MARCH1 Interacts with β-Catenin as a New Oncogene for Gastric Cancer

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Abstract

Background:

Membrane-associated RING-CH 1 (MARCH1) is an E3 ubiquitin ligase that plays an important role in antigen presentation. The latest research found that MARCH1 can either promote or suppress cancer progression in ovarian, liver, and bladder cancers, but its function in gastric cancer has not been addressed. This study aimed to investigate the role of MARCH1 in gastric cancer.

Methods:

The Cancer Genome Atlas (TCGA) database was used to evaluate the expression level and biological function of MARCH1 in gastric cancer. We down-regulated and up-regulated the expression level of MARCH1 in gastric cancer cell line AGS by transfecting siRNAs and overexpression plasmids. Then we detected cell proliferation, migration, invasion and apoptosis using CCK-8 assay, transwell chamber assay and flow cytometry respectively. The relationship between MARCH1 and β-catenin was analyzed using western blotting assay.

Results:

The results showed that the expression of MARCH1 in gastric cancer was elevated and significantly related to clinical stage, tumor grade, and lymph node metastasis. It is worth noting that there was no significant correlation between the increase in MARCH1 expression level and overall survival. In addition, knocking down and upregulating the expression level of MARCH1 significantly affected the proliferation, migration, invasion, and apoptosis of gastric cancer cells. Furthermore, the study found that MARCH1 and β-catenin positively regulated each other, suggesting that MARCH1 may participate in the malignant biological behavior of tumors through the Wnt/β-catenin pathway.

Conclusions:

In summary, this study shows the function of MARCH1 in the progression of gastric cancer and provides unique insights into the regulatory mechanism of MARCH1 and β-catenin, which indicates that MARCH1 may be a new target molecule for gastric cancer.

Background

Gastric cancer is one of the most common cancers worldwide. Among different cancer types, the incidence of gastric cancer ranks fourth, after lung, breast, and colorectal cancers. The mortality rate in gastric cancer patients is second only to lung cancer[1, 2]. Due to the unclear pathogenesis of this disease and lack of effective treatment methods, the rate of late diagnosis and mortality are very high. Gastric cancer is biologically and genetically heterogeneous, and its carcinogenic mechanism is not clear at the molecular level[3]. Therefore, studying the mechanisms of gastric cancer progression, identifying
biodiagnostic markers for gastric cancer metastasis, and developing targeted therapy drugs using specific molecules are of great significance for the prevention and treatment of gastric cancer.

In our previous study, we screened the differentially expressed genes in gastric cancer and adjacent tissues using the Cancer Genome Atlas (TCGA) database. We found that the expression level of MARCH1 was higher in gastric tumor samples than in paired normal tissues. The MARCH family is a family of E3 ubiquitin ligases that was discovered in recent years, and consists of MARCH1-11[4]. MARCH family proteins control the stability, transportation, and function of important immune receptors[5]. In addition to their role in immunity, MARCH family proteins also play key roles in physiological processes such as spermatogenesis, membrane transport, and lipid synthesis[6, 7]. As a member of this family, MARCH1 is highly expressed in secondary lymphoid tissues, B cells, monocytes, and immature dendritic cells[7]. It downregulates protein expression level by guiding MHC-II and other protein molecules to ubiquitination and degradation in the lysosomal compartment, thereby controlling the antigen presentation process[8]. This shows that MARCH1 plays an important role in the immune system regulation.

Early studies found that MARCH1 played an important role in antigen presentation mainly by downregulating the expression levels of MHC-II and CD86 on the surface of antigen presenting cells[9]. However, in recent years, people have gradually discovered that MARCH1 also seemed to play a role in both tumor promotion and tumor suppression[10–12]. A previous study by our group found that MARCH1 mRNA expression was higher in gastric cancer tissues than in adjacent tissues. We also found that the expression level of MARCH1 was significantly higher in the gastric cancer cell line AGS than in the normal gastric mucosa epithelial cell line GES-1. However, the specific functions of MARCH1 and the underlying mechanism of its involvement in gastric cancer progression have not been fully clarified. In this study, bioinformatics analysis was performed to explore the prognostic value of MARCH1 in gastric cancer. In addition, its biological functions were also evaluated to determine how MARCH1 is involved in gastric cancer progression. The results of this study proved the importance of MARCH1 in the development and progression of gastric cancer and laid the foundation for further research.

**Methods**

**TCGA database**

Gastric cancer tissue samples with mRNA expression data and corresponding clinicopathological data were downloaded from the cancer genome atlas (TCGA) database. Samples were divided into tumor (n=375) and healthy tissues (n=32) to evaluate the differential expression of MARCH1. Some patients had incomplete clinicopathological characteristics data. These patients were included in the analysis of clinicopathological characteristics they possessed, but excluded in the analysis of information they lacked.

**Cell culture**
AGS cell line were originally owned by the laboratory, which was cultured in RPMI-1640 medium (Biological Industries, China) supplemented with 10% fetal bovine serum (Biological Industries, China) and 1% penicillin-streptomycin solution (HyClone, US) at 37°C in 5% CO₂ atmosphere. 0.25% trypsin (Biological Industries, China) was used to digest and passage the cell until they reached 90% confluence.

Cell transfection

Cells grown in the logarithmic phase were plated with 1×10⁵ per well in a six-well plate, or 5×10³ in a 96-well plate the day before transfection. After 24h the cell density is about 60%~80% and then the cells were transfected with siRNAs or plasmids using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Two siRNAs against MARCH1, negative control (NC) siRNAs and the MARCH1 overexpression plasmid were synthesized by Genepharma (Shanghai, China). The sequences for the MARCH1 siRNA were: siMARCH1-1, 5′-CAGGAGGUCUUGUCUUAUTT-3' and 5'-AUGAAGACAAGACCUCUGT-3'; siMARCH1-2, 5'-GGUAGUGCCUGUACCACAATT-3' and 5'-UUGUGGUAGGACACUAACCTT-3'. The NC siRNA sequences were: 5′-UUCUCCGGAACGUUCAGUTT-3' and 5′-ACGUGACACGUUCGGAGATT-3'.

Western blotting

Whole-cell protein lysates were extracted using cell lysis buffer (Beyotime, China), and the protein concentrations were determined by the BCA assay (Solarbio, China). The lysates were boiled in SDS polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (EpiZyme, China) for 5-10 minutes at 99°C and separated on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) by electroblotting, and after blocking in 5% nonfat milk (Sangon Biotech, China) for 2-3h. Then the membrane was incubated with primary antibodies against anti-MARCH1 (immunoway, China), anti-β-catenin (immunoway, China), anti-tubulin α (immunoway, China) at 4°C overnight and then left with the secondary antibodies peroxidase-conjugated goat anti-rabbit (BOSTER, China) for 60 minutes at room temperature. Finally the membranes were quantified using an enhanced chemiluminescence signal (EpiZyme, China). Photometric analyses of immunoblots were carried out using the Image Lab software package. The quantitative analysis through ImageJ software.

Cell proliferation assay

Cell proliferation assays were performed using a Cell Counting Kit-8 (CCK-8, MCE, China). According to the manufacturer's instructions, 5000 cells were seeded in 96-well plates overnight before treatment and per group set 5 auxiliary holes. Transfect the plasmid or siRNA according to the instructions, stop the culture at 0h, 24h, 48h, 72h after transfection. CCK-8 reagent was added to each well, and after incubation with the reagent for 2h at 37°C. The absorption and reference wavelength was measured at 450nm. Then we draw proliferation graphs of different groups over time.
Transwell assay

Cell migration and invasion assay were performed using 6.5mm transwell insert chambers with a 8.0µm pore polycarbonate membrane. The cells (3000), which were transfected after 24h were cultured in serum-free medium, placed in the upper chambers and coated with Matrigel basement membrane matrix (Corning, USA) for 2h at 37°C before the cells were added. The medium with 20% FBS was added to the down chamber. The cells were incubated for 48h, and cells which did not migrate through the pores were removed with a cotton swab. Then the upper chambers were fixed in 4% paraformaldehyde, stained with 0.5% crystal violet (Sangon Biotech, China) and counted under a photo microscope. However, there was no need for the Matrigel coating for the cellular migration assay.

Cell apoptosis assay

The apoptosis assays were performed using an Annexin V, FITC Apoptosis Detection Kit (Dojindo, Japan) according to the manufacturer’s instructions. We collected at least 10,000 cells after transfection, washed them twice with cold PBS, and used 100µL Annexin V Binding Solution to make cell suspension. The cell suspension was incubated with 5µL of Annexin V-FITC and 5µL of PI for 15 minutes, followed by apoptosis analysis by flow cytometry (BD Biosciences, USA).

Statistic analysis

Statistical analysis was performed by SPSS 26.0. GraphPad Prism7.0 software was used for statistical drawing. The Mann-Whitney U test was used to compare MARCH1 expression in normal and tumor tissues. The Wilcoxon signed-rank test was used to compare MARCH1 expression in paired tissues. To examine the association between clinicopathological characteristics and MARCH1 expression (after excluding normal tissue sample data), the Wilcoxon signed-rank test was performed for comparison between two groups [distant metastasis (M stage) and alive status], and the Kruskal-Wallis test was performed for multi-group comparison [TNM Stage, Grade, tumor stage (T stage) and lymph node metastasis (N stage)]. The median value of MARCH1 expression was selected as the cut-off value, and patients with gastric cancer were subsequently divided into high and low-expression groups. Kaplan-Meier survival curves were constructed and log rank tests performed to evaluate the association between MARCH1 expression and overall survival (OS). The independent-sample t-test is used for comparison between the two groups, the one-way analysis of variance (ANOVA) is used for the comparison between multiple groups. *p < 0.05, **p < 0.01 and ***p < 0.001 were considered statistically significant.

Results

Upregulation of MARCH1 in gastric cancer
To explore the expression level of MARCH1 in gastric cancer, MARCH1 mRNA expression data were extracted from the TCGA database. The results showed that MARCH1 was significantly upregulated in gastric cancer tissues compared with normal tissues (P < 0.001) (Fig. 1A). In addition, gastric tumors expressed significantly higher levels of MARCH1 than paired normal tissues did (P < 0.001) (Fig. 1B).

**Correlation Of March1 Expression With Clinicopathological Factors**

Using the median of the expression value of MARCH1 as the cut-off value, patients with gastric cancer were divided into high and low expression groups. The association between MARCH1 expression and the clinicopathological characteristics was reviewed and analyzed in 375 cases (Table I). Results showed that 188 cases (50.1%) exhibited high expression levels of MARCH1, while 187 cases (49.9%) exhibited low expression levels of MARCH1. As indicated in Fig. 2A-F, the upregulation of MARCH1 was significantly associated with TNM stage, histological grade, tumor stage, lymph node metastasis, and distant metastasis (Fig. 2A-F).

In addition, these data revealed that MARCH1 expression was not significantly correlated with clinical outcomes (Fig. 2G). The correlation between MARCH1 levels and patient prognosis was investigated revealing that high MARCH1 expression was not correlated with poor overall survival.

**Construction Of March1 Knockdown And Overexpression Ags Cell Lines**

To further study the role of MARCH1 in gastric cancer, we used small RNA interference (siRNA) technology to reduce the expression level of MARCH1 in a gastric cancer cell line (AGS). The results showed that siMARCH1-1 and siMARCH1-2 could efficiently downregulate the expression level of MARCH1 protein compared with siNC (P < 0.01) (Fig. 3A). Next, we transfected the MARCH1 overexpression plasmid (pEX-3-MARCH1) to the AGS cell line; AGS cells transfected with an empty vector plasmid (pEX-3) were used as control. Accordingly, the expression level of MARCH1 protein was significantly higher in the pEX-3-MARCH1 group than in the pEX-3 group (P < 0.001) (Fig. 3B).

**The effect of knockdown and overexpression of MARCH1 on cell proliferation**

We analyzed the effects of knockdown and overexpression of MARCH1 on the proliferation of AGS cells. The CCK-8 assay results showed that MARCH1 knockdown significantly inhibited proliferation of the AGS cells (P < 0.01) (Fig. 4A), whereas overexpression of MARCH1 promoted proliferation in these cells (P < 0.05) (Fig. 4B).

**The effect of knockdown and overexpression of MARCH1 on cell migration**
To further explore the role of MARCH1 in cell migration, we used a transwell assay to evaluate the migration of the AGS cell line after knockdown and overexpression of MARCH1. The results of the Matrigel-free transwell assay showed that the average number of cells that migrated in the siNC, siMARCH1-1, and siMARCH1-2 groups were 239.75 ± 29.44 cells/HP, 163.75 ± 17.97 cells/HP, and 80.5 ± 6.66 cells/HP, respectively (P < 0.01) (Fig. 5A). The average number of cells that migrated in the pEX-3 group and pEX-3-MARCH1 group were 30.00 ± 8.88/HP and 50.17 ± 6.37 cells/HP, respectively (P < 0.01) (Fig. 5B). Therefore, knockdown and overexpression of MARCH1 were observed to inhibit and promote the migration of AGS cells, respectively.

The effect of knockdown and overexpression of MARCH1 on cell invasion

We used a Matrigel-covered transwell chamber to perform the cell invasion assay. The results showed that the average number of cells that migrated in the siNC group, siMARCH1-1, and siMARCH1-2 groups were 70.00 ± 5.00 cells/HP, 26.67 ± 2.52 cells/HP, and 44.00 ± 3.61 cells/HP, respectively (P < 0.01) (Fig. 6A). After overexpression of MARCH1, the average number of cells in the pEX-3-MARCH1 group that migrated through the chamber was 64.00 ± 5.57 cells/HP, which was greater than that in the empty vector pEX-3 group (42.33 ± 4.73 cells/HP) (P < 0.01) (Fig. 6B).

The effect of knockdown and overexpression of MARCH1 on cell apoptosis

To investigate whether MARCH1 had an effect on cell apoptosis, cell apoptosis assays were performed using flow cytometry. The apoptosis rates in the siMARCH1-1 and siMARCH1-2 groups were significantly higher than that in the siNC group (P < 0.01) (Fig. 7A). Moreover, pEX-3-MARCH1 group had a lower apoptosis rate than the pEX-3 group (P < 0.01) (Fig. 7B).

MARCH1 and Wnt/β-catenin pathway form a feedback loop to regulate each other

The Wnt/β-catenin signaling pathway is one of the most commonly dysregulated pathways in tumors. To verify the effect of MARCH1 on the Wnt/β-catenin pathway, we used western blotting to test the expression level of β-catenin after downregulating and upregulating MARCH1. The results showed that compared to the siNC group, the siMARCH1-1 and siMARCH1-2 groups had reduced β-catenin expression levels (P < 0.01) (Fig. 8A). The β-catenin expression levels observed in the pEX-3-MARCH1 group were higher than those in the pEX-3 group (P < 0.001) (Fig. 8B). These results indicated that MARCH1 may be involved in the regulation of the Wnt/β-catenin pathway. To further explore the association between MARCH1 and β-catenin, we used XAV-939, an inhibitor of the Wnt/β-catenin pathway. We treated AGS cell line with two different doses of XAV-939 (5 µM and 10 µM). The western blotting assay was performed to detect the expression level of MARCH1 after administering XAV-939 to the AGS cell line. Interestingly, the results showed reduction in MARCH1 protein level in the AGS cell line after XAV-939 treatment (P < 0.05) (Fig. 8C). Inhibiting the Wnt/β-catenin pathway was observed to inhibit the expression of MARCH1, indicating that Wnt/β-catenin can regulate the expression of MARCH1.

Discussion
In the present study, MARCH1 was shown to be upregulated in gastric cancer. Although it had little effect on the survival in gastric cancer patients, it was closely related to the tumor pathological grade and clinical stage. MARCH1 may be an independent indicator of poor prognosis in gastric cancer. Experimental studies found that downregulating or upregulating the expression level of MARCH1 in gastric cancer cells could significantly change tumor progression.

A previous study found that MARCH1 was highly expressed in ovarian cancer, which was the first report on MARCH1 function in tumors[10]. The research reported that MARCH1 promoted the proliferation and invasion of liver cancer cells through the PI3K-AKT-β-catenin pathway. In vivo experiments have also suggested that MARCH1 may be a potential oncogene in liver cancer[11]. Other studies have found that resveratrol affected the biological characteristics of liver cancer by downregulating the expression level of MARCH1[13]. In contrast, MARCH1 exerted a tumor suppressor effect in bladder cancer, and ciRs-6 increased the expression level of MARCH1 by sponge adsorption of miR-653 to inhibit the growth of bladder cancer[12]. These studies suggest that MARCH1 can be a potential target for cancer treatment; however, its function in gastric cancer has not been reported. Our research demonstrated that changing the expression level of MARCH1 could affect malignant biological functions in gastric cancer cells such as cell proliferation, migration, invasion, and apoptosis, which is consistent with studies on liver and ovarian cancers. The present study provides evidence for the role of MARCH1 in the occurrence and progression of gastric cancer.

However, it is important to clarify the underlying mechanism of MARCH1 action and its downstream target in gastric cancer. The Wnt signaling pathway is a well-studied signaling pathway that regulates cell growth, migration, and differentiation during development[14]. Activation of the Wnt/β-catenin signaling pathway has been found in approximately 30–50% of gastric cancer tissues and various types of gastric cancer cell lines[15]. Our study found that knocking down and over-expressing MARCH1 reduced and increased the expression level of β-catenin, respectively, and further affected cellular functions, which suggests that β-catenin may be a downstream target of MARCH1. To further explore the mechanism of interaction between MARCH1 and β-catenin, we used XAV-939, a Wnt/β-catenin pathway inhibitor[16, 17]. The results showed that XAV-939 reduced MARCH1 expression in gastric cancer cells, and the higher the concentration of XAV-939, the lower the expression of MARCH1. This suggests that inhibiting the Wnt/β-catenin pathway inhibits the expression of MARCH1, implying that the Wnt/β-catenin pathway can regulate the expression of MARCH1. To the best of our knowledge, this study is the first to reveal the molecular mechanism of MARCH1 regulating β-catenin levels in gastric cancer. Further studies are needed to determine the precise mechanisms of MARCH1 and β-catenin in the occurrence of gastric cancer.

In conclusion, this study showed that MARCH1 plays a role in gastric cancer progression by upregulating β-catenin and may be a new tumorigenesis biomarker for the disease. Therefore, MARCH1 can be a potential target for the treatment of gastric cancer. In addition, these data indicate that further research on the association between MARCH1 and β-catenin may be helpful for targeted therapy of gastric cancer.
Conclusion

In summary, our study evaluated the expression level, biological function, and prognostic value of MARCH1 in gastric cancer. We found that MARCH1 expression level was higher in patients with gastric cancer and this was significantly associated with clinical stage, tumor grade, and lymph node metastasis. Moreover, our findings suggest that MARCH1 is involved in the regulation of cell proliferation, migration, invasion, and apoptosis in gastric cancer cells. Apart from that, our findings also indicate that MARCH1 may regulate tumor malignancy via Wnt/β-catenin pathway.

Abbreviations

TCGA: The Cancer Genome Atlas; MARCH1: Membrane-associated RING-CH 1; MHC-II: MHC class II gene; FBS: fatal bovine serum; FITC: fluorescein isothiocyanate; siRNA: small RNA interference

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analysed during the current study are available in the Cancer Genome Atlas database. https://portal.gdc.cancer.gov/

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NW, LY, CL designed this study. NW, DY, XJ, and JD completed the experiment. NW, AW, and KW analyzed the data. NW interpreted the data and wrote the manuscript. LY and CL substantively revised the manuscript. All authors reviewed and agreed to submit the manuscript. All authors declared no conflict of interests in this study.

Acknowledgments
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Tables

Table I. Relationship between expression of MARCH1 and clinicopathologic features of patients with gastric cancer.
| Variables          | MARCH1 high | MARCH1 low | P-value |
|--------------------|-------------|------------|---------|
|                   | n=188       | n=187      |         |
| n                  | %           | n          | %       |
| TNM Stage<sup>a</sup> |             |            | 0.006   |
| StageI             | 14          | 8.2        | 39      | 21.5    |
| StageII            | 56          | 32.7       | 55      | 30.4    |
| StageIII           | 81          | 47.4       | 69      | 38.1    |
| StageIV            | 20          | 11.7       | 18      | 9.9     |
| Histological grade<sup>b</sup> |          | 0.001      |         |
| G1                 | 4           | 2.1        | 6       | 3.4     |
| G2                 | 51          | 27.3       | 86      | 48.0    |
| G3                 | 132         | 70.6       | 87      | 48.6    |
| T stage<sup>c</sup> |             | 0.043      |         |
| T1                 | 4           | 2.2        | 15      | 8.0     |
| T2                 | 37          | 20.6       | 43      | 23.0    |
| T3                 | 83          | 46.1       | 85      | 45.5    |
| T4                 | 56          | 31.3       | 44      | 23.5    |
| N stage<sup>d</sup> |             | 0.042      |         |
| N0                 | 43          | 24.4       | 68      | 37.6    |
| N1                 | 55          | 31.2       | 42      | 23.2    |
| N2                 | 37          | 21.0       | 38      | 21.0    |
| N3                 | 41          | 23.3       | 33      | 18.2    |
| M stage<sup>e</sup> |             | 0.755      |         |
| M0                 | 167         | 92.3       | 163     | 93.7    |
| M1                 | 14          | 7.7        | 11      | 6.3     |
| Alive status<sup>f</sup> |         | 0.658      |         |
| Alive              | 110         | 58.5       | 114     | 61.3    |
| Dead               | 78          | 41.5       | 72      | 38.7    |
Seventeen samples of the TNM stage of MARCH1 high expression group were missed and six samples of the TNM stage of MARCH1 low expression group were missed. One sample of the grade of MARCH1 high expression group were missed and eight samples of the grade of MARCH1 low expression group were missed. One sample of the T stage of MARCH1 high expression group were missed. Twelve samples of the N stage of MARCH1 high expression group were missed and six samples of the N stage of MARCH1 low expression group were missed. Seven samples of the M stage of MARCH1 high expression group were missed and thirteen samples of the M stage of MARCH1 low expression group were missed. One sample of the status of MARCH1 low expression group were missed. Bold values are statistically significant (P<0.05).

T stage, tumor stage; N stage, lymph node metastasis; M stage, distant metastasis.

Figures

Figure 1

Comparison of MARCH1 expression between gastric cancer tissues and normal tissues using data from TCGA. (A) The expression of MARCH1 in gastric cancer tissues (n=375) was significantly higher compared with normal tissues (n=32). (B) Compared with 27 paired normal tissues, gastric cancer tissues exhibited significant upregulation of MARCH1. ***P<0.001.
Figure 2

Analysis of TGCA datasets was conducted to determine expression of MARCH1 and its association with clinicopathological factors. (A) TNM stage; (B) Histological grade; (C) T stage; (D) N stage; (E) M stage; (F) Alive status; (G) Kaplan Meier curves.
The CCK-8 assay detects the cell viability of knockdown group (A) and overexpression group (B) in AGS cell line at 0, 24, 48, 72h. Results were presented as mean ± SD of three independent experiments.

Figure 5
The transwell assay detects the effect of knockdown and overexpression of MARCH1 on cell migration. Magnification 100×. Results were presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6

A

![Images of siNC, siMARCH1-1, siMARCH1-2](image1.jpg)

B

![Images of pEX-3, pEX-3-MARCH1](image2.jpg)

Figure 6

The transwell assay detects the effect of knockdown group (A) and overexpression group (B) on cell invasion at 48h. Magnification 100×. Results were presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 7

The cell apoptosis rate of knockdown group (A) and overexpression group (B) in AGS cell line at 48h. Results were presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.