

Ubiquitin E3 Ligase MARCH7 promotes proliferation and invasion of cervical cancer cells through VAV2-RAC1-CDC42 pathway

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Abstract. Ubiquitin E3 Ligase MARCH7 is involved in T cell proliferation and neuronal development. In our previous study, we demonstrated MARCH7 promoted malignant behavior of ovarian cancer via the nuclear factor (NF)- κ B and Wnt/ β -catenin signaling pathway. However, the expression and function of MARCH7 in cervical cancer remains unknown. The present study aimed to unravel the expression and function of MARCH7 in cervical cancer to elucidate its potential role in the diagnosis and pathogenesis of cervical cancer. Results indicated that the expression of MARCH7 was abnormally high in cervical cancer tissues than normal cervical tissues. However, silencing the expression of MARCH7 in HeLa cells resulted in decreased cell proliferation and invasion. Mechanistic investigations revealed that MARCH7 interacted with VAV2. Silencing the expression of MARCH7 in HeLa cells inhibited the VAV2-RAC1-CDC42 signaling pathway. Overall, the results of the present study identified MARCH7 as a candidate oncogene in cervical cancer, and a potential target for cervical cancer therapy.

Introduction

Cervical cancer is the third most common cancer and the fourth leading cause of cancer death among women. Cervical squamous cell carcinoma (SCC) is a common cervical cancer type, which is attributed to the persistent infection of high-risk human papillomaviruses (1,2). The cellular effectors and mediators of inflammation are crucial constituents of the local environment of tumors. In some types of carcinoma, inflammatory environment are present before a malignant

change occurs. In other types of cancer, the oncogenic factor induces an inflammatory microenvironment which promotes the development, invasion and progression of carcinoma (3-6). Many studies indicates that chronic inflammation may be an crucial co-carcinogenic factor for HPVs associated cervical cancer (7-9). Therefore, the underlying mechanisms for cervical carcinogenesis and progression are still under investigation, in order to identify new target molecules that could lead to improved diagnosis and treatment.

The membrane-associated RING-CH (MARCH) proteins belong to the RING finger protein family of E3 ubiquitin ligases, which consisting of eleven members in mammals. MARCH proteins have a lot of cellular functions, including in immune regulation, protein quality control, membrane trafficking and spermatogenesis (10). MARCH7 is member of MARCH family, which contains an approximately 690-amino-acid-protein with a single RING finger domain (11). In our previous study, we found MARCH7 promoted malignant behavior of ovarian cancer involving in nuclear factor (NF)- κ B and Wnt/ β -catenin pathway (12). However, little is known about the cellular localization and function of MARCH7 in the cervical carcinoma.

In the present study, we investigated MARCH7 expression and its potential role in cervical carcinoma, trying to clarify the possible function and mechanism of MARCH7 in the cancer malignancy, progression.

Materials and methods

Tissue specimens. The tissue microarray slides containing malignant and benign cervical tissues (n=70) were provided from US Biomax Inc. cancer tissue bank collection (US Biomax Inc., Rockville, MD, USA). The utilization of archived cancer samples used in this study was approved by the Ethics Commission of Chongqing Medical University (Chongqing, China), and informed consent was obtained from the patients.

Immunohistochemistry. Immunohistochemistry was performed according to the SP kit instructions (SP-9000; ZSGB-BIO, Beijing, China). After dewaxing and hydration, the sections were heated in citrate buffer (pH 6.0; Sigma-Aldrich, St. Louis, MO, USA) in a microwave oven for 20 min for antigen

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retrieval. The sections were then cooled naturally to room temperature. The sections were washed for 3 min x 3 cycles. The sections were then incubated in 3% aquea hydrogenii dioxidi for 15 min at room temperature and washed with PBS for 3 min x 3 cycles. The sections were blocked in 5% donkey serum (ab7475; Abcam Inc., MA, USA) for 30 min at 37°C. Anti-MARCH7 rabbit polyclonal antibody (1:100; bs-9341R; Bioss Biotechnology, Beijing, China) was incubated with the sections overnight at 4°C. Negative controls included omission of primary antibody and use of irrelevant primary antibodies. The corresponding secondary antibodies, which were conjugated to horseradish peroxidase (Bioss Biotechnology), were incubated with the sections for 1 h at room temperature. The sections were washed in PBS for 3 min x 3 cycles. The sections were incubated in horseradish enzyme labeled chain avidin solution (Bioss Biotechnology) for 30 min at 37°C and washed in PBS for 3 min x 3 cycles. The proteins were visualized by diaminobenzidine (DAB). All of the sections were observed by three independent pathologists using a light microscope. A total of 22 representative high power fields (x40) were chosen, and the positively-stained cells were counted for each sample. The intensity of MARCH7-positive cells was scored as follows: 0, 1, 2, or 3, for negative, weak, moderate, and strong intensity, respectively. The percentage of MARCH7-positive cells was scored as follows: 0 for no cytoplasm expression, 1 for 1-25% positive tumor cytoplasm, 2 for 26-50% positive tumor cytoplasm, 3 for 51-75% positive tumor cytoplasm, and 4 for 76-100% positive tumor cytoplasm (13). The multiply of the intensity and percentage scores led to as the final MARCH7 staining score and was defined as follows: Staining score <6 considered as low expression, while staining score of ≥7 was considered as high expression. An average of 22 fields was observed for each tissue. All values were represented as the mean ± standard error (mean ± SEM).

Plasmid construction. The cDNA was reverse transcribed from the total RNA of HeLa cells extracted using TRIzol® reagent according to the manufacturer's protocol, using dNTP Mix, 5X RT buffer, HiFiScript 1st Strand cDNA Synthesis kit and RNase-free water. Then the full coding sequence of vav2 was amplified from the cDNAs derived from HeLa cells with a forward primer (F, 5'-GCCATGGGCCATATGATGGAGCAGTGGCGGCAG-3') and a reverse primer (R, 5'-CCGGATCCTCTAGATCACTGGATGCCCTCCTCTTCT-3'). March7 was subcloned using the same strategy as vav2 and the primers were as follows: F, 5'-atggagtctaaacctcaaggattc-3') and R, 5'-ttaggcaatatcaaatgcctgtta-3'. The amplification products of vav2 and March7 were 2637 and 2115 bp in length, respectively, and then were subcloned into pCMV5 vector with Flag or HA tags.

Detection of protein expression by western blotting. Expression of MARCH7, VAV2, CDC42 and RAC1 protein was analyzed by western blotting as described (14). The primary antibodies used included polyclonal rabbit anti-MARCH7 (1:1,000; ab84130; Abcam Inc.); monoclonal rabbit anti-VAV2 (1:1,000; ab52640; Abcam Inc.); monoclonal rabbit anti-CDC42 (1:1,000; 187643; Abcam Inc.); monoclonal rabbit anti-RAC1 (1:1,000; ab180683; Abcam Inc.); and polyclonal rabbit anti-GAPDH (1:1,000; AB10016; Sangon Biotech, Shanghai,

China). The densities of bands were analyzed by a gel imaging system and calculated compared to the internal control.

Co-immunoprecipitation. Co-immunoprecipitation samples were prepared as follows: Cells were harvested after being transfected for 48 h, and was dissolved in IP buffer [1% Triton X-100, Complete Mini protease inhibitor cocktail (Roche Applied Sciences, Basel, Switzerland), 1 M phosphate-buffered saline (PBS) pH 7.4] for 30 min on ice. Cell lysates was centrifuged at the maximum rpm for 30 min at 4°C to get the supernatant. Soluble fractions were incubated with anti-HA antibody overnight on a rotator at 4°C. After three washes of TBST, Dynabeads protein A (Invitrogen AG, Switzerland) were added and incubated for 2 h by rotation at room temperature. Protein complexes were eluted by boiling with a loading buffer.

Cell culture, transfection procedure, and reagents. Human HeLa cervical cancer cells were obtained from the Cell Bank of the Chinese Academic of Science (CBP600232; Shanghai, China). The cells cultured in RPM1 1640 containing 10% FBS and antibiotics in atmosphere of 5% carbon dioxide at 37°C. Double-strand oligonucleotides corresponding to the target sequences were synthesized by Genepharma Co., Ltd. (Shanghai, China). The following sequences were targeted for human MARCH7: MARCH7-1: 5'-GCACUUGGGAGUAAUUUGA-3'; MARCH7-2: 5'-GCACACGUGUCCGAUUUAU-3' and NC (negative control) siRNA: 5'-UUCUUCGAAGGUGUCACGUTT-3'. Lentiviral vector expressing shRNA targeting MARCH7 (named LV3-shMarch7-1 and LV3-shMarch7-2) were provided from Genepharma Co., Ltd..

Cell proliferation assay. Cell proliferation was determined by EdU assay was carried out using the Cell-Light™ EdU imaging detecting kit according to the manufacturer's instructions (Ruibo Biotechnology, Guangzhou, China). EdU is a thymidine analog whose incorporation can be used to label cells undergoing DNA replication (15). The EdU-positive cells are proliferate cells. The cell proliferation rate is EdU-positive cells ratio the highest-positive cells (12,16-18).

Cell proliferation was assessed by the CCK-8 assay (C0037; Beyotime, Shanghai, China) following to the manufacturer's instructions. Cells were seeded into 96-well plates and cultured for an additional 24 h. After treatment with OT, 10 μl of the kit reagent was added and then incubated for another 2 h. OD value was read at 450 nm to obtain the final results (19,20).

Immunofluorescence for F-actin staining. HeLa cells were seeded (1x10⁴) on the cover slip and allowed to adhere overnight. Twenty-four hours after transfection, cells were fixed, permeabilized and then stained with Tetramethylrhodamine (TRITC)-conjugated phalloidin (Sigma-Aldrich) for 1 h. Nuclei were stained with Hoechst (Beyotime) for 5 min. The results were analyzed with fluorescence (Nikon E800, Tokyo, Japan)) (21).

Matrigel invasion assays. For Transwell invasion assays, the upper side of an 8-μm pore, 6.5-mm polycarbonate Transwell filter (Corning, New York, NY, USA) chamber was uniformly coated with Matrigel basement membrane matrix

Table I. Association of MARCH7 expression with clinicopathological characteristics in 70 patients of cervical cancer.

Characteristics	No. of patients (n=70)	MARCH7 expression		P-value
		Low no. (%)	High no. (%)	
Age (years)				>0.05
<50	38	25 (65.79)	13 (34.21)	
≥50	32	22 (68.75)	10 (31.25)	
Normal cervical Cancer tissues	10	9 (90.00)	1 (10.00)	<0.05
FIGO stage	70	47 (67.14)	23 (32.86)	
I/II	66	46 (69.70)	20 (30.30)	<0.05
III/IV	4	1 (25.00)	3 (75.00)	
Grade				
1	44	38 (86.36)	6 (13.64)	
2	16	6 (37.50)	10 (62.50)	
3	10	3 (30.00)	7 (70.00)	
		Grade 2-3 vs. 1		<0.05
Tumor type				
Squamous carcinoma	60	40 (66.67)	20 (33.33)	
Adenosquamous carcinoma	4	3 (75.00)	1 (25.00)	
Adenocarcinoma	6	4 (66.67)	2 (33.33)	
		Squamous vs. non-serous		>0.05

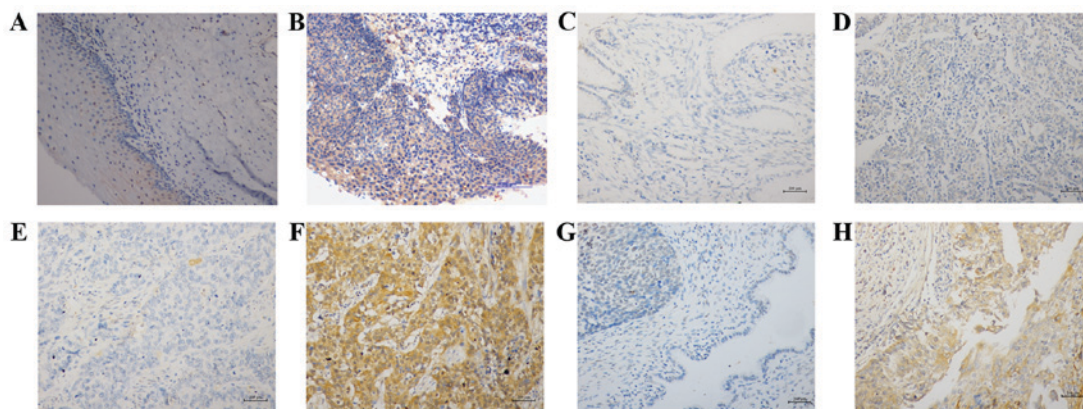


Figure 1. Immunohistochemical analysis of MARCH7 expression in cervical cancer. MARCH7 expression was not detectable in epithelium and stromal cells in normal cervical tissues (A). MARCH7 was predominantly localized in the (B) cytoplasm, (D) plasma membrane, (F) cytoplasm. The expression of MARCH7 in different types of cervical cancer samples. (A) normal cervical tissues; (B) CINII; (C) adenocarcinoma (stage I); (D) adenocarcinoma (stage II); (E) adenosquamous carcinoma (stage IB); (F) adenosquamous carcinoma (stage IIIB); (G) squamous cell carcinoma (stage I); (H) squamous cell carcinoma (stage IIIB). Original magnification, x200. MARCH7, membrane-associated RING-CH; moderate atypical hyperplasia (CINII), in which cells show noticeable heteromorphism in a disordered arrangement and the abnormally proliferated cells occupy the lower 2/3 part of the cervical epithelial layer.

(BD Biosciences, Bedford, MA, USA) for 2 h at 37°C before cells were added. A total of 5×10^4 cells were seeded into the top chamber of a Transwell filter (in triplicate) and incubated for 48 h. Invasive cells on the lower side of the filter, were fixed in 4% paraformaldehyde, stained in 0.5% crystal violet (Beyotime), and counted using a microscope. A total of five fields were counted for each Transwell filter. Each field was counted and photographed at magnification, x200.

Statistical analysis. All statistical analyses were performed using SPSS software, version 17.0 (Chicago, IL). Three or

more separate experiments were performed for each experiment. Statistical analysis was performed by Student's t-test or ANOVA. Data are presented as means \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Aberrant expression of MARCH7 in cervical carcinoma tissues. The expression profile of MARCH7 is not understood. We examined the expression pattern of MARCH7 in normal

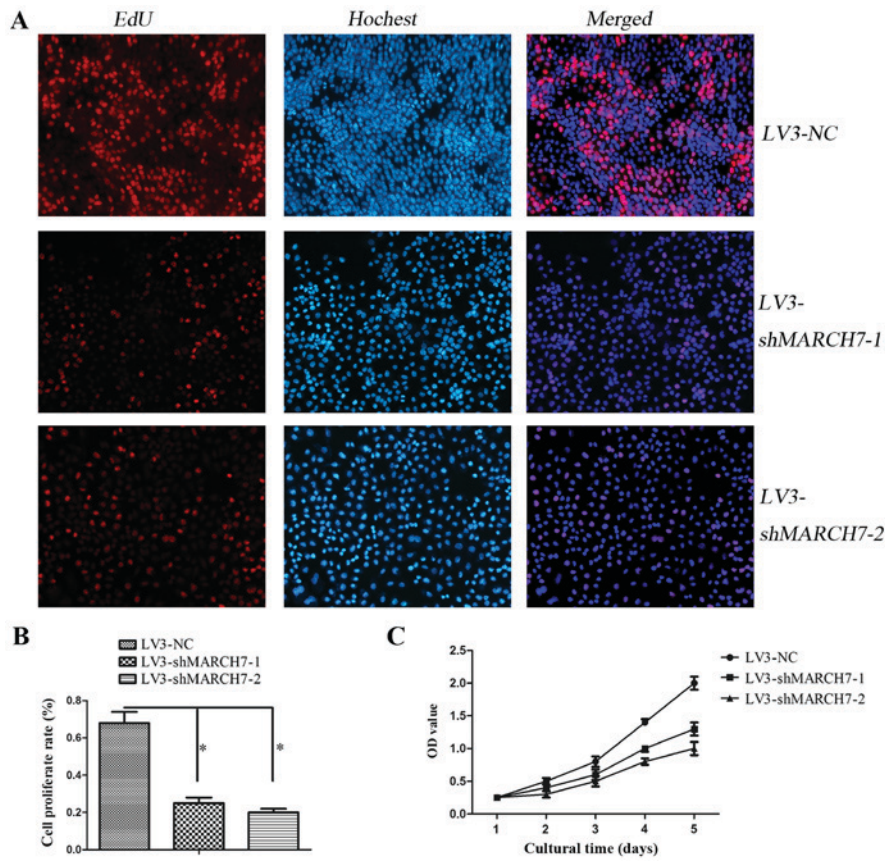


Figure 2. Silencing of MARCH7 inhibited the proliferation of cervical cancer HeLa cells. (A and B) HeLa cells were infected with LV3-NC, LV3-shMARCH7-1 and LV3-shMARCH7-2. Cell proliferation was assessed by EdU. The proliferation rate of LV3-shMARCH7-1 and LV3-shMARCH7-2 cells was lower than that of LV3-NC cells. Original magnification, x200. (C) Cell proliferation was determined by CCK-8 assay. Error bars represented standard error. *P<0.05, and **P<0.001. LV3-shMARCH7-1, lentiviral vector expressing shRNA targeting MARCH7; NC, negative control; LV, lentiviral vector.

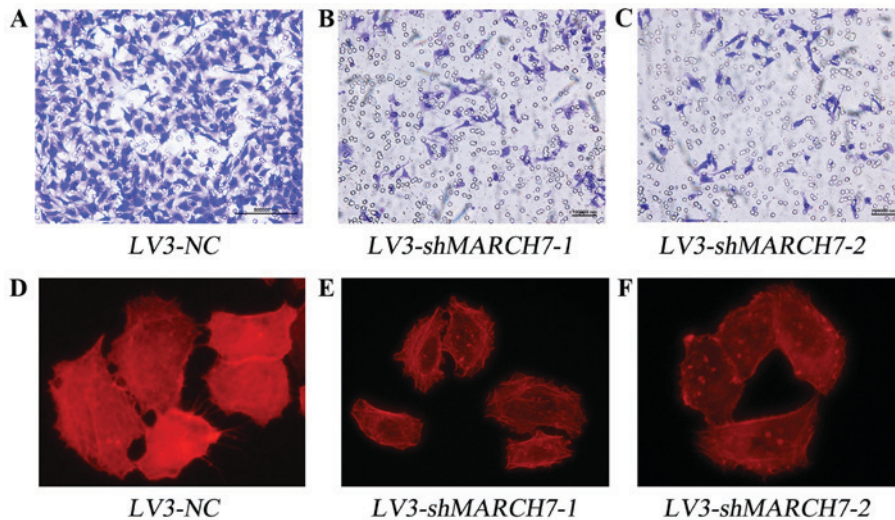


Figure 3. Silencing of MARCH7 inhibited the invasion of cervical cancer HeLa cells. (A-C) Cervical cancer HeLa cells invasion ability was detected by Matrigel invasion assays. The invasion ability of LV3-shMARCH7-1 and LV3-shMARCH7-2 infected HeLa cells was decreased compared with LV3-NC infected cells (D-F). F-actin staining. Original magnification, x400. LV3-shMARCH7-1, lentiviral vector expressing shRNA targeting MARCH7; NC, negative control; LV, lentiviral vector.

cervical tissues and cervical cancer tissue samples using immunohistochemistry. MARCH7 expression at a low level in normal cervical samples, while cervical carcinoma samples showed a significantly higher MARCH7 expression (Fig. 1A-H). MARCH7 was predominantly localized on the plasma

membrane, and cytoplasm (Fig. B and E). Control immunoglobulin did not show any immunoreactivity (Fig. 1A). To determine the correlation of MARCH7 expression with cancer type and cancer stage, all cancer samples were grouped into histologic types (SCC, adenosquamous carcinoma and adenocarcinoma).

Table II. The relative expression of protein detected by western blot.

Group	P-value			
	CDC42	RAC1	VAV2	MARCH7
LV3-NC	0.95	0.98	0.91	0.95
LV3-shMARCH7-1	0.62	0.42	0.41	0.51
LV3-shMARCH7-2	0.55	0.33	0.32	0.46

LV3-shMARCH7-1, lentiviral vector expressing shRNA targeting MARCH7; LV, lentiviral vector; NC, negative control.

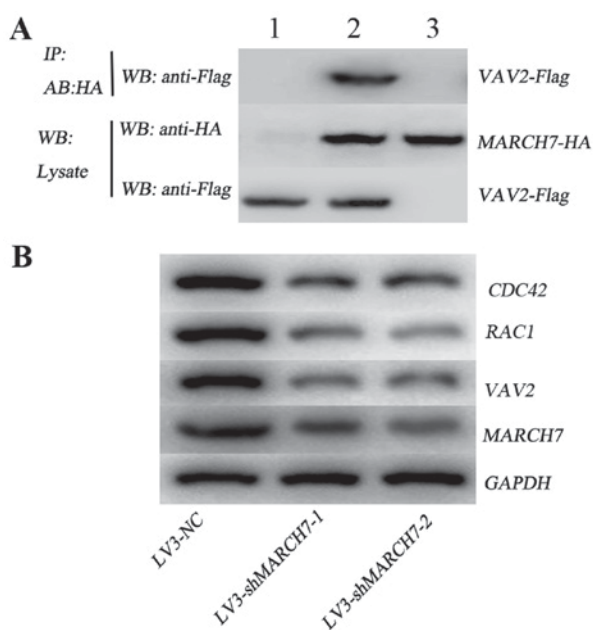


Figure 4. MARCH7 regulated VAV2-RAC1-CDC42 pathway. (A) The interaction between MARCH7 and VAV2 was identified by Co-IP assay. Cells were co-transfected with Flag-VAV2 and HA-MARCH7, and control group was established simultaneously, cells were then harvested 24 h later. Anti-HA antibodies were used to pull the interaction protein. Then, they were detected by anti-Flag antibodies. Results showed that Flag bands could not be detected in the cells transfected with Flag-VAV2 (lane 1) or HA-MARCH7 (lane 3) only. However, it can be detected in cells co-transfected with both Flag-VAV2 and HA-MARCH7 (lane 2), which indicated that there existed interaction between MARCH7 and VAV2 *in vivo*. (B) The expression of VAV2, RAC1 and CDC42 were determined by western blot analysis. The VAV2, RAC1 and CDC42 expression in cells infected with LV3-shMARCH7-1 or LV3-shMARCH7-2 was significantly lower than in control cells. LV3-shMARCH7-1, lentiviral vector expressing shRNA targeting MARCH7; LV, lentiviral vector.

However, the associations between MARCH7 expression and cancer type were not significant ($P>0.05$; Table I). MARCH7 expression was significantly higher ($P<0.05$) in cervical cancer samples compared with normal cervical samples. The Mann-Whitney U test was used to assess the associations between MARCH7 overexpression and clinicopathologic variables of 70 cervical cancer samples (Table I). MARCH7 immunostaining was significantly higher in tumor samples with advanced stage (stage III/IV) compared to early stage

(stage I/II) disease ($P<0.05$). Moreover, the staining intensity significantly correlated with the tumor grade (grades 2-3 vs. 1; $P<0.05$). However, the associations between MARCH7 expression and age were not significant ($P>0.05$; Table I).

MARCH7 knock-down decreased proliferation in cervical cancer cells. To discover the role MARCH7 played in cervical cancer cells growth, we conducted a knockdown of MARCH7 using Lentiviral vector expressing shRNA targeting MARCH7 (named LV3-shMARCH7-1 and LV3-shMARCH7-2) in cervical cancer cells. EdU assay and CCK-8 assay were both performed to detect cell proliferation. The proliferation rate of cells infected with LV3-shMARCH7-1 or LV3-shMARCH7-2 decreased compared to the control cells as judged by EdU staining (Fig. 2A and B). Similar tendencies, results of CCK-8 assay demonstrated that cell proliferation ability was weakened after downregulation of MARCH7 (Fig. 2C).

MARCH7 knock-down decreased invasion in cervical cancer cells and induced F-actin remodeling. Ability of invasion is a special characteristic of cancer cells that affects cancer progression and metastasis. To study whether MARCH7 modulated cervical cancer cell invasion, we performed a Matrigel invasion assay. Transwell assay suggested that the invasion capabilities of the cervical cancer cells infected with LV3-shMARCH7-1 and LV3-shMARCH7-2 were significantly inhibited ($P<0.05$) (Fig. 3A-C). Cellular invasion is mainly dependent on the actin polymerization at the leading edge of the cells. Since the silence of MARCH7 in cervical cancer cells caused such a striking inhibition in the cellular invasion, we detected the changes of the F-actin in cervical cancer cells. We found that LV3-NC infected HeLa cells, F-actin staining was predominantly localized in the cellular outgrowth and projections. In contrast, in LV3-shMARCH7-1 or LV3-shMARCH7-2 infected HeLa cells, F-actin staining was homogenous throughout the cytoplasm, and the formation of membrane ruffles and lamellipodia was prevented (Fig. 3D-F). These results implied that MARCH7 can modulate cellular dynamics by reorganizing the actin cytoskeleton.

MARCH7 knock-down inhibited VAV2-RAC1-CDC42 pathway. Our data suggested that MARCH7 might played an important role in cellular proliferation and invasion. Therefore, we speculated that MARCH7 might be involved in the signal pathway, which is associated with tumor progression. Bioinformatics predicted that MARCH7 may interact with VAV2 (<https://thebiogrid.org/>). In order to identify the interaction between MARCH7 and VAV2, we designed a Co-immunoprecipitation (Co-IP) experiment. Cells were co-transfected with Flag-VAV2 and HA-MARCH7. Control group was established simultaneously, and then were harvested 48 h later. Anti-HA antibodies were used to pull the interaction protein. Then, they were detected by anti-Flag antibodies using western blot analysis. Our results indicated that Flag bands could not be detected in cells infected with Flag-VAV2 or HA-MARCH7 only. However, It can be detected in cells that co-transfected with Flag-VAV2 and HA-MARCH7. We also found that silencing MARCH7 inhibited the expression of VAV2, CDC42 and RAC1 (Fig. 4B and Table II).

Discussion

In the present study, we found elevated MARCH7 expression in cervical cancer tissues, compared to normal cervical tissues. We also observed that increased expression of MARCH7 in cervical cancer was correlated with tumor stage and histological grades. Our results show that upregulation of the expression of MARCH7 in cervical cancer increased cellular invasion and cell proliferation *in vitro*. These results suggest that higher MARCH7 expression is closely associated with cervical cancer progression and aggressive behaviors, indicating that MARCH7 may function as a novel tumor marker and a potential therapeutic target for cervical cancer. Our data also demonstrate that MARCH7 can regulate VAV2/RAC1/CDC42 pathway.

In addition to the investigation of MARCH7 in cervical cancer, we explore the role of MARCH7 in cervical cancer. We found that knocking down MARCH7 expression in cervical cancer HeLa cells suppressed cancer cell growth and invasion.

For a living cell, the actin cytoskeleton, motion skeleton of cells, keeps remodeling at a constant rate. Cellular migration and invasion is dependent on localized actin polymerization at the leading edge of the cells. Polymerization of globular actin leads to the formation of long fibrous molecules, F-actin. In eukaryotic cells, cell migration requires the formation of cell membrane extensions containing actin filaments (22). In this study, silencing of MARCH7 expression in HeLa cells caused a marked decrease in the cellular migration and invasion, we analyzed the alterations in the pattern of the F-actin in HeLa cells by silencing expression MARCH7. In LV3-NC infected HeLa cells, F-actin staining was predominantly localized in the cellular outgrowth and projections. In contrast, in LV3-shMARCH7-1 or LV3-shMARCH7-2 infected HeLa cells, F-actin staining was homogenous throughout the cytoplasm, and the formation of membrane ruffles and lamellipodia was prevented. These results suggest that MARCH7 can modulate cellular dynamics by reorganization of the actin cytoskeleton. These results indicated that MARCH7 is a crucial regulator of glioma progression by its effects on actin cytoskeletal changes.

The VAV family serves as a guanine nucleotide exchange factor (GEF) for the Rho family of GTPases (23). VAV2 is important for tumor growth, neo-angiogenesis and metastasis in several cancers (24-26). VAV2 and VAV3 are also required for skin tumor initiation and promotion (27). Vav2 is an activator of Cdc42 and Rac1 (23,28). VAV2-Rac1 pathway regulates cancer cell adhesion through controlling focal adhesion kinase activity (26). Our study confirms that silencing MARCH7 suppressed the expression of VAV2, RAC1 and CDC42. Hence, we believed that there might be interactions between MARCH7 and VAV1/RAC1/CDC42 pathway. Indeed, our results of co-immunoprecipitation proved that MARCH7 interacted with VAV2. Based on these findings, we concluded that the increased expression of MARCH7 in cervical cancer promotes cancer progression through VAV1/RAC1/CDC42 pathway.

In conclusion, the present study provides evidence identifying MARCH7 as a tumor promoting gene for human cervical cancer. Our study also suggests that MARCH7 may be a potential therapeutic target in patients with cervical cancer.

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