in CRC patients using a tissue microarray containing 41 CRC patient samples. Cell Counting Kit-8 (CCK-8), colony formation, and transwell assays were used to quantify the impact of ADAMTS12 on cell proliferation and migration in ADAMTS12-overexpressing and ADAMTS12-deficient cell lines. The signaling pathways that ADAMTS12 mediated were identified by dual-luciferase reporter assays, and confirmed by western blotting and quantitative teal-time polymerase chain reaction (qRT-PCR).

Results: The ADAMTS12 mRNA level was upregulated in CRC tissues, and CRC patients with a high level of ADAMTS12 showed worse prognosis when compared with the patients with a low level of ADAMTS12. *In vitro* functional assays demonstrated that overexpression of ADAMTS12 significantly boosted cell proliferation and migration while ADAMTS12 deficiency remarkably impaired both tumor cell behaviors. Mechanical studies further verified that ADAMTS12 overexpression enhanced the transcriptional activity of β-catenin in the Wnt/β-catenin signaling pathway. In the ADAMTS12-deficient context, the downstream gene expression of myc and cyclin D1 was significantly reduced compared with that in wild-type cancer cells.

Conclusions: ADAMTS12 fulfills the tumor-promotor role by activating Wnt/β-catenin signaling pathway in colon cells and may represent a new option in CRC target treatment.

Keywords: ADAMTS12; Wnt/β-catenin; colorectal cancer (CRC); migration; proliferation

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Introduction

Colorectal cancer (CRC) is ranked as one of most prevalent malignant neoplasms in the digestive system (1). In 2017, it was responsible for about 19.0 million disability-adjusted life-years (DALYs), 1.8 million newly diagnosed cases, and 896,000 cancer-associated deaths (2). Currently, key interventions to the high burden of CRC have been adopted which include the removal of polyps and early diagnosis, and these have been reported to improve prognosis and prolong survival (3). However, the treatment and prevention of CRC is still a global challenge due to the dynamic genomic alternations during CRC progression. For these

reasons, the identification of critical molecules in CRC is urgently needed to improve treatment.

ADAMTS12 is a member of the ADAMTS family and contains 19 genes encoding zinc metalloproteinase in mammals. First, described in 1997, this protease family is a group of extracellular, multifunctional enzymes, which may function as a critical regulatory factor implicated in the reciprocal interactions of cells, or cells with extracellular matrices (4,5). So far, numerous studies have demonstrated that ADAMTS metalloproteinase participates in diverse physiological and physiopathological processes in human acquired disorders such as osteoarthritis (6), cardiovascular disease (7), platelet coagulopathy (8), and cancer (9). In cancer-associated progression, ADAMTS12 shows a dual pro or/and anti-tumoral role in proteolytic or non-proteolytic ways. In renal cell carcinoma, ADAMTS12 is listed as 1 of 7 extracellular matrix (ECM) genes, and upregulated and high expression of ADAMTS12 may contribute to metastases (10). The pro-tumoral function has also been demonstrated in human choriocarcinoma JEG-3 cells with the enhancement of tumor invasion in the ADAMTS12-overexpressing cell line (11). Inversely, *in vitro* and *in vivo* findings have demonstrated that Adamts12-deficiency displays an increasing angiogenic response and an enhanced tumor invasion, suggesting that ADAMTS12 is a potential antitumogenic factor (12). In CRC, ADAMTS12 has been reported as a factor affecting prognosis of patients (13,14), and epigenetic silence of ADAMTS12 may promote the proliferative properties of tumor cells (15). However, the concrete function and underlying mechanism that ADAMTS12 exerts in CRC have not been sufficiently clarified.

Herein, we are first to report that ADAMTS12 is increased in CRC and, through an analysis of The Cancer Genome Atlas (TCGA) data, we discovered that the increased expression of ADAMTS12 is related to worse prognosis. Mechanically, we found that ADAMTS12 influenced CRC proliferation and migration by targeting Wnt/β-catenin signaling in vitro. Thus, our findings highlight the critical role of ADAMTS-12 for CRC progression and suggest that ADAMTS-12 is a potential therapeutic target for CRC patients.

Methods

Reagents, antibodies, and cell lines

Anti-ADAMTS12, anti-flag, anti-GAPDH, and anti-rabbit horseradish peroxidase (HRP) secondary antibodies were obtained from indicated companies. A β-catenin gene luciferase reporter plasmid was constructed according to the standard molecular biology experiments and applied to determine which signaling pathway the candidate gene modulated. HEK293T and HCT116 cell lines were kept in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U penicillin/streptomycin in standard conditions. All cell lines were purchased from TCGA.

Constructing plasmids and transfection

To obtain Flag-ADAMTS12, cDNA-encoding ADAMTS12 was cloned into a p3×FLAG-CMV-10 vector. An ADAMTS12 knockout plasmid was established by CRISPR/Cas9 hitKO technology. Transient transfection was completed by Lipofectamine 2000 according to the manufacturer's instructions. The post-transfected cells were incubated and harvested for subsequent assays.

Immunohistochemical analysis

To validate the expression of ADAMTS12 in CRC tissues, we purchased a CRC microarray that included tissues from 41 cancer patients and 41 paracancerous tissues from Wuhan Aiwei Biotechnology Co., Ltd. and carried out an immunohistochemical assay with EnVision kit. Briefly, the deparaffinized and rehydrated slides were pretreated with 0.3% hydrogen peroxidase, followed by blockage with 1% bovine serum albumin. Next, the slides were incubated successively with anti- ADAMTS12, the second antibody, and 3,3-diaminobenzidine (DAB). Finally, HIC staining was performed with Mayer's hematoxylin before visualization.

Western blot analysis

To confirm the overexpression of indicated proteins, the total cell lysates were harvested, and the protein concentration was determined by Bradford assay. Equivalent protein was resolved by SDS-PAGE and transferred to PVDF membranes. After being placed in blocking buffer for 1 h, the membrane was blotted by the matched antibody for 24 h at 4 ℃, followed by secondary antibodies for 1 h. The blotted protein was visualized by HRP Color Development Solution.

Cell proliferation assay

Cell count KIT-8 assay was performed to determine the proliferative capacity of ADAMS12-overexpressing or -deficient HCT116 cells, and 2×10^3 cells were cultured in a 96-well plate at 37 ℃ with the 450 nm absorption being recorded every 24 h. Experimental procedures were performed in triplicate.

Clonogenic assay

A colony formation experiment was used to examine the clonogenic potency of tumor cells. The cells were harvested by trypsinization, detached by pipette and quantified using a hemocytometer. Next, 1×10^3 seed cells were incubated in dishes at 5% CO_2 and 37 ℃ for 10 days. The colonies were counted with a stereomicroscope after fixation and staining.

In vitro cell migration assay

The motility of metastatic cancer cells was examined in a 24-well format transwell apparatus with an 8 μm pore size insert. After trypsinization, the transfected cells were suspended in 200 μL serum-free DMEM and seeded in the upper compartment. Next, the transwell inserts were added to the lower compartment containing DMEM with 0.5% FBS. After incubation at 37 ℃ and 5% CO_2 for 10 h, the insert was taken out and the remaining cell debris was removed with a cotton swab. The cells on the lower side of the insert were counted under a microscope after fixation and cystal violet staining.

Luciferase reporter assays

Since Wnt/β-catenin signaling is closely associated with CRC progression, we examined the impact of the ADAMTS12 expression to the transcriptional activity of b-catenin by luciferase reporter assays (16). HEK293T was transfected with ADAMTS12 expression plasmids or control plasmids and subsequently cotransfected with the β-catenin luciferase reporter vectors and pR-TK control vectors via Lipofectamine 2000. Then, 36 h after transfection, the cells were harvested and prepared so they could go undergo detection for changes in reporter luciferase activities.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

To further confirm the signaling pathway the protein directly modulated, we carried out real-time quantitative polymerase chain reaction (RT-qPCR) to examine the classical downstream target genes of Wnt/β-catenin signaling cascade. The cells were lysed with Trizol reagent. Total RNA was extracted and reverse transcribed according to a traditional molecule clone protocol. The expression level of myc and cyclin D1 was measured with the $2^{-\triangle\triangle Ct}$ method.

Statistical analysis

GraphPad Prism 7.0 Windows was used for all analyses. For continuous variables, the values are expressed as mean ± SEM. Unpaired two-tailed Student's *t*-test was carried out to compare the differences of the ADAMTS12 expression level between tumor tissues and the matched normal adjacent tissues. Prognostic values of colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) patients were analyzed by TCGA database. The viability and migration capacity of the investigated cells were subjected to Mann-Whitney U test. Spearman's correlation analysis was performed to examine the ADAMTS12 and Wnt/β-catenin signaling pathway. One-way analysis of variance (ANOVA) was applied to compare the difference of the key gene expression level in Wnt/β-catenin signaling pathway. A P value of less than 0.05 was regarded as significant.

Results

Overexpression of ADAMTS-12 in colorectal carcinoma

Firstly, we extracted and analyzed the data of CRC and the corresponding adjacent normal tissues from TCGA database. As illustrated in *Figure 1A*, the mRNA expression of ADAMTS12 in tumor tissues was significantly elevated in comparison with that in their matched benign samples. Kaplan-Meier analysis was further employed to verify the prognostic role of ADAMTS12 expression. The results showed that the overexpression of ADAMTS12 corresponded to the unfavorable prognosis, while no significant association was found between its expression and COAD prognosis (*Figure 1B*). In addition, a tissue microarray consisting of 41 colorectal tissue samples was adopted to examine the expression of ADAMTS12. *Figure 1C* illustrates the remarkably increased expression of ADAMTS12 in tumor tissues, which is consistent with the result of TCGA analysis. All the above findings suggest that

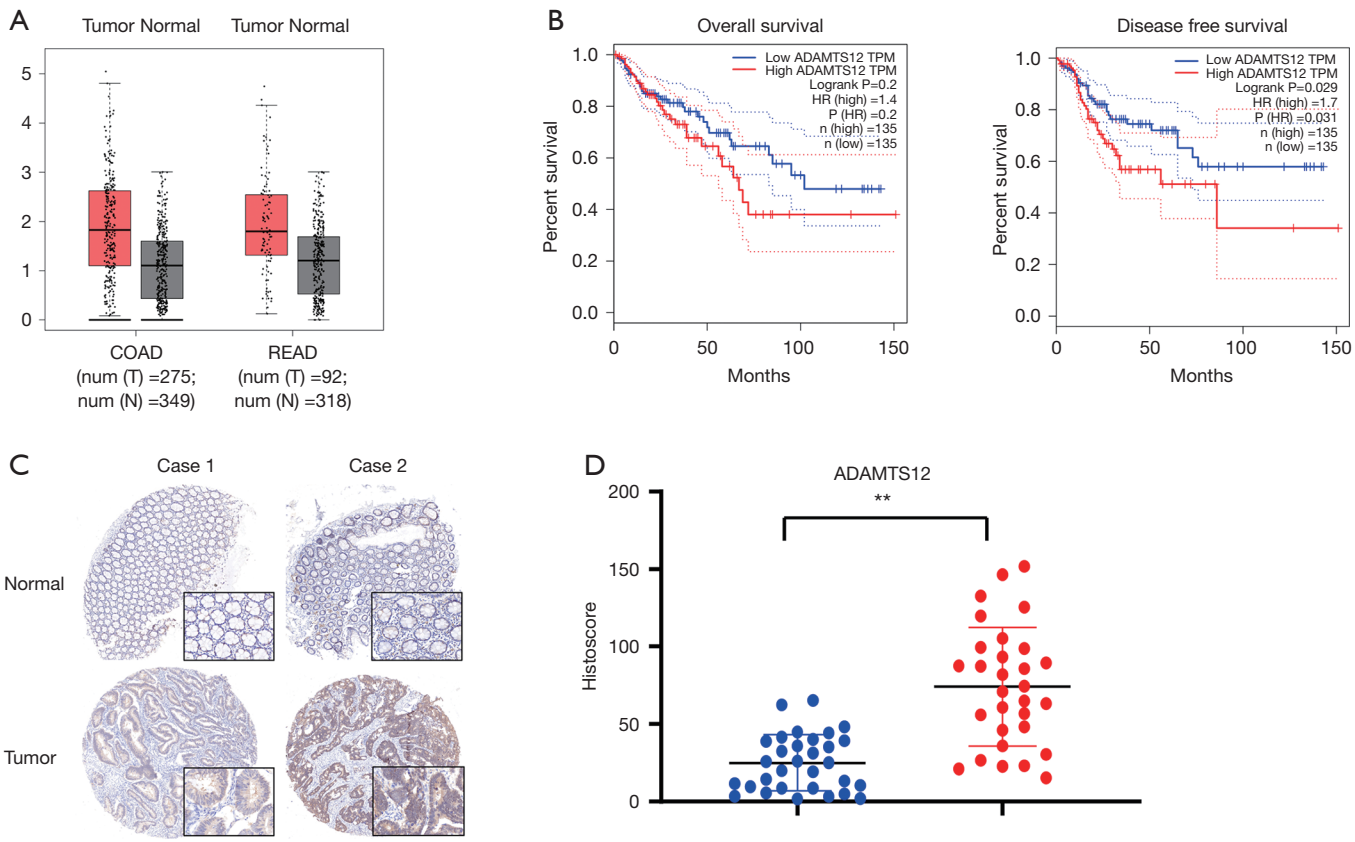

Figure 1 ADAMTS12 expression in CRC cancers. (A) ADAMTS12 expression in 275 COAD and 92 READ cancers tissues in comparison with paired normal adjacent tissues from TCGA; (B) ADAMTS12 gene on CRC patient survival assessed via online Kaplan-Meier-plotter; (C) typical example of ADAMTS12 expression in CRC tissues and adjacent tissues via immunohistochemical staining (IHC) (Magnification: ×4); (D) ADAMTS12 IHC histoscore assessed on the CRC tissues microarray consisting of 41 CRC tissues and 41 adjacent tissues. CRC, colorectal cancer; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; TCGA, The Cancer Genome Atlas; IHC, immunohistochemistry. **, P<0.01.

ADAMTS12 was increased in COAD and READ tissues and may play a pro-oncogenic role in COAD and READ progression.

Ectopic ADAMTS-12 expression promotes tumor proliferation and migration in vitro

To further determine the tumorigenic properties of ADAMTS12, we carried out a series of *in vitro* assays in cells with stable ectopic expression of ADAMTS12. We constructed a flagged ADAMTS12 expression plasmid and transfected it into HCT116 cells, with western blot being used to validate the transfection efficiency with flag-antibody (*Figure 2A*). Using CCK8 and colony formation assay, we found that overexpression of ADAMTS12 in HCT116 cells significantly promoted the cell viability in comparison with parent cells (*Figure 2B,C*). In addition, transwell assay was used to test the influence of ADAMTS-12 to the migratory activity of HCT116 cells. In cells of overexpressed ADAMTS12, we also observed a marked increase in migratory potency when compared with the parental cells (*Figure 2D*). These data suggest that overexpression of ADAMTS12 significantly increase colon cell proliferation and migration.

ADAMTS12 deficiency restrains tumor growth and migratory activity in vitro

On account of the obvious overexpression of ADAMTS12 in CRC, we used the HCT116 cell lines to construct

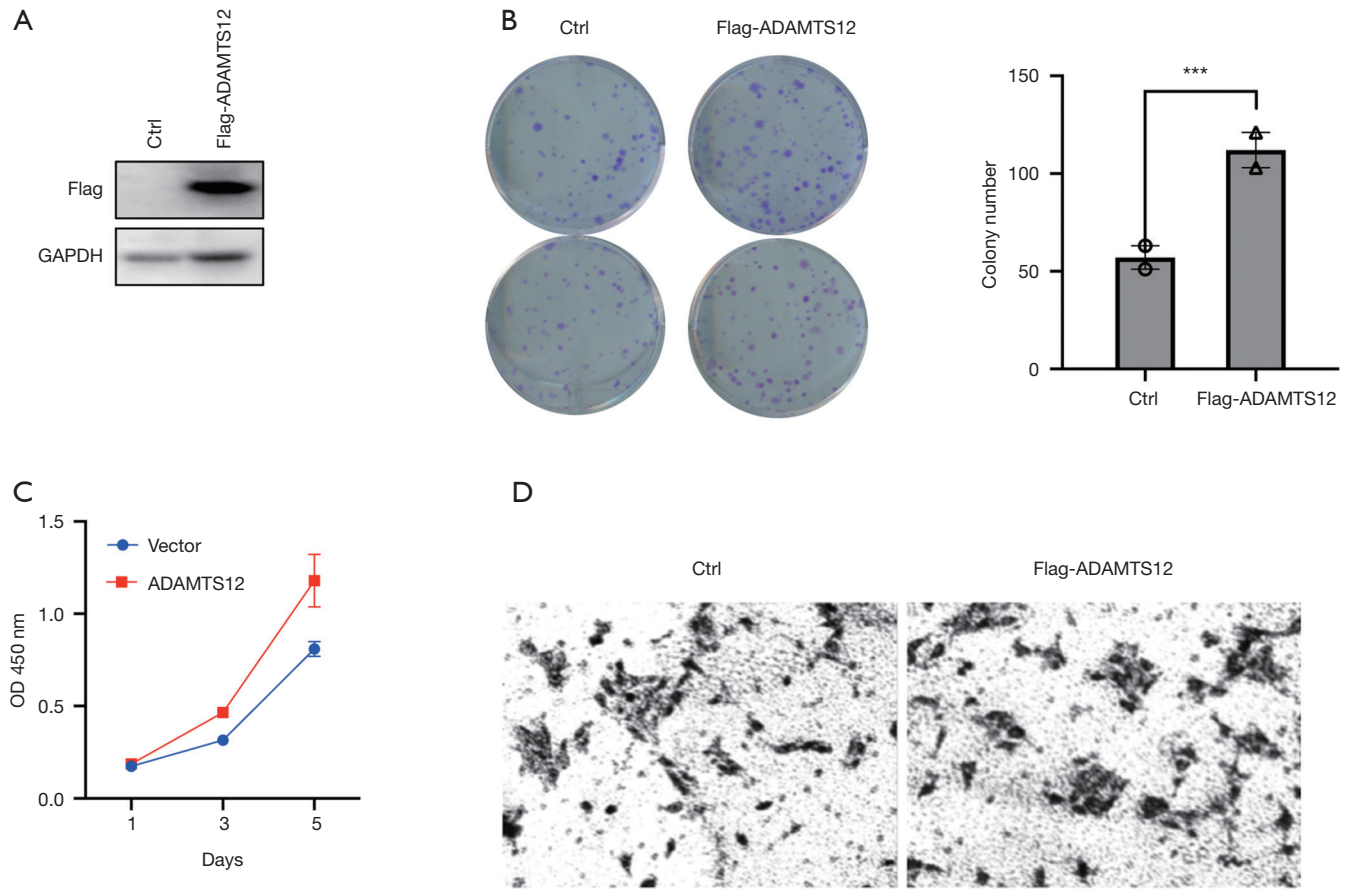

Figure 2 Overexpression of ADAMTS12 promotes the proliferation and migration of CRC cells. (A) Western blot analysis of the expression of ADAMTS12 with anti-flag; (B,D) clone formation assay and transwell assay for cancer cells with ADAMTS12 overexpressing and the control. The cells were imaged (×20); (C) the proliferation curve of HCT116 after transfection with flag-ADAMTS12 compared with the parental cells. CRC, colorectal cancer. ***, P<0.001.

ADAMTS12-deficient cells by CRISPR/Cas9 hitKO technology. Western blot was performed for knockout validation (*Figure 3A*). In the ADAMTS12-deficient background, the colony formation capacity was remarkably reduced, and the number of migratory cells displayed a noticeable decrease (*Figure 3B,C,D*). Taken together, the genetic inactivation of ADAMTS12 significantly relieved the malignant phenotypes of HCT116 cells.

ADAMTS12 activates Wnt/β-catenin signaling by targeting β-catenin

Subsequently, we implemented a luciferase reporter assay to assess the potential changes in the wnt/β-catenin signaling cascades that ADAMTS12 might have affected in HEK239 cells. As illustrated in *Figure 4A*, the transcriptional activity of Wnt/β-catenin was stimulated significantly in ADAMTS12-overexpressing cell lines. To confirm the modulation of ADAMTS12 in Wnt/β-catenin signaling cascade, we subsequently examined the key downstream molecules in the Wnt/β-catenin signaling event. As seen in *Figure 4B,C,D*, myc and cyclin D1, 2 classical target genes of Wnt/β-catenin, were dramatically decreased in ADAMTS12 knockdown HCT116 cell lines, suggesting that ADAMTS12 might be implicated in the malignancy of CRC by directly modulating Wnt/β-catenin signaling pathway.

Discussion

In recent years, several studies have suggested that ADAMTS12 plays a dual pro or/and anti-tumoral role in

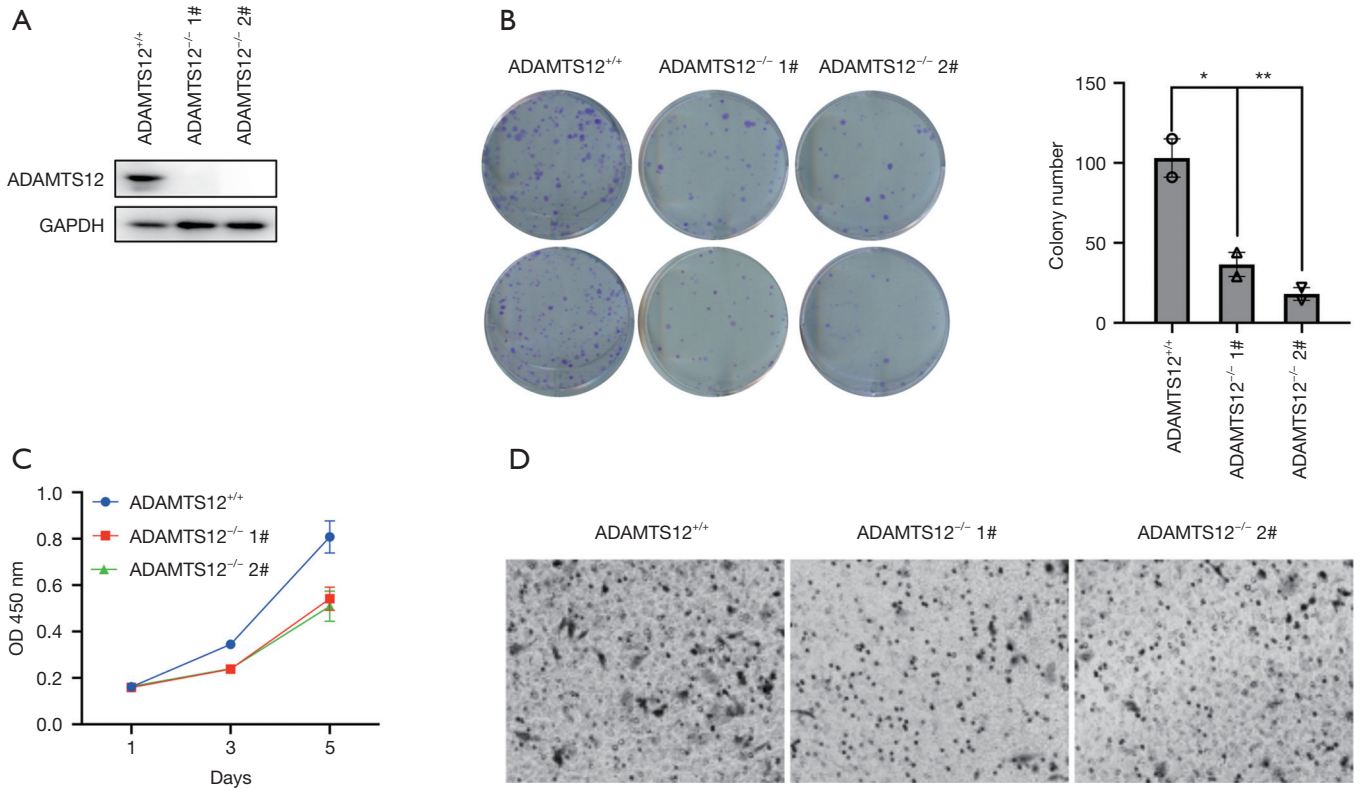

Figure 3 Knockout of ADAMTS12 gene inhibits the proliferation and migration of CRC cells. (A) Western blot analysis of the expression of ADAMTS12 with the antibody of anti-ADAMTS12; (B,D) clone formation assay and transwell assay for cancer cells with ADAMTS12 deficiency and the parental cells. The cells were imaged (×20); (C) the proliferation curve of HCT116 cells with ADAMTS12-defiencient groups compared with the parental cells. CRC, colorectal cancer. *, P<0.01; **, P<0.001.

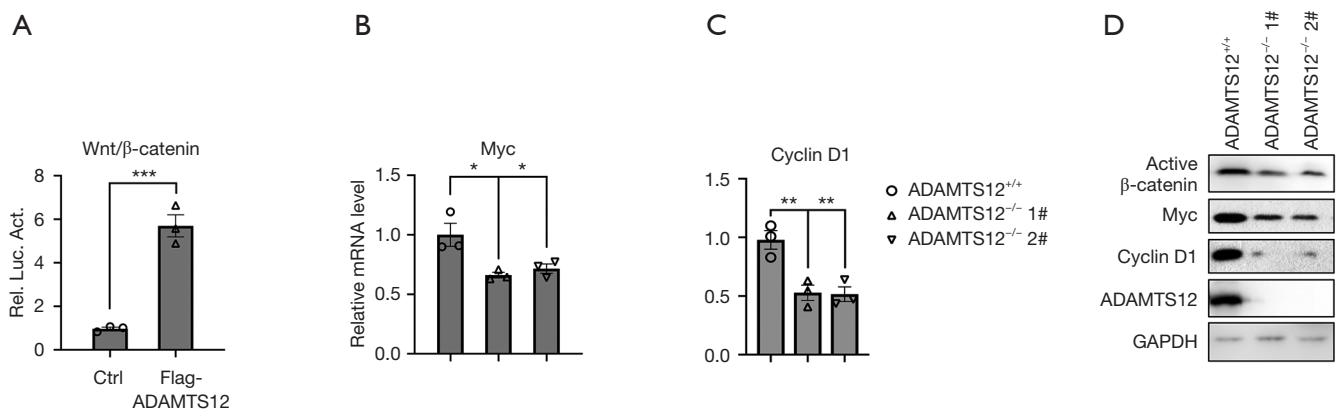

Figure 4 Overexpression of ADAMTS12 promotes Wnt/-b signaling activity. (A) The luciferase reporter gene assay was carried out to screen the overexpression of ADAMTS12 on transcriptional activities of β-catenin, which is the critical regulator of Wnt/β-catenin signaling pathway; (B) Western blot and qRT-PCR were performed to verify the downstream gene of Wnt/-b signaling activity (cylin D1 and myc). qRT-PCR, quantitative teal-time polymerase chain reaction. *, P<0.01; **, P<0.01; ***, P<0.001.

cancer progression, including in tumorigenesis. However, the precise mechanisms of ADAMTS12 in CRC have not yet been fully understood. Here, we underscored the pro-tumor role of ADAMTS12 in CRC in vitro. Subsequent mechanical findings suggest that ADAMTS12 promoted the viability and migratory behaviors of CRC cells via Wnt1/β-catenin signaling pathway.

ADAMTS12, a secreted metalloproteinase, is a critical regulator in key ECM remodeling events related to tumor malignant behaviors and its aberrant expression is associated with numerous diseases including cancer (12,17). However, its role in CRC is much less understood. First, we compared the ADAMTS12 expression in CRC tissues and its paired adjacent normal tissues by analyzing the data from TCGA and hydrophobic interaction chromatograph (HIC) assays, and the results showed that ADAMTS12 expression was significantly upregulated in tumor tissues. In this regard, our findings were consistent with those of Moncada-Pazos *et al.* (13), who pointed out that *ADAMTS12* gene was activated in stroma surrounding malignant cells. Next, we carried out Kaplan-Meier survival analysis using the download data, and the results revealed that the high expression of ADAMTS12 was relevant with shorter overall survival (OS) and disease-free survival (DFS), suggesting that ADAMTS12 may serve as an unfavorable prognostic indicator for CRC progression. Yet, these results were contrary to a report from Wang and his colleagues. They tested the ADAMTS12 expression of 112 cases of CRC tissues by immunohistochemical staining (14), and their results demonstrated that absence of ADAMTS12 expression in CRC tissues was a poor indicator for CRC patients, indicating ADAMTS12 may serve as a tumor-protective role in CRC progression. A possible explanation for these differences are that the stroma can induce a pro-tumor-anti-tumor response switch when interacting with the neoplastic cells (18).

To further explore the concrete role of ADAMTS12 in CRC progression, we employed a series of *in vitro* function assays. The results showed that exogenous expression of ADAMTS12 could remarkably promote CRC cell proliferation and migration while silencing ADAMTS12 via CRISPR/Cas9 hitKO technology impaired the proliferative and migratory activity of CRC cells. It is universally known that activating sustained proliferation and metastasis are the typical hallmarks of cancers. As for ADAMTS, evidence has shown that these metalloproteinases interact with cells and ECM-related factors to functionally influence tumor cells migration, proliferation, and invasion. For example, ADAMTS12 was upregulated in invasive human trophoblastic cells while in poorly invasive JEG-3 cells the overexpression of ADAMTS12 could enhance the invasion (11). Accordingly, ADAMTS12 may function as a pro-tumor factor in CRC development and progression *in vivo*.

Numerous previously published reports have also highlighted the importance of Wnt/β-catenin signaling pathway in the CRC progression (16). Aberrant activation of Wnt/β-catenin signaling pathway enhances the expression of myc and cyclin D1, and eventually contributes to the malignant behaviors of tumor cells. We conducted a dual-luciferase reporter gene assay in order to explore the underlying mechanism of ADAMTS12-mediated cancer proliferation and metastasis. These results indicate that ADAMTS12 positively regulates the transcriptional activity of β-catenin, which was reconfirmed by the lower expression of the downstream target genes including myc and cyclin D1 in cells deficient of ADAMTS12. Our findings suggest that, ADAMTS12 mediated the proliferation and migration of CRC cells via Wnt/β-catenin signaling pathway, which is has been shown to have an important impact on epithelial-mesenchymal transformation (EMT) in HCT116 cells (19). On the other hand, ADAMTS has been reported to play a dominant role in degrading ECM components, and modulating migration and angiogenesis, which may be further trigger epithelial-to-mesenchymal transition (EMT) (20). For example, Adamts18 and ADAM15 are known to interact with E-cadherin which is a critical factor to EMT, modulating the malignant phenotypes of tumor cells (21,22). Thus, we speculated that ADAMTS12 may mediate EMT by Wnt/β-catenin signaling pathway to increase colon tumorigenesis. To our knowledge, we are first to report ADAMTS12 exerting its potency in a non-enzymatic way. Although our study supports the association of ADAMTS12 and EMT, the ADAMTS12-regulated mechanism still needs to clarify in further detail.

In addition, previous studies reported that ADAMTS12 was epigenetically silenced in CRC, acting as a tumor-suppressor enzyme in CRC progression (13,15). However, our results verified the contradictory role of ADAMTS12 in CRC cell lines. These results suggest that ADAMTS12 may indeed play a dual role in CRC progression. The dual properties of ADAMTS12 can also be observed in ADAMTS1 and is caused by the equilibrium of different fragments (23). As for ADAMTS12 in colon cells, it may be reinstated with stimulation (13), which may give us an explanation for the switching between the 2 roles. In

addition, transcriptional modification of ADAMTS12 may offer us some clues to explain the dual roles in CRC progression (15). Therefore, subsequent studies will need to elucidate the mechanical balance of ADAMTS12's dual role induced by CRC.

In conclusion, we demonstrated ADAMTS12 is significantly unregulated in CRC tissues and that ADAMTS12-high CRC patients showed poor survival. Based on the overexpression and knockdown experiment in cells, ADAMTS12 influenced the proliferative and migratory potency of CRC cells by Wnt/β-catenin signaling pathway. Our findings offer new insight into ADAMTS12's role in CRC carcinogenesis, and suggest that ADAMTS12 may be a valuable therapeutic target for CRC.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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