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Original Paper

Sh-MARCH8 Inhibits Tumorigenesis via PI3K Pathway in Gastric Cancer

Junfeng Yin[a] Ze Ji[b] Yidong Hong[c] Ziyan Song[c] Nan Hu[c]
Min Zhuang[c] Baoxiang Bian[c] Yi Liu[d] Fenglei Wu[c]

[a]Department of General Surgery, The Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, [b]Department of Respiratory Medicine, Suzhou Kowloon Hospital, Shanghai Jiaotong University School of Medicine, Suzhou, [c]Department of Oncology, Affiliated Lianyungang Hospital of Xuzhou Medical University, Lianyungang, [d]Department of Pathology, Affiliated Lianyungang Hospital of Xuzhou Medical University, Lianyungang, China

Key Words
MARCH8 • Gastric cancer • Apoptosis • PI3K pathway • ß-catenin stat3 pathway

Abstract
Background/Aims: To identify new treatment strategies for gastric cancer and to elucidate the mechanism underlying its pathophysiology, we transfected sh-MARCH8 into the human gastric cancer cell lines MKN-45 and AGS to investigate the roles of MARCH8 in gastric cancer. ***Methods:*** We used genetic engineering to construct the sh-MARCH8 interference plasmid and transfected it into gastric cancer cells. Colony formation assays and cell viability measurements were performed to detect the viability and proliferation of cancer cells. Wound healing assays were performed to estimate the migration and proliferation rates of the cells. Cell invasion assays were used to estimate the invasive abilities of the cells. Cell apoptosis analysis was performed by using flowing cytometry. Western blot analysis was performed to estimate the expression levels of proteins. Statistical analysis was performed using the SPSS 18.0 software. Student's t-test was used to determine the significance of all pairwise comparisons of interest. ***Results:*** We observed that the transfection of sh-MARCH8 inhibited the survival and proliferation of MKN-45 and AGS cells. The migration and invasion of the MKN-45 and AGS cells were significantly decreased, and apoptosis was induced in comparison with the control cells. These results were further confirmed by data showing that sh-MARCH8 increased the BAX/BCL2 ratio in MKN-45 and AGS cells. We also observed that sh-MARCH8 inactivated the PI3K and ß-catenin stat3 signaling pathways by changing protein expression levels or the phosphorylation of related proteins. ***Conclusion:*** These data suggested that sh-March8 reduced viability and induced apoptosis of the MKN-45 and AGS cells through the PI3K and ß-catenin stat3 signaling pathways. Taken together, our data revealed that transfection of sh-MARCH8 into the MKN-45 and AGS gastric cancer cell lines inhibited their growth, and this approach may be useful as a novel strategy for gastric cancer therapy.

J. Yin, Z. Ji and Y. Hong contributed equally to this work.

Fenglei Wu
Department of Oncology, Affiliated Lianyungang Hospital of Xuzhou Medical University
182 Tongguan Road north Lianyungang Jiangsu (China)
Tel. +86 0518 85456700, Fax +86 0518 85456700, E-Mail fengleiw@126.com

Introduction

Since researchers determined the genetic basis of cancer in 1914 [1], the carcinogenesis of many types of cancer has been studied, and the genetic theory of cancer has been accepted [2]. Gastric cancer (GC) is a highly heterogeneous disease with different molecular and genetic changes, including several chromosomal alterations [3, 4], variations in epigenetics [5-9], and miRNA involvement [10]. GC is the fifth most common cancer and the third most common cause of cancer deaths worldwide, accounting for 8.8% of all cancer-related deaths (723, 000 deaths) [11, 12]. Even today, in east Asia, Latin America and eastern Europe, as well as specific subgroups in the United States, the incidence and mortality of gastric cancer remains disproportionately high [11, 12].

Over the past several decades, the study of GC has provided information on its genetics, epigenetic inheritance, transcriptome, and metabolic changes [13-18]. Based on Laurence's histopathological classification, a new molecular classification system was proposed to better classify gastric tumors [19-22].

The MARCH (membrane-associated RING-CH) family of E3 ubiquitin ligases was found with the aid of the viral protein homolog to interfere with host defenses [23]. Similar to most ubiquitin ligases, members of the MARCH family target various membrane proteins for degradation [24-27]. To date, eleven members of the MARCH family have been identified and characterized. Most of these ligases share a basic structure with c-MIR, the founding member of the family. Now known as MARCH8, this protein contains an N-terminal RING finger domain that potentially interacts with an E2 enzyme, as well as two trans-membrane domains [23, 25, 28]. These proteins usually contain several (two, four, or twelve) trans-membrane (TM) domains; however, MARCH7 and MARCH10 do not contain any predicted TM domains [29].

The functions of MARCH8 and its close relative MARCH1 have been studied mostly in immune cells, where these proteins mediate the ubiquitination and downregulation of immune-regulatory cell surface molecules, including MHC-II, Fas, and CD86 (B7.2) [24, 25, 30-33]. MARCH8 also controls cell surface expression of some additional proteins [34, 35]. It has been observed that MARCH8 is expressed in the early embryos of zebrafish and *Xenopus*, suggesting that this protein might have a role in embryogenesis [37]. MARCH1 may be involved in dendritic cell maturation by promoting the ubiquitination and degradation of MHC-II and CD86 [29, 32, 38, 39]. MARCH10 and MARCH11 are highly expressed in the testes and are predicted to play a pivotal role in spermatogenesis and the organization of spermatid flagella [40-42]. Several other MARCH family members are widely expressed in various tissues, but their physiological roles are largely unknown.

In the present study, we aimed to investigate the roles of MARCH8 in human GC and to elucidate the underlying mechanisms. In this study, we report that knockdown of MARCH8 reduced viability and induced apoptosis in MKN-45 and AGS cells through the PI3K and ß-catenin stat3 signaling pathways. These data suggest that MARCH8 inhibition may be a novel strategy for gastric cancer therapy.

Materials and Methods

Agents

The RPMI-1640 medium was from HyClone Company (Cat# SH30809.1, Logan, Utah, USA). The fetal goat serum was from Gibco (Cat# A31608-02,USA). The antibiotics, 0.25% trypsin, CCK-8 and DMSO were from Beijing Solarbio Science and Technology Company (Cat# P1400, T1300, CA1210, D8370, Beijing, China). Lipofectamine2000 was obtained from Invitrogen (Cat# 1777190, USA). The Annexin V-FITC/PI Apoptosis Kit was ordered from the 4A Biotech Company (Cat# FXP018-100, Beijing, China). The primary antibodies, including AKT CST (Cat# 9272, 1:1000); p-AKT CST (Cat# 4060, 1:1000); mTOR CST (Cat# 2972,

1:1000); p-mTOR CST (Cat# 5536, 1:1000); BCL2 Abcam (Cat# ab692, 1:1000); BAX Abcam (Cat# ab32503, 1:1000); active-caspase-3 Abcam (Cat# ab13585, 1:1000); β-catenin PTG (Cat# 51067-2-AP, 1:1000); E-CAD PTG (Cat# 20874-1-AP, 1:1000); CYDLIN D1 Abcam (Cat# ab40754, 1:1000); P-STAT3 Abcam (Cat# ab30647, 1:1000); GAPDH PTG (Cat# 60004-1-Ig, 1:5000); and all rabbit anti-human antibodies were from Abcam (Cambridge, United Kingdom). The HRP sheep anti-rabbit/mouse secondary antibodies (1:5000) were from PTG (USA).

The ultrapure RNA extraction kit (HiFiScript cDNA Synthesis Kit), fluorescence quantitative PCR kit (UltraSYBR Mixture), RIPA lysis buffer, BCA protein assay kit, and protease inhibitor cocktail were all from Beijing Kangwei Century Company (CwBio, Beijing, China). Additionally, the primers were synthesized by Genewiz (Beijing, China). The Matrigel was from BD Biosciences (354230, USA), and the Transwell cell culture plates were from Millipore (M160439, USA). The cell culture equipment was from Eppendorf (Hamburg, Germany). The LDS sample buffer was from Invitrogen (NP0007, USA). The protein marker was from ThermoScientific (SM1881, USA), and the ECL developer was from PTG (B500022, USA).

Construction of sh-MARCH8 interference plasmid

The MARCH8 interference sequence is 5′-CTTGAGCTGAATGAGAGAATA-3′. For insertion into the p-Super vector, *Bgl* II and *Hind* III restriction enzyme sites were fused to each terminus. The plasmid containing the MARCH8 interference sequence was confirmed by enzyme digestion and sequencing.

Cell culture and transfection

The MKN-45 and AGS gastric cancer cell lines were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were incubated in RPMI-1640 medium containing 10% FBS, 100U/ml penicillin and 0.1mg/ml streptomycin at 37°C with 5% CO_2. The cells were trypsinized during the logarithmic phase and were re-plated into a fresh 6-well plate. The sh-MARCH8 transfection was performed using Lipofectamine2000 following the manufacturer's instructions. The p-Super empty vector was used as the control.

Fluorescence quantitative PCR

The total RNA was extracted using the Ultrapure RNA Extraction Kit, and the cDNA was synthesized with the HiFiScript cDNA Synthesis Kit. The expression of MARCH8 was detected by fluorescence quantitative PCR, and the primers used are listed below:

MARCH8:

Forward: 5′-GGGAGAAGTTGCAGATGAC-3′

Reverse: 5′-GCACATACAAGGACCAGAC-3′

β-actin was used as standard control:

Forward: 5′-CCCGAGCCGTGTTTCCT-3′

Reverse: 5′-GTCCCAGTTGGTGACGATGC-3′

The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

The transfected cells were cultured in a 6-well plate to 95% confluence. Forty-eight hours after transfection, the cells were lysed with ice-cold RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0 and protease inhibitors), and the protein concentration was measured using the BCA method. The protein samples were denatured by heating at 95°C for 5 minutes, and approximately 20 μg was loaded into each lane in a 10% SDS-PAGE gel. After transferring the protein to the PVDF membrane, it was blocked with 5% non-fat milk for 1 hour at room temperature and incubated with primary antibodies in blocking solution at 4°C overnight. The membrane was then washed three times for 5 minutes with TBST and was later incubated with the relevant secondary antibody in blocking buffer at room temperature for 1 hour. After washing, the membrane was covered with the ECL substrate for signal development. The images were acquired using darkroom development techniques for chemiluminescence.

Cell viability and proliferation assays

After transfection for 24 h, the cells were trypsinized and counted to make a suspension. Approximately 1000 cells were seeded into each well of a 96-well plate. Cell viability was checked every 24 hours by adding 10 µl of CCK8 reagent. After incubation at 37 °C for 90 minutes, an OD value of excitation light was detected using enzyme standard instrument with 450 nm. A proliferation curve was plotted using the OD values.

Colony formation assays

After transfection for 24 h, the cells were trypsinized to produce a single-cell suspension and were counted. Approximately 500 cells were seeded into each 10 cm dish with 5 ml of medium. The dishes were placed in an incubator at 37°C with 5% CO_2 and left there until the cells formed sufficiently large clones. The medium was removed, and the cells were rinsed carefully with PBS. The cells were then fixed with 5 ml 4% paraformaldehyde for 30 minutes followed by staining with 0.1% crystal violet for 30 minutes. The crystal violet was carefully removed followed by rinsing with tap water. The dishes with the colonies were left to dry in normal air at room temperature. The numbers of colonies were counted and compared to the control group.

Wound healing assays

Wound healing assays were carried out to estimate the migration and proliferation rates of the cells. The cells were plated in a 6-well plate at 5×10^5 cells per well and incubated at 37 °C overnight to obtain a 100% confluent cell monolayer. Twenty-four hours after incubation, a line was scratched with a pipette tip to destroy a small area of the cell layer. After washing with PBS, the cells were incubated in a serum-free medium. The open gaps were later examined using a microscope over a time range of 0, 24 or 48 hours, and the sizes of the gaps were measured with ImageJ software at 6 to 8 different points.

Cell invasion assays

A coating buffer (containing 0.01M Tris pH 8.0, 0.7% NaCl and filtered through a 0.2-µm sterile filter unit) was prepared before the experiment. Any pipettes, syringes, or containers that came into contact with the Matrigel were chilled prior to use. The aliquot of the Matrigel matrix was placed on ice at 4°C overnight. The Matrigel was subsequently diluted with serum-free 1640 medium at a 1:6 ratio, and 100 µl were applied to each permeable support well of a 24-well plate. This plate was incubated at 37°C for 4 hours. The remaining coating buffer was removed from the permeable support membrane without disturbing the Matrigel layer. Next, 100 µl and 600 µl of serum-free 1640 medium were added to the inside and outside of the support well, respectively, followed by incubation at 37°C for 30 minutes. The coated invasion chambers were then ready for use. The cells were trypsinized and re-suspended in serum-free culture medium. After that step, 100 µl of the cell suspension (containing 1×10^4 cells) was added to each of the 24-well invasion chambers and 600 µl of medium containing 10% FBS was added to the outside of the chamber.

Twenty-four hours after incubation at 37°C, the non-invading cells were removed using a cotton swab and both sides of the chambers were washed twice with 1X PBS. The cells were fixed with 4% paraformaldehyde at room temperature for 15 minutes and stained with 0.1% crystal violet for 5 minutes. After washing with 1X PBS, the filters were cut off and mounted on slides. The cells were observed and imaged, and the numbers of invaded cells were counted under a microscope.

Flow cytometric analysis of cell apoptosis

After 24 more hours of incubation in serum-free medium for starvation, the cells were trypsinized with EDTA-free trypsin. After washing with PBS, the cells were re-suspended in 1X binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$). The cell density was adjusted to 3 5 x 10^5 cells/ml. After that step, 5 µl of Annexin V-FITC solution was added to the 100 µl of cell suspension and incubated at room temperature in the dark. Next, 10 µl of 20 µg/ml propidium iodide (PI) was added for two more minutes of double staining. The results were analyzed with a flow cytometer (BD FACSC anto II, BD Biosciences, San Jose, CA, USA). The viable cells were negative for both PI and Annexin V, while the apoptotic cells were positive for Annexin V and negative for PI. Late apoptotic cells showed both Annexin V and PI positivity. The apoptotic rate was calculated using the BD FACSDiva software.

Immunohistochemical analysis

An EliVisionTMplus kit (KIT-9902) was used for the immunohistochemical analysis. The tissue sections were dewaxed using xylene and rehydrated with an ethanol gradient. Antigen retrieval was completed by boiling the sections two times in sodium citrate for 10 minutes. The slides were treated with 3% H_2O_2 for 10 minutes followed by washing with TBS. After blocking for 1 hour at room temperature, primary antibodies were applied to the sections, and they were incubated for 1 hour at room temperature. The slides were developed with DAB developer and doubled stained with hematoxylin, dehydrated with gradient ethanol and mounted with neutral resins. The slides were examined under an OPTIKA B-150 microscope and the images were analyzed with Image Pro Plus6.0 (Media Cybernetics).

Under the 40X objective, 3–5 fields of view were randomly chosen. The staining was scored as A: 0 for no staining, 1 for light yellow, 2 for light brown, and 3 for dark brown. The ratios of the stained cells were scored as B: 1 for 1%–10%, 2 for 11%–50%, 3 for 51%–80%, and 4 for over 80%. The final score was calculated by multiplying A x B. A score of 0 would be considered negative, 1–4 as light positive and over 4 as strong positive.

Statistical analysis

The statistical analysis was completed using the SPSS 18.0 software. The results were expressed as means ± standard deviations (SD), as indicated in the figure legend. The data are representative of three independent experiments performed in triplicate. Student's t-test was used to determine the significance for all pairwise comparisons of interest. The differences were considered to be statistically significant when $P<0.05$.

Results

MARCH8 expression in gastric cancer

MARCH8 was reported to mediate the ubiquitination and downregulation of immune regulatory cell surface molecules in immune cells [24, 25, 30-33] and to control cell surface expression of some additional proteins [34, 35]. We looked up the expression of MARCH8 in the online tool Gene Expression Profiling Interactive Analysis (GEPIA), which is based on the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) dataset for transcriptomic analysis [36], which includes 408 GC samples and 211 controls. As shown in Fig. 1, the expression level of MARCH8 is significantly higher in GC samples at $P<0.05$. This observation suggests the possibility that MARCH8 is involved in the tumorigenesis of GC and is a potentially good candidate for GC treatment.

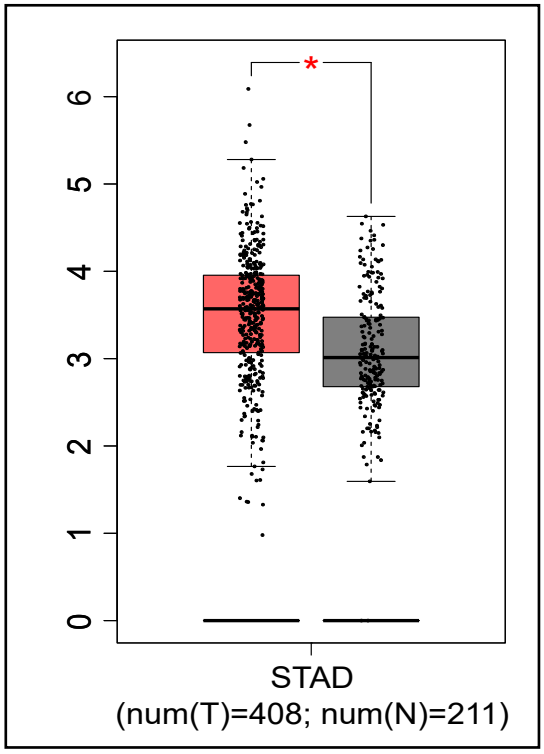

Fig. 1. Boxplot of the mRNA expression levels of MARCH8 in gastric cancer and controls. The red and gray boxes represent gastric cancer and normal tissues, respectively. The data were obtained from the TCGA database, including 408 gastric cancer samples and 211 controls. The y-axis indicates the $\log_2$-transformed gene expression levels.

MARCH8 could be used as a prognostic marker in gastric cancer

The online Kaplan-Meier plotter (www.kmplot.com) can assess the survival effect of a gene or a combination of genes in lung, breast, ovarian and gastric cancer. Gene expression data and relapse-free and

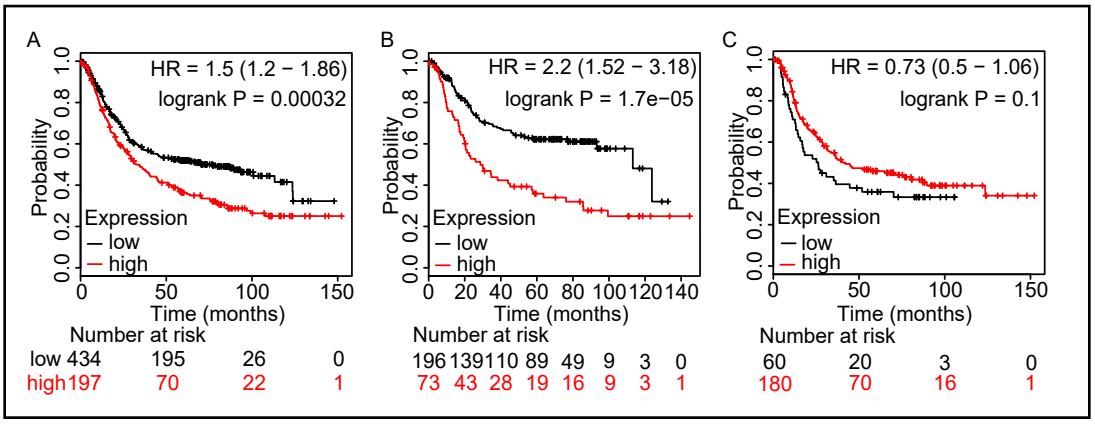

Fig. 2. Survival probability of the gastric cancer patients with high and low MARCH8 mRNA expression levels. The data were obtained from www.kmplot.com. (A) Survival curves are plotted for all gastric cancer patients (n = 876). (B) Survival curves are plotted for intestinal-type cancer patients (n = 320). (C) Survival curves are plotted for diffuse-type cancer patients (n = 241).

Table 1. The correlation of MARCH8 mRNA expression with clinical factors in gastric cancer patients.

Sample information		Cases	HR	95% CI	P
HER status	negative	641	1.53	1.17-2	0.0017
	positive	425	1.65	1.13-2.39	0.0082
pathological grades	Ⅰ	166	1.39	0.86-2.24	0.18
	Ⅱ	67	1.49	0.77-2.87	0.23
clinical stages	1	69	1.81	0.58-5.64	0.3
	2	145	2.04	1.1-3.8	0.021
	3	319	1.64	1.12-2.4	0.01
	4	152	1.59	1.06-2.38	0.025
Treatment	surgery alone	393	1.45	1.08-1.95	0.013
	5-FU-based adjuvant	158	0.65	0.21-1.96	0.44
	other adjuvant	80	0.33	0.13-0.79	0.0086

overall survival information were downloaded from the GEO (Affymetrix microarrays only), EGA and TCGA databases. The effect of MARCH8 mRNA expression on the survival of GC patients was determined using the Kaplan-Meier plotter; 876 GC samples were included, of which there were 320 intestinal-type cancer samples and 241 diffuse-type cancer samples with 33 months of follow-up. The valid Affymetrix ID of MARCH8 is 231933_at. The Kaplan-Meier survival curves for the low and high MARCH8 expression patient groups are shown in Fig. 2. The plots showed poorer prognosis rates for the GC (HR=1.5, 95%CI 1.2–1.86, *P*=0.00032) and intestinal-type GC patients (HR=2.2, 95%CI 1.52–3.18, *P*=1.7e-5) with higher MARCH8 expression.

The correlation of MARCH8 expression with various clinical factors was analyzed, including clinical stage, HER2 status, pathological grade and treatment method (Table 1). The MARCH8 mRNA expression level is significantly correlated with HER2 status, clinical stage, and surgery treatment at P<0.05.

Expression of MARCH8 is remarkably increased in gastric cancer tissues

We first detected the endogenous level of MARCH8 in para-carcinoma tissues and GC tissue samples obtained from the same patient using a tissue microarray. There is strong immunohistochemical staining of MARCH8 in GC tissue, while its expression is considerably lower in normal tissues (Fig. 3). The correlation of MARCH8 expression and the relationship

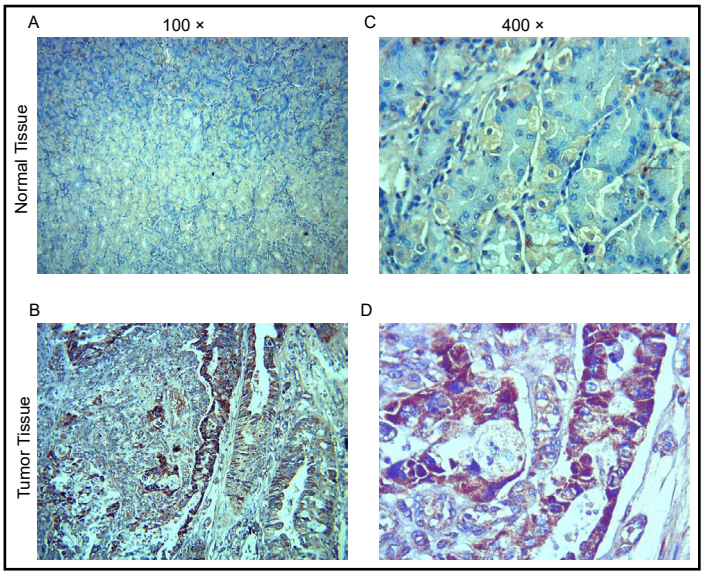

Fig. 3. Endogenous levels of MARCH8 in para-carcinoma tissues and gastric cancer tissues were detected by tissue microarray. (A) MARCH8 expression in the tissue adjacent to carcinoma in patients with gastric cancer (100); (B) MARCH8 expression in cancer tissue in patients with gastric cancer (100); (C) MARCH8 expression in the tissue adjacent to the carcinoma in patients with gastric cancer (400); (D) MARCH8 expression in cancer tissue in patients with gastric cancer (400).

Table 2. MARCH8 expression and the relationship between clinical pathological features of gastric cancer patients. * In some cases, the clinical pathological information is not complete, so the total cases of lymph-node metastasis and pathological grade was less than 31 cases, but no influence on the reliability of the experimental results

Clinicopathologic feature	Cases (n)	MARCH8 level High-expression (n)	Low-expression (n)	P
Age				
≤60	17	7	10	0.2001
>60	14	9	5	
Sex				
Male	18	9	9	0.654
Female	13	7	5	
Tumor sizes				
≤5cm	20	7	13	0.0285
>5cm	11	9	3	
Lymph-node metastasis*				
With	13	7	6	0.8548
Without	10	5	5	
Pathological grade*				
Ⅰ - Ⅱ	11	8	3	0.1333
Ⅲ	12	5	7	

between the clinical pathological features of GC patients were analyzed and listed in Table 2. MARCH8 expression is significantly higher in patients with tumors greater than 5 cm than in patients with tumors less than or equal to 5 cm (P=0.0285).

Transfection of the sh-MARCH8 plasmid efficiently inhibited the expression of MARCH8 in MKN-45 and AGS cells

The efficiency of the MARCH8 knockdown by the p-Super sh-MARCH8 plasmid was determined in two GC cell lines, MKN-45 and AGS. The MARCH8 RNA levels in both sh-MARCH8-transfected cell lines were significantly reduced (Fig. 4 A). Accordingly, the protein levels in both cell lines were decreased, as demonstrated by the protein band intensities (Fig. 4 B, left), which were standardized to GAPDH as shown in the bar graph (Fig. 4 B, right).

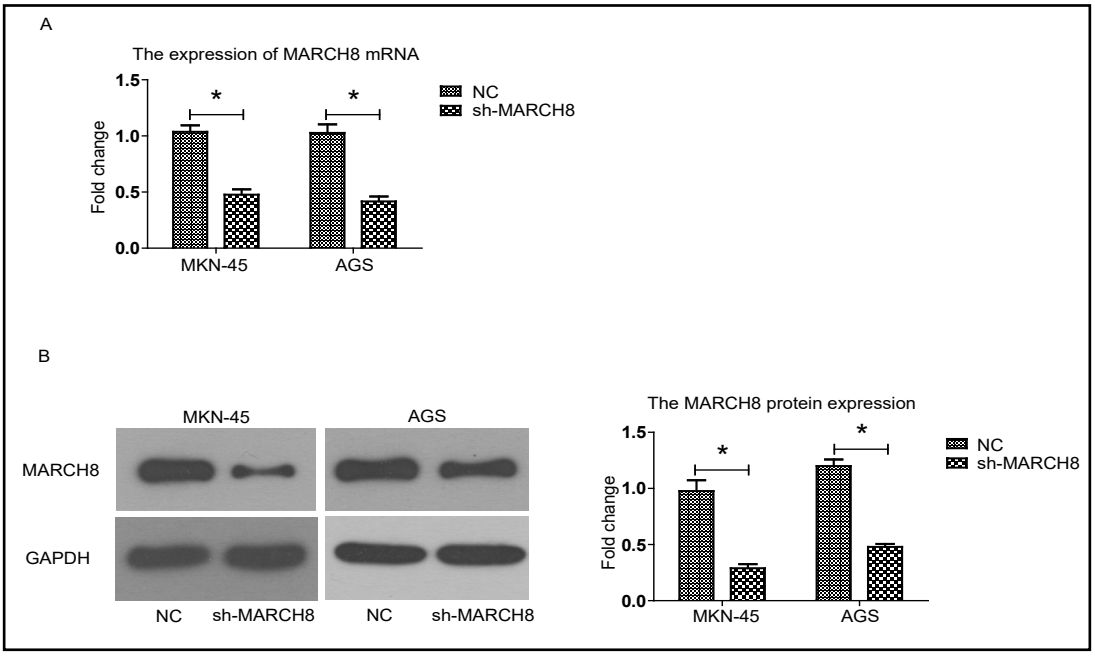

Fig. 4. Sh-MARCH8 plasmid can inhibit MARCH8 expression in MKN-45 and AGS cells. (A) The expression of MARCH8 mRNA in sh-MARCH8-transfected MKN-45 and AGS cells was detected by quantitative PCR. (B) The protein levels of MARCH8 in both cell lines were detected by Western blot analysis (left) and were standardized to GAPDH (right).

Reduction of MARCH8 significantly inhibited gastric cancer cell proliferation

As sh-MARCH8 efficiently decreases the expression of MARCH8, we next measured the effect of MARCH8 knockdown on the proliferation of the MKN-45 and AGS GC cells. CCK8 assays and colony formation assays were performed to evaluate whether the reduction of MARCH8 may affect the proliferation of MKN-45 and AGS cells. As shown by the CCK8 assays, MARCH8 knockdown significantly decreased the viability of both MKN-45 and AGS cells (Fig. 5 A and B). Also, the colony formation assays showed that transfection of the sh-MARCH8 vector resulted in decreased proliferation of both MKN-45 and AGS cells in comparison to the si-NC transfection control (Fig. 5 C and D).

Knockdown of MARCH8 inhibited migration and invasion of MKN-45 and AGS cells

Transwell assays were performed to investigate the effect of MARCH8 knockdown on cancer cell migration and invasion *in vitro*. The migration and invasion assays provide an *in vitro* system to study cell invasion by malignant and normal cells. The numbers of crystal violet-stained cells in sh-MARCH8-transfected wells are considerably lower than those of the control groups (Fig. 5 E and F). The numbers of migrated cells and invasive cells were significantly decreased in the sh-MARCH8-transfected wells for both the MKN-45 and AGS cells (Fig. 5 G and H).

Down-regulation of MARCH8 induced apoptosis of gastric cancer cells and affected the expression of apoptosis-related proteins

GC cell apoptosis was analyzed by flow cytometry. The cells were transfected with the sh-MARCH8 plasmid or the control plasmid (sh-RNA) for 48 hours and later double stained with Annexin V and PI. Early apoptotic cells were identified by Annexin V-positive and PI-negative staining. Late apoptotic cells were positively stained by both dyes. The transfection of sh-MARCH8 significantly increased the rate of apoptosis of both the MKN-45 and AGS cells compared with the control cells (Fig. 6 A and B).

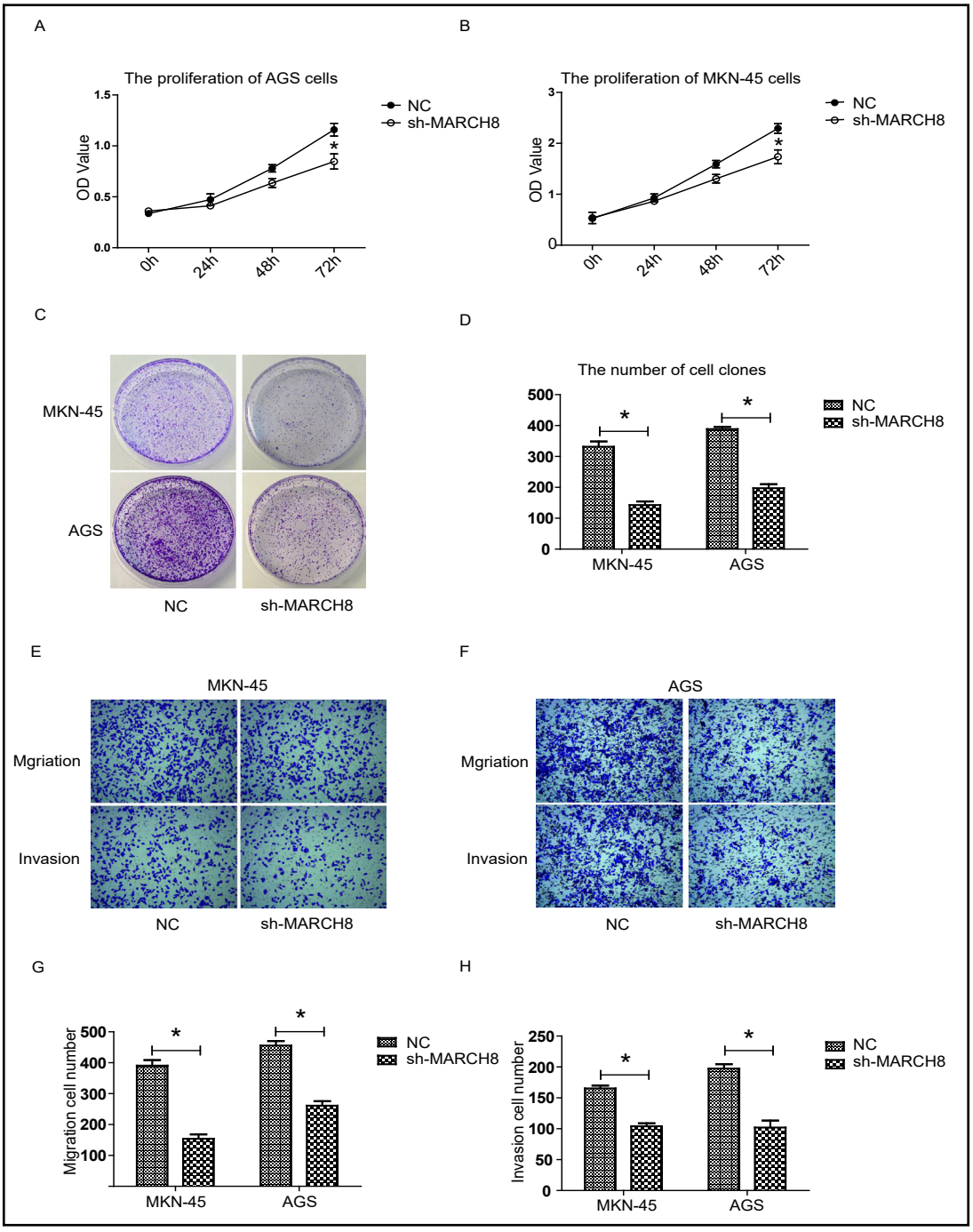

Fig. 5. Down-regulation of MARCH8 significantly inhibited cell proliferation, migration and invasion in gastric cancer. (A) and (B) The time curves showed that the proliferation of both MKN-45 and AGS cells was decreased after MARCH8 knockdown. (C) The viability of the MKN-45 and AGS cells and (D) the cologenic ability. (D) The influence of MARCH8 on clone numbers was evaluated by a clone formation efficiency assay. The error bars indicate ± SDs. *P<0.01 by Student's t-test. (E) and (F) Crystal violet-stained cells show the migration and invasion of cells transfected with sh-MARCH8 and the corresponding sh-NC control. (G) and (H) The numbers of migrating and invading cells were significantly decreased in sh-MARCH8-transfected MKN-45 and AGS cells (P<0.01).

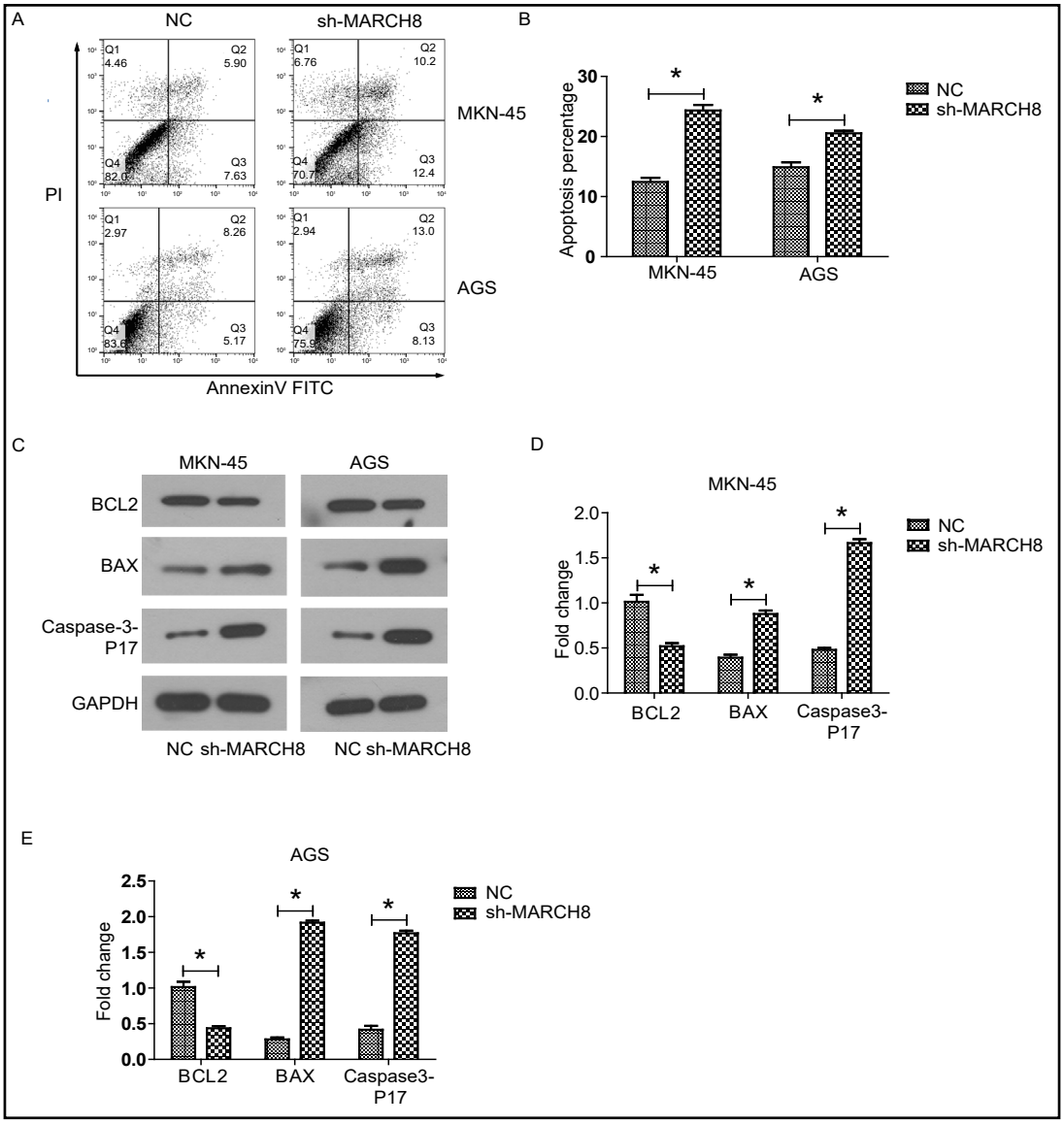

Fig. 6. sh-MARCH8 induced apoptosis of gastric cancer cells and affected the expression of apoptosis-related proteins. (A) MKN-45 (upper) and AGS (lower) cells were harvested and stained with Annexin V-fluorescein isothiocyanate (FITC) and PI, and cell apoptosis was analyzed by flow cytometry. Flow cytometry staining revealed a significant shift in Annexin V-FITC-positive cells in the sh-MARCH8 transfected cells. (B) The percentage of apoptotic cells was significantly increased by sh-MARCH8 (*P<0.01). (C) MKN-45 (left) and AGS (right) cell lysates were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies. GAPDH was used as a loading control. (D and E) The values of the band intensities represented the densitometric estimation of each band normalized to GAPDH (*P<0.01).

To determine the mechanism of apoptosis induction by sh-MARCH8, a Western blot analysis was performed. As previous studies have shown that the expression of the anti-apoptotic BCL2 and pro-apoptotic BAX proteins are critical factors for initiating apoptosis via the mitochondria [43, 44], we examined the expression of BCL2 and BAX in the sh-MARCH8 plasmid-transfected MKN-45 and AGS cells by Western blot analysis. Compared with the control group, the sh-MARCH8-transfection decreased BCL2 expression while simultaneously increasing BAX expression (Fig. 6 C, D and E). These results indicated that sh-MARCH8 increased the BAX/BCL2 ratio in gastric cancer cells and suggested that MARCH8 knockdown may trigger the mitochondrial apoptotic pathway in these cells. Next,

we measured the expression levels of caspase-3 p17 and showed that transfection of sh-MARCH8 robustly increased the expression of caspase-3 p17 (Fig. 6 C, D and E).

sh-MARCH8 suppressed the activation of the PI3K and ß-catenin stat3 signaling pathways in gastric cancer cells

To elucidate the molecular mechanism of sh-MARCH8 on GC inhibition, we examined the effect of sh-MARCH8 on the PI3K pathway in both MKN-45 and AGS cells. The PI3K pathway is implicated in many cellular processes including cell growth, survival, and the promotion of angiogenesis. After being activated at the cell membrane, PI3K phosphorylates PIP2, leading to an accumulation of PIP3 [45]. This lipid second messenger recruits AKT and PDK1 to the cell membrane, where AKT is phosphorylated by PDK1 [46]. Phosphorylated AKT regulates cellular processes by phosphorylation of a number of substrates, including BclxL/BCL2-associated death promoter (BAD) [47]. Another AKT substrate, mTOR, has the most significant role in tumorigenesis [48]. Once activated, mTOR increases mRNA translation by phosphorylation of the downstream molecule P70-S6 kinase [49]. The PI3K-AKT-mTOR pathway can be negatively regulated by the tumor suppressor PTEN [50].

Our results showed that in the sh-MARCH8-transfected group, there was no change in the expression of AKT; however, the level of the phosphorylated form p-AKT decreased (Fig. 7 A, C and D). mTOR has the same expression pattern as AKT (Fig. 7 A, C and D). These data suggested that MARCH8 knockdown inhibited PI3K and caused the decreases in the

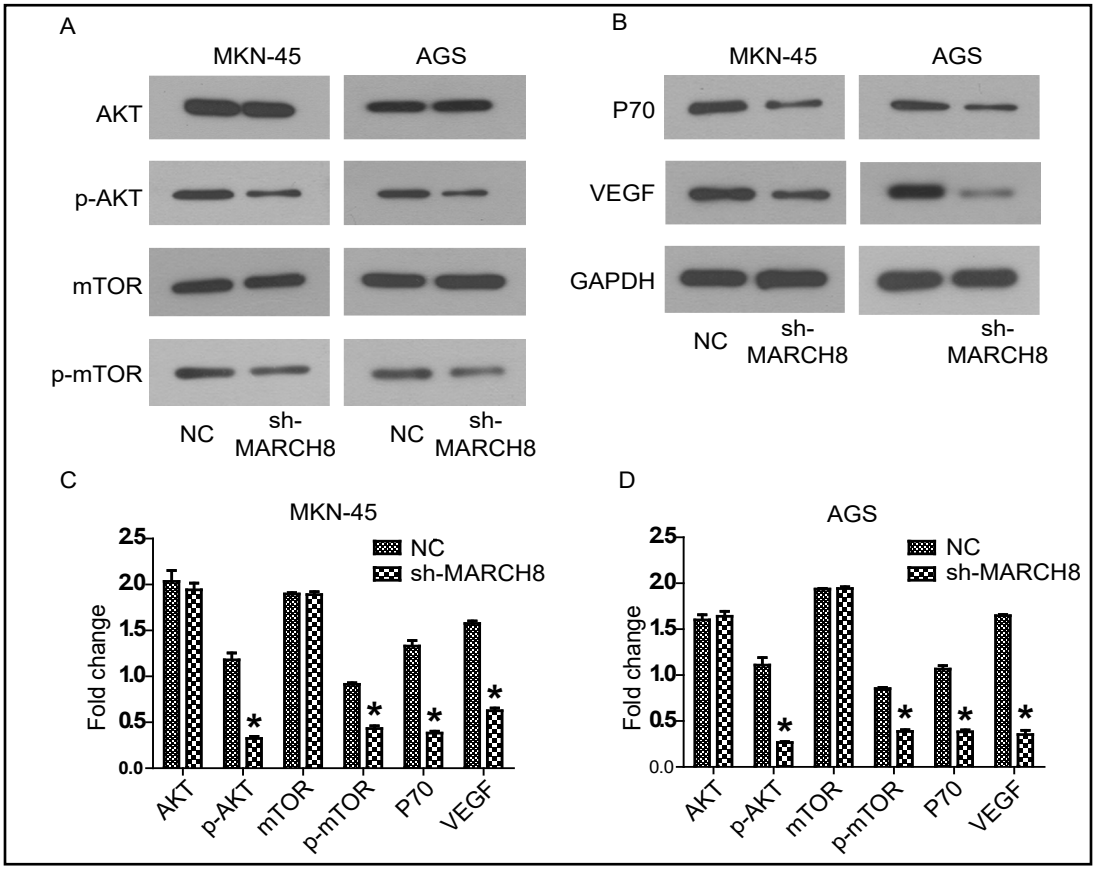

Fig. 7. sh-MARCH8 suppressed the activation of the PI3K signaling pathway in gastric cancer cells. (A) and (B) MKN-45 (left) and AGS (right) cell lysates were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies. GAPDH was used as a loading control. (C) and (D) The values of the band intensities represented the densitometric estimation of each band normalized to GAPDH from the MKN-45 (C) and AGS (D) cells (*P<0.01).

phosphorylated forms of AKT and mTOR. The expression levels of P70 and VEGF were also decreased after transfection of sh-MARCH8 (Fig. 7 B, C and D).

To further confirm this conclusion, we examined the effect of sh-MARCH8 on the expression levels of members of the ß-catenin stat3 signaling pathway, including ß-catenin, E-cadherin, Cyclin D1 and p-STAT3. Our results showed increased expression of E-cadherin and decreased expression of ß-catenin, Cyclin D1 and p-STAT3 (Fig. 8). The decreased Cyclin D1 expression in the sh-MARCH8-treated group was consistent with AKT inhibition and mTOR phosphorylation. Since PI3K activation inhibits GSK3β, the increased expression of GSK3β suggested that the PI3K expression was inhibited by sh-MARCH8 transfection.

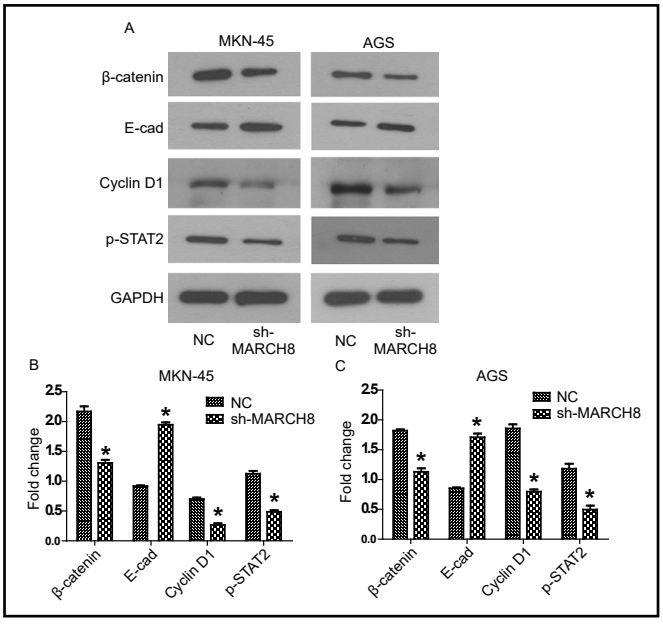

Fig. 8. sh-MARCH8 inhibited the activation of the β-catenin stat3 signaling pathway in gastric cancer cells. (A) MKN-45 (left) and AGS (right) cell lysates were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies. GAPDH was used as a loading control. (B) and (C) The values of the band intensities represented the densitometric estimation of each band normalized to GAPDH from the MKN-45 (B) and AGS (C) cells (*P<0.01).

Discussion

In this study, we report the effects of sh-MARCH8-induced knockdown of endogenous MARCH8 in human GC cells, indicating that appropriate levels of MARCH8 expression are essential for the survival and maintenance of GC cells. Cancer is a multifactorial process, and many molecular alterations have been shown to influence tumor initiation and development through abnormal gene expression or protein alterations. In 2017, more than 1.5 million cases of GC are expected in the world, 38% of which are expected to occur in China and 44% in 24 countries that belong to the International Agency for Research On Cancer (IARC) [51]. Although the prevalence is declining, GC is the fourth most common cancer worldwide, accounting for approximately 10% of invasive cancers, making it the third leading cause of cancer mortalities.

Due to the difficulty of GC diagnosis and its low survival rate [51], new treatment strategies are needed. MKN-45 and AGS are human GC cell lines that are resistant to tumor necrosis factor and to other cytotoxic drugs. MARCH8 is a member of the MARCH family of membrane-bound E3 ubiquitin ligases (EC 6.3.2.19). MARCH enzymes add ubiquitin (see Online Mendelian Inheritance in Man [OMIM] database, omim.org, ID is 191339) to their target lysine residues in substrate proteins, thereby signaling these proteins' vesicular transport between membrane compartments. MARCH8 induces the internalization of several membrane glycoproteins [23, 24]. In our study, we used MKN-45 and AGS cells to investigate the effects of sh-MARCH8 on their proliferation, migration and invasion. Our results showed a remarkable increase in MARCH8 expression in GC, which makes it a potential prognostic marker of GC. In MKN-45 and AGS cells, transfection of the sh-MARCH8 plasmid efficiently inhibited the expression of MARCH8. The reduction of MARCH8 significantly inhibited the proliferation of MKN-45 and AGS GC cells, and decreased both their migration and invasion.

Down-regulation of MARCH8 induced apoptosis of GC cells and altered the expression of apoptosis-related proteins. Our results suggested that the mechanism underlying these phenotypes involves sh-MARCH8 suppressing the activation of the PI3K and ß-catenin stat3 signaling pathways in gastric cancer cells. These results reflected a potential pathological role of this protein in GC.

Mitochondria-mediated cell apoptosis is the major apoptotic pathway and is most commonly mediated by a variety of protein-membrane and protein-protein interactions of the B-cell lymphoma 2 protein (BCL2) family. BCL2-associated X (BAX), a member of the BCL2 family, is a pro-apoptotic protein. Overexpression of BAX triggers the release of mitochondrial proteins that cleave caspase-3, thereby activating it to induce apoptosis [52]. In our study, we found that sh-MARCH8 transfection increased the BAX/BCL2 ratio in both MKN-45 and AGS cells and also increased the expression of caspase-3 p17. These results suggest that the loss of MARCH8 triggered apoptosis via a mitochondrial pathway. However, the BCL2 family proteins are regulated by many signaling pathways such as PI3K, wnt/β-catenin and p53. In our study, MARCH8 knockdown changed the expression of BCL2 family proteins; however, there was no evidence showing that MARCH8 could directly regulate their expression levels. We concluded that MARCH8 regulated the apoptosis proteins indirectly through other signaling pathways.

To elucidate the mechanism of MARCH8's inhibition of MKN-45 and AGS cells, we studied the effects of sh-MARCH8 on PI3K pathway members. The PI3K/AKT/mTOR pathway is implicated in numerous cellular processes ranging from cell growth and survival to the promotion of angiogenesis [44, 53]. The serine/threonine kinase AKT inhibits apoptosis and mediates cell survival by activating PI3K [54]. Another kinase, mTOR, is expressed in most mammalian cells [55], and it is involved in the inhibition of autophagy and acts as a cellular sensor of nutrients and growth factors. Furthermore, mTOR is also an important effector in the PI3K signaling pathway [56]. ß-catenin stat3 signaling proteins belong to a family of secreted proteins that plays important roles in the development and maintenance of many tissues, and include ß-catenin E-cadherin, Cyclin D1 and stat3. ß-catenin stat3 signaling proteins also control kinase-signaling pathways. We discovered that sh-MARCH8 transfection decreased the amounts of the phosphorylated forms of p-AKT and mTOR via the phosphorylation of PI3K. Since PI3K activation inhibits GSK3β, the increased expression of GSK3β and the decreased expression of P70 further confirmed this conclusion.

Conclusion

Our findings indicated that MARCH8 could be considered a potent GC inhibitor and could be potentially used as a supplementary agent in GC therapy. The underlying mechanism of the interaction between MARCH8 and conventional cancer therapies requires further research in the future.

Disclosure Statement

No conflicts of interest exist.

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