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Original Article

The Role and Function of Secretory Protein Matrix Metalloproteinase-3 (*MMP3*) in Cervical Cancer

Lei Shao [1], ***Wanqiu Liu*** [1], ***Chunyan Zhang*** [1], ***Wei Ma*** [2], ***Xiao Yu*** [3], ***Jing Han*** [1], ****Xiaojuan Wang*** [1]

1. *Department of Gynecology, the Second Affiliated Hospital of Qiqihar Medical University, Qiqihar, China*
2. *Qiqihar City Hospital of Traditional Chinese Medicine South Department of Gynecology, Qiqihar, China*
3. *Department of Obstetrics and Gynaecology, the Second Affiliated Hospital of Qiqihar Medical University, Qiqihar, China*

***Corresponding Author:** Email: wangxiaojuan2021@163.com

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Abstract

Background: We started with RNA-seq analysis and aimed to investigate the possibility of secretory protein matrix metalloproteinase-3(*MMP3*) as a new diagnosis and therapeutic target in cervical cancer.

Methods: The study was conducted on Nov 2021 at the Second Affiliated Hospital of Qiqihar Medical University, Qiqihar, China. Through conjoint analysis of gene expression data as well as survival rate data, we explored the potential secretary proteins associated with cervical cancer carcinogenesis. One hundred patients aged 38-72 years with clinical stage I-IV cervical cancer, and 100 age-matched healthy women were included. The expression changes in serum of clinical patients was detected. We knockdown or overexpressed the secretory proteins then explored its influence on biological function of cervical cancer cells.

Results: By cross-analysis of The Cancer Genome Atlas (TCGA) database and MetazSecKB database, *MMP3* gene was most significantly upregulated in cervical cancer patients ($P < 0.05$). Furthermore, *MMP3* protein was remarkably increased in the serum of clinical cervical cancer patients and decreased after receiving treatment. Overexpression of *MMP3* in HT-3 cells or culturing new cells using the supernatant of the medium after *MMP3* overexpression could increase cell viability ($P < 0.05$) as well as proliferation ($P < 0.05$). Knockdown of *MMP3* reduced the phosphorylation of PI3K as well as AKT proteins, while the PI3K phosphorylation inhibitors could suppress the impact of *MMP3* on increasing cell proliferation as well as viability.

Conclusion: *MMP3* could be an underlying target for early diagnosis and treat cervical cancer in the future.

Keywords: Cervical cancer; Diagnosis; Matrix metalloproteinase-3

Introduction

Cervical cancer is a malignant tumor occurred in the epithelium of the uterine cervix in female. The incidence and mortality rates of cervical cancer are significantly higher than those of other gynecological tumors, and seriously threaten the health of women worldwide (1). The current treatments for cervical cancer could not solve the characteristics of high metastasis and chemotherapy resistance of cervical cancer (2). Therefore, it is a greatly clinical implication to investigate new

therapeutic approaches and cervical cancer-related molecular mechanisms.

Matrix metalloproteinase3 (*MMP3*) is one of the major members of the MMPs family (3) and is involved in the regulation of tumor cell invasion, immunosuppression, and metastasis (4). The PI3K-Akt signaling pathway is a crucial regulator of cellular apoptosis and shows considerable functions in tumor malignant proliferation, tumor metastasis, and resistance to radiotherapy and chemotherapy (5). The activation of PI3K could lead to phosphorylation and activation of Akt (p-Akt), which next activate its downstream target proteins to regulate tumor cell apoptosis and promote tumor cell survival (6).

In this article, we focused on the function of the secretory protein *MMP3* in cervical cancer and explored the relationship between *MMP3* and the PI3K/Akt signaling pathway in regulating cervical cancer of cell growth as well as apoptosis. We provided a possible blood biomarker for the clinical diagnosis of cervical cancer. We aimed to hint an underlying clinic therapeutic target for future treatments of cervical cancer.

Methods

TCGA/MetazSecKB data analysis and KEGG pathway enrichments

Gene expression data associated with cervical cancer patients was downloaded from the Cancer Genome Atlas (TCGA) database, and the detectable secreted proteins in the peripheral blood of cervical cancer patients were obtained from the MetazSecKB database on Nov 2021 at the Second Affiliated Hospital of Qiqihar Medical University, Qiqihar, China. The healthy tissues (n =3) and cervical cancer tissues (n =306) from the TCGA database were analyzed with R package "limma" to search genome-wide differential expression genes, the significantly differential expression genes were defined as adjusted *P* value < 0.05 as well as |log2FC|≥1. Meanwhile, cervical cancer associated clinical data from the TCGA database was analyzed with Kaplan-Meier survival analysis to get significantly gene-related overall survival rate (HR > 1, $P < 0.05$). KEGG pathways analysis was performed to explore the key pathways associated with targeted genes.

Clinical samples

All experiments were proved by the medical Ethics Committee of the Second Affiliated Hospital of Qiqihar Medical University (NO. LHYD-2021101), and all patients and healthy volunteers included in the study were informed the detail of all trials and signed the informed consent before the trial.

One hundred patients aged 38-72 years with clinical stage I-IV cervical cancer, and 100 age-matched healthy women were included here. The peripheral blood of before-surgery and post-surgery and excision tissues of patients were collected and the peripheral blood and biopsy tissues of healthy people were collected. The excluded criteria were as follows: [1] not receive any treatment before surgery; [2] patients with psychiatric diseases including depression and anxiety; [3] pregnant women; [4] acute and critical illnesses; [5] patients with infections, inflammatory diseases, liver and kidney dysfunction, and autoimmune diseases.

Cell lines and cell culture

The human cervical cancer cell lines HT-3 and HeLa were from American type culture collection (ATCC). The cell culture conditions and methods were as follows: the cells were planted in DMEM (11995065, Gibco, USA) medium with 10% fetal bovine serum (FBS, 16140071) and 1 x Pen/Strep (15140122) in the incubator at 37 ℃ with 5% CO2.

ELISA

The human *MMP3*-ELISA kit was purchased from Wuhan Huamei Company, and the standards, washing solution, binding solution, and antibody working solution were prepared based on the reagent instructions. The samples were thawed and balanced at room temperature for half an hour, then add the dilution solution and fertilized at 25 ℃. The OD values were detected

at 450 nm and the concentrations of samples were calculated and qualified to strander's OD values.

RNA extraction and RT-qPCR amplification

The RNeasy Plus Mini Kit (Qiagen, MD, USA) was used to extract the tatal RNAs according to the manufacturer's instructions. The PrimeScript RT reagent Kit (Promega, WI, USA) was used to perform the reverse transcription. After the reverse transcription step, gene expression was quantified using SYBR Green Master Mix (Life Technologies, CA, USA). The primer sequences were shown in the table below (Table 1). Relative gene expression were calculated by the $2^{-\Delta\Delta}$Ct method. β-actin was used as an internal reference for gene qRT-PCR normalization.

Table 1: Primer Sequences

Gene	*Forward primers*	*Reverse primers*
MMP3	CGGTTCCGCCTGTCTCAAG	CGCCAAAAGTGCCTGTCTT
β-actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCAC-GAT

Western blot

The cultured cells were washed with 1 x PBS twice, then collected and lysis with RIPA buffer for extraction total proteins. The concentrations of proteins were detected and quantified through BCA kit (P0012, Beyotime, China). The proteins were added 5 x SDS Loading (P0015, Beyotime, China) and denatured at 95 ℃ for 10 min. The protein samples were running in SDS-PAGE gels, then transferred it to PVDF membrane, the membranes were blocked with 5% non-fat milk for 1 hour. Then fertilized the membrane with primary antibodies overnight at 4 ℃. The second day, the memberance was washed by TBST for three times and incubated with HRP-labeled secondary antibody for 1 h. After that, the membranes were added the solution of ECL Fluorescence Assay Kit (Item BB-3501, Ameshame, UK) and then exposed for imaging in a gel imager. The relative protein content or β-actin protein values was expressed as grayscale values, each experiment was repeated three times.

CCK-8 assay

The cell viabilities were determined by CCK-8. The brief steps were as follow: Cultured cells were incubated in 96-well plates with 1000 cells per well, then CCK-8 reaction solution was added to the well and cultivated for 1 h. The cell viabilities were measured at 450 nm and were calculated using the OD values.

TUNEL assay

The DeadEnd Fluorometric TUNEL System kit (Promega, USA) was used to perform the TUNEL assay. The cells were cultivated in a 4 °C incubator with 4% paraformaldehyde for 25 min, then washed twice by PBS. Then cells were treated with 0.2% TritonX-100 for 5 min for penetrating cell membrane. Then the samples were fertilized by TUNEL staining solution for half an hour at room temperature, also washed three times by PBS and observed in the microscope. The numbers of TUNEL staining positive cells are calculated and recorded by two individual researchers individually and the mean is recognized as the TUNEL cells of the sample.

Statistical analysis

We used SPSS version 21.0 (IBM Corp., Armonk, NY, USA) to perform the statistical analysis. The measurement data were expressed as mean ± standard deviation (SD), and the unpaired t-test was used to match the data of two groups that obeyed normal distribution. The comparisons of data between the groups were analyzed by One-way ANOVA with Tukey's test. What's more, ANOVA with Bonferroni post hoc for comparing the data between groups at differ-

ent time points. The statistical significant was mean $P < 0.05$.

Results

MMP3 is closely related to the development and progression of cervical carcinoma

The differential expression genes analysis find 619 genes were highly expressed in cervical cancer patients (|log2FC| > 1), TCGA database analysis find 811 genes were related to the survival rate of cervical cancer patients (HR > 1).
We also found 2594 secreted proteins could be detected in the human peripheral blood (Fig. 1A).

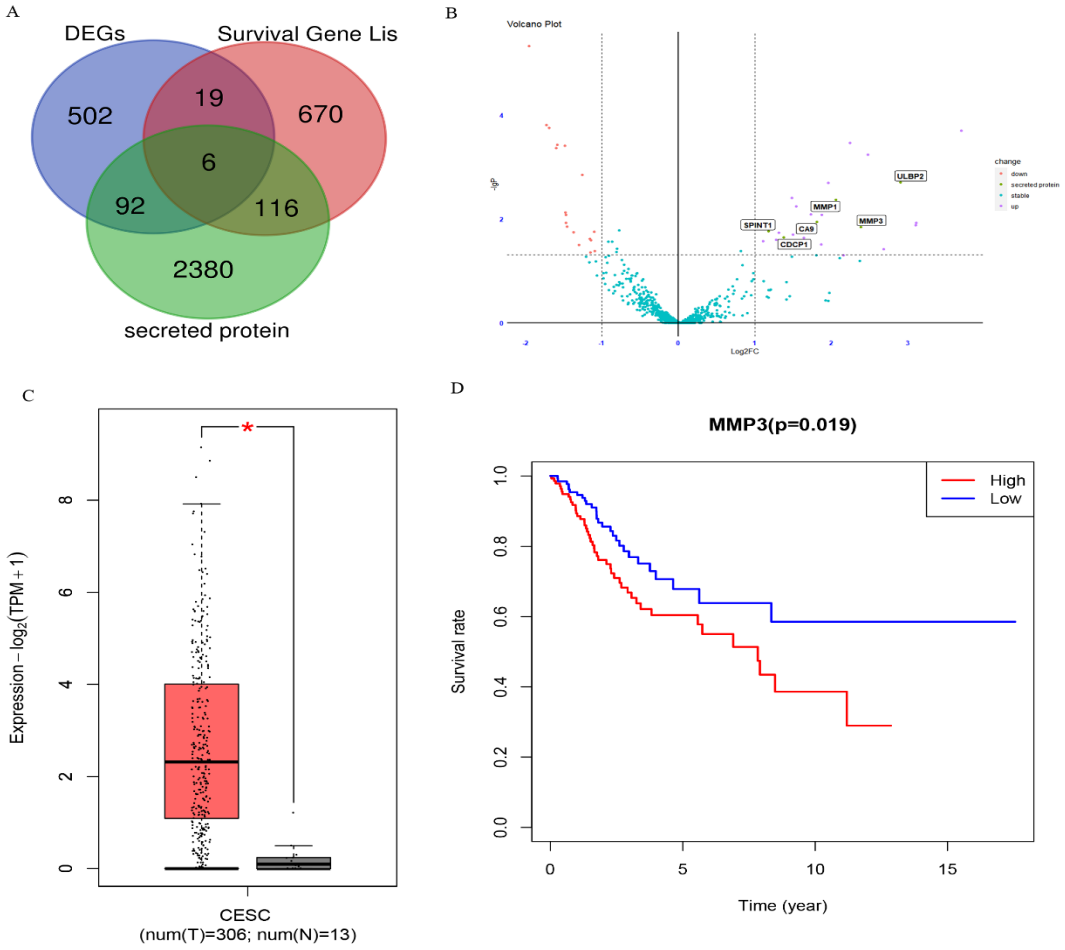

Fig. 1: Analysis of targets related to the development and progression of cervical carcinoma
A: The cross-comparison of DEGs from the TCGA database, survival-related genes from the TCGA database, and detectable secreted proteins from the MetazSecKB database. B: Volcano plots of significantly differential expression genes in cervical cancer. C: GEPIA2 website analysis of *MMP3* in para-cancer tissues and normal tissues of cervical cancer patients. D: Kaplan-Meier survival analysis of the two groups of high as well as low *MMP3* expression in cervical cancer patients. * indicates $P < 0.05$

After cross-comparison, six secreted proteins with high expression in cervical cancer tissues and associated with survival rate were identified: *CA9*, *MMP1*, *MMP3*, *ULBP2*, *SPINT1*, and *CDCP1*. The expression of *MMP3* in cervical cancer tissues shows up-regulated compared with normal tissues, and the difference was more significant compared with the other five genes (Fig.

1B). Through analysis of the GEPIA2 website, the RNA-seq data of 306 para-cancer tissues as well as 13 normal tissues from TCGA cervical cancer sets showed the expression of *MMP3* was higher in para-cancer tissues than normal tissues (HR > 1) (Fig. 1C). Furthermore, the survival rate of cervical cancer patients with high *MMP3* was significantly lower than those who with low *MMP3* expression (Fig. 1D). These findings indicated MMP3 is closely related to the development and progression of cervical carcinoma.

Serum MMP3 concentration is highly associated with disease status

The expression of *MMP3* protein in the serum of cervical cancer patients before and after surgery was detected by ELISA kit. The results showed *MMP3* expression was remarkably increased in the serum of cervical cancer patients (n = 100) compared with the normal volunteers (n = 100) (Fig. 2A).

Also, the *MMP3* levels in serum were significantly lower after treatment compared to those before treatment ($P < 0.01$) (Fig. 2B).ROC correlation analysis showed serum *MMP3* concentration was highly correlated with cervical cancer with the 0.9196 of AUC value (95% CI: 0.7642-0.9543) (Fig. 2C). At the same time, the follow-up results showed the survival rate of patients with high *MMP3* expression in serum before operation was significantly lower than those who with low *MMP3* expression in serum (Fig. 2D). In addition, we collected the clinical information of cervical cancer patients in the hospital (Table 2), both the univariate factors analysis (Table 3) and multivariate factors analysis (Table 4) showed serum *MMP3* concentrations were highly associated with survival rates of patients.

Table 2: Cervical Cancer Patient Clinical Information Form

Patient characteristics	***N (Percentage)***
Age (y)	
< 50	56 (56.00)
≥50	44 (44.00)
Tumor size (cm)	
<4	62 (62.00)
≥4	38 (38.00)
HPV	
Positive	3 (3.00)
Negative	97 (97.00)
Histology	
Adenocarcinoma	46 (46.00)
Squamous cell carcinoma	43 (43.00)
other	11 (11.00)
FIGO stage	
I	39 (39.00)
II	49 (49.00)
III	6 (6.00)
IV	6 (6.00)
Grade	
I	16 (16.00)
II	29 (29.00)
III	51 (51.00)
IV	4 (4.00)
MMP3	
High	50 (50.00)
Low	50 (50.00)

Table 3: Univariate Analysis of Survival Rate

Characteristic	*HR*	*95% CI*	*P Value*
Age (≤50 vs >50)	1.162	0.986-1.623	0.437
Tumor size (<4 vs ≥4)	2.316	1.125-2.412	0.015
HPV (Positive vs Negative)	0.867	0.612-1.298	0.638
Histology			
Adenocarcinoma vs Squamous cell carcinoma	0.968	0.678-1.879	0.694
Adenocarcinoma vs Other	0.932	0.543-1.638	0.732
FIGO Stage			
I vs II	0.763	0.648-1.584	0.032
II vs III	0.482	0.326-1.038	0.008
II vs IV	0.869	0.759-1.793-	0.043
Grade			
I vs II	0.468	0.367-1.022	0.044
II vs III	0.689	0.598-1.128	<0.001
II vs IV	1.348	1.026-1.687	0.003
MMP3 (High vs Low)	0.729	0.628-1.694	0.012

Table 4: Multifactorial Variable Analysis of Survival Rate

Characteristic	*HR (95% CI)*	*P Value*
Tumor size		0.832
<4	NA	
≥4	NA	
FIGO stage		0.032
I vs II	0.844 (0.691-0.918)	
II vs III	0.546 (0.257-0.832)	
II vs IV	0.328 (0.224-0.768)	
Grade		0.036
I vs II	0.573 (0.691-0.918)	
II vs III	0.467 (0.364-0.893)	
II vs IV	0.332 (0.156-0.845)	
MMP3		0.0073
High	1	
Low	0.532 (0.459-0.752)	

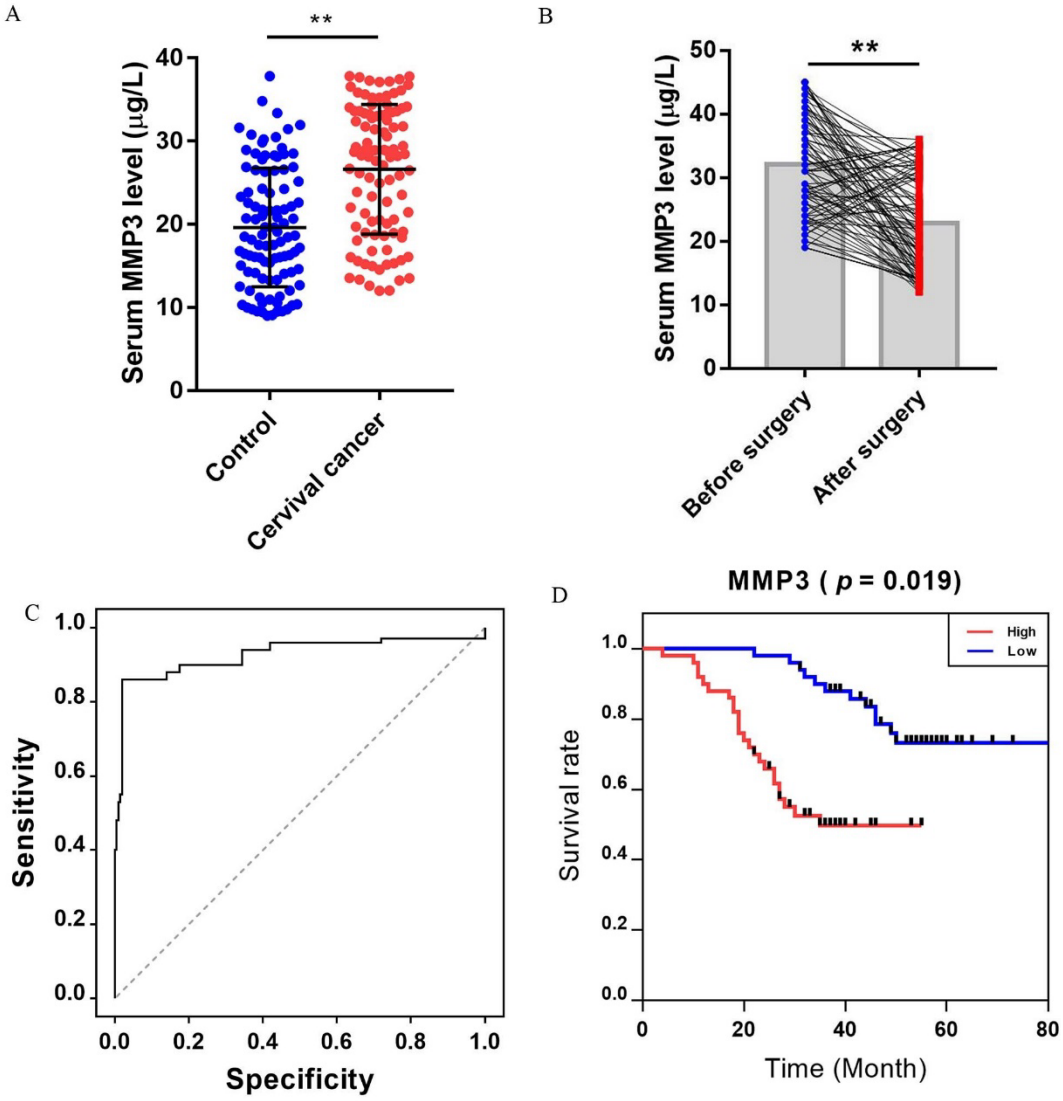

Fig. 2: Expression of *MMP3* in the serum of CCA patients
A: ELISA results of *MMP3* expression in serum of cervical cancer patients and normal controls. B: The serum *MMP3* expression levels in cervical cancer patients before and after surgery. C: ROC analysis of serum *MMP3* concentration and cervical caner status. D: Survival analysis of patients with high and low *MMP3* expression. ** indicates $P < 0.01$

These results suggested *MMP3* was highly expressed in the serum of cervical cancer patients compared to healthy people and highly correlated with survival rates of cervical cancer patients.

MMP3 specifically regulates the proliferation of CCA cells

The CRISPR score after *MMP3* knockdown in cervical cancer cell lines was significantly lower compared with that of other background cell lines, which hint that *MMP3* might play a vital role in the cervical cancer cells (Fig. 3A). To verify this hypothesis, we knocked down *MMP3*, QRT-PCR results showed the mRNA of *MMP3* was much lower in the si-*MMP3* group compared to si-Ctrl in both cell lines after 48 hours of transfection (Fig. 3B). Western blotting results also showed the protein of *MMP3* was reduced in

the si-*MMP3* group compared with the si-Ctrl group in HT-3 and HeLa cells (Fig. 3C-D). These findings showed *MMP3* siRNA could effectively knock down the expression of *MMP3* in HT-3 and HeLa cells. Next, CCK-8 showed the proliferation rate of the si-*MMP3* group was significantly declined compared to the si-Ctrl group both in HT-3 as well as HeLa cells after 5 days of transfection (Fig. 3E). Furthermore, the decreased proliferation level at 5 days in HT-3 cells was more significant compared to HeLa cells (Fig. 3F). Thus, knockdown *MMP3* could significantly affect the proliferation viability of tumor cells, and HT-3 cells showed more sensitivity to the *MMP3* expression.

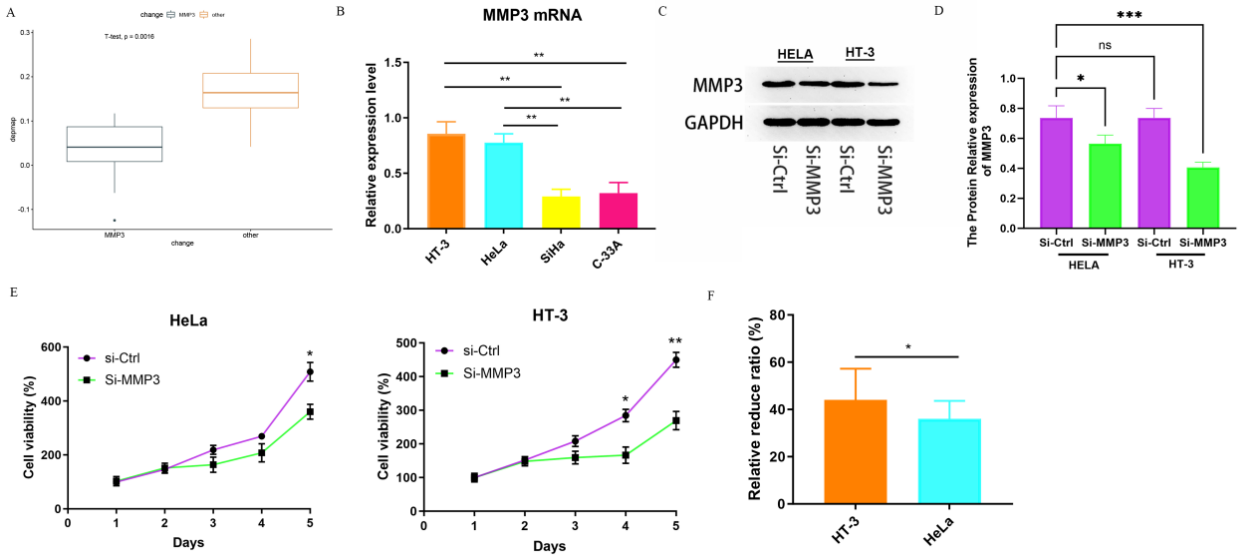

Fig. 3: The role of *MMP3* in CCA

A: The cell scores of secreted protein *MMP3* knockdown in cell lines; B: qRT-PCR detected *MMP3* mRNA levels in HT-3 as well as HeLa after transfection with si-Ctrl or si-*MMP3* siRNA; C-D: Western blotting results of *MMP3* protein expression in each group. E: The cell viability of each group after siRNA transfection by CCK-8 kit; F: The level of decrease in cell viability of si-*MMP3* group compared with si-Ctrl group at day 5 of transfection in each group. *$P < 0.05$, **$P < 0.01$

Overexpression of MMP3 increases cell viability in HT-3 cells

Western blotting showed that after 2 days of transfection, the *MMP3* expression level of the oe-*MMP3* group was highly significantly increased (Fig. 4A-B), also the cell viability and BrdU positive cells were greatly elevated in oe-*MMP3* group (Fig. 4C-D). Considering that the *MMP3* is a secreted protein, which may be highly expressed in culture medium, the ELISA assay was performed and the results showed that *MMP3* was higher in the supernatant of the culture medium in the oe-*MMP3* group compared with the oe-GFP group (Fig. 4E). Then, we collected the culture medium supernatant from oe-GFP and oe-*MMP3* groups and transferred it to newly passaged HT-3 cells. We found that the cell viability (Fig. 4F) and the number of BrdU-positive cells (Fig. 4G) of HT-3 cells in the oe-*MMP3*-Sup group were higher than those in the oe-GFP-Sup group. Therefore, the findings hint high intracellular and extracellular expression of *MMP3* could promote cervical cancer cell viability and proliferation.

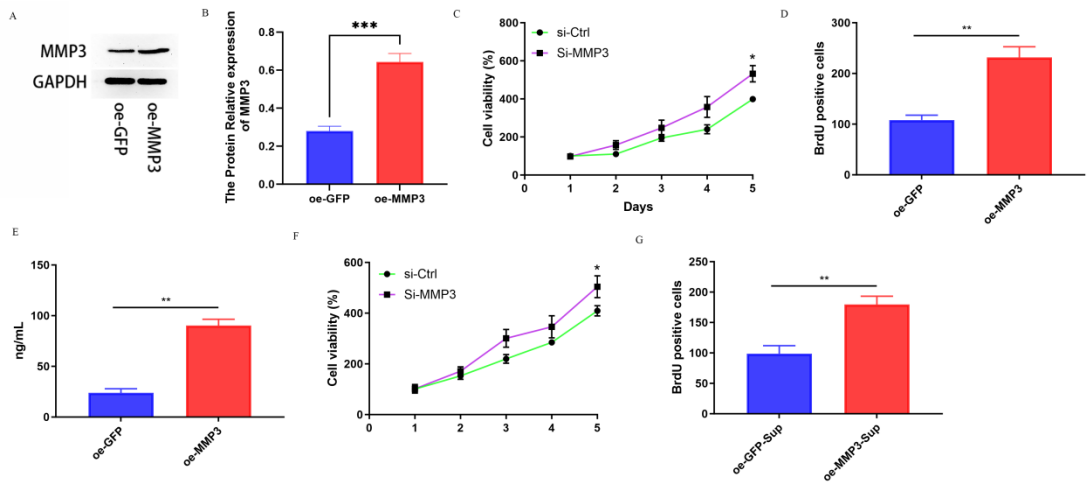

Fig. 4: *MMP3* overexpression could promote HT-3 cell proliferation

A-B: Western blotting detection of *MMP3* protein expression level in HT-3 cells after overexpression of GFP or *MMP3*; C: CCK-8 assay detection of cell viability in the group after oe-GFP or oe-*MMP3* plasmids transfected; D: BrdU-positive cells in two groups; E: Extracellular *MMP3* levels in the supernatant of the culture medium of two groups were detected through ELISA kit. F: CCK-8 assay detected the cell viability of two groups. G: Number of BrdU positive cells in each group. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Knockdown MMP3 increases apoptosis in HT-3 cells

We knocked down *MMP3* expression using siRNA in HT-3 cells and found that TUNEL-positive cells were significantly increased in the si-*MMP3* group than those in si-Ctrl group (Fig. 5A). What's more, the western blot showed the apoptosis-related proteins Bcl-2 were significantly decreased, Cyt-C and Cleaved-Caspase 3 were increased in the si-*MMP3* group (Fig. 5B-C). The above results suggested knock down *MMP3* could lead to enhanced apoptosis levels in HT-3 cells.

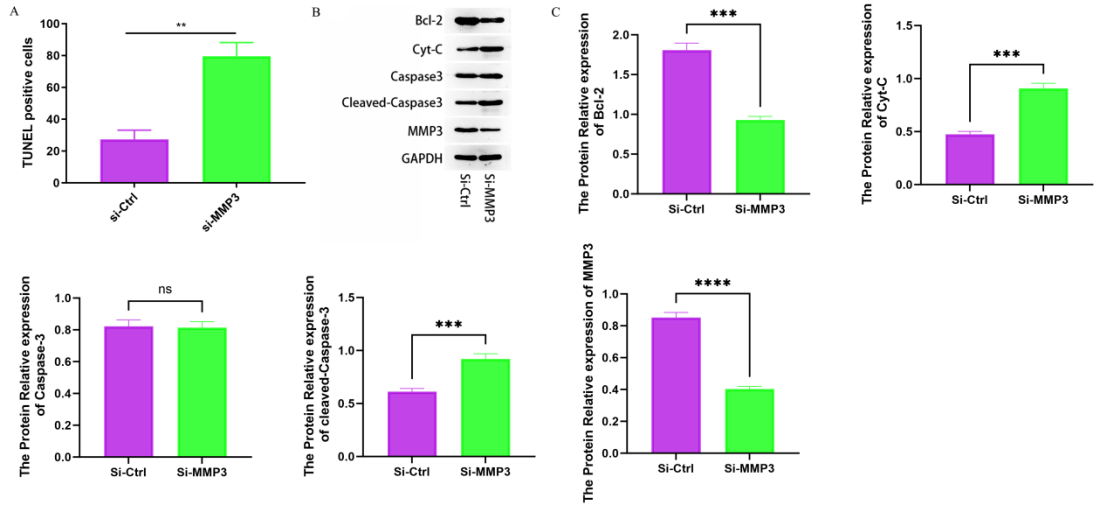

Fig. 5: *MMP3* deficiency could increase apoptosis in HT-3 cells

A: TUNEL positive cells in si-Ctrl and si-*MMP3* groups; B-C: Western blotting of apoptosis-related proteins Bcl-2, Cyt-C, Caspase3, Cleaved-Caspase3 and *MMP3* in two groups. ** $P < 0.01$,*** $P < 0.001$,**** $P < 0.0001$,ns indicated not significant

MMP3 inhibits cell apoptosis through PI3K/Akt signaling pathway

The pathway enrichment analysis of DEGs between low-*MMP3* and high-*MMP3* expression patients showed PI3K/Akt pathway was up-regulated in high-*MMP3* expression group (Fig. 6A). Western blotting results showed that *MMP3* protein was decreased in the supernatant of the culture medium of si-*MMP3* group (Fig. 6B), the PI3K and Akt proteins were not significantly changed between si-*MMP3* and si-Ctrl group (*P* more than 0.05), but the phosphorylation levels of PI3K and Akt present decreased in si-*MMP3* group (Fig. 6C-D). Then, we found that although *MMP3* protein was highly expressed in oe-*MMP3* groups' supernatant of culture medium (Fig. 6E), the p-PI3K and p-Akt were greatly decreased in oe-*MMP3*/wortmannin group compared to those in oe-*MMP3*/DMSO group (Fig. 6F-G). And the TUNEL positive cells were increased in oe-*MMP3*/wortmannin group (Fig. 6H). These results revealed that MMP3 inhibits cell apoptosis through PI3K/Akt signaling pathway.

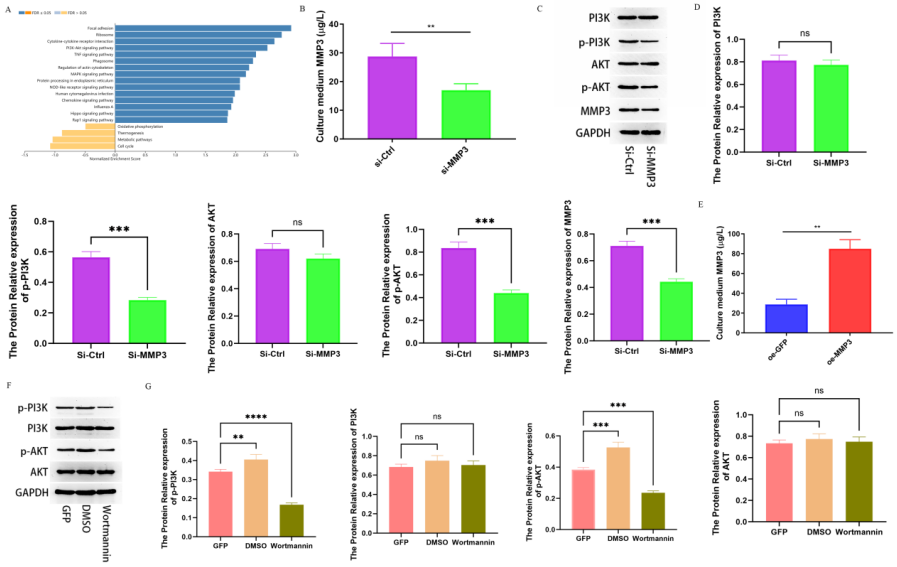

Fig. 6: The relationship between *MMP3* and PI3K/Akt pathway

A: The KEGG pathways enrichment analysis showed that PI3K/Akt pathway was up-regulated in the high-*MMP3* expression group; B: ELISA detected the *MMP3* concentrations in the supernatant of culture medium of two groups; C-D: PI3K/Akt pathway associated proteins expression levels in two groups; E:ELISA results of *MMP3* proteins in two groups; F-G: Western blotting detected the PI3K/Akt pathway associated proteins expression in each group; H: TUNEL assay results of three groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns indicates not significant

Discussion

MMP3 was identified as a biological target closely associated with cervical carcinogenesis and progression and apoptosis of cervical cancer cells, and KEGG enrichment analysis showed *MMP3* was closely associated with PI3K/Akt signaling pathway. Overexpression of *MMP3* significantly inhibited cervical cancer cell apoptosis, while in vitro treatment of cervical cancer cells with wortmannin. The results suggested *MMP3* inhibits apoptosis through the PI3K/Akt signaling pathway and it was very vital in the development and progression of cervical cancer.

MMPs proteins can be divided into four subclasses according to their structural domains with different kinds of biological functions in animals like cell autophagy, proliferation, together with angiogenesis (7). The proteins of MMPs in tumor tissues is consistent with the expression of different MMPs proteins, and is associated with the function of different MMPs proteins together

with the type of tumor. For example, MMP7 protein is selectively expressed in tumor epithelial cells, while stromal cells express stromelysin-3, stromelysin-1 and gelatinase A (8,9). Stromelysin-3 as well as MMP13 is predominantly expressed in stromal cells in breast cancer (10), and MMP2 is expressed in malignant tumors (11). MMP9 is expressed in brain malignant tumor cells (12). *MMP3* expression was strongly related to tumor progression (13).

In fact, positive correlations between *MMP3* expression and tumor infiltration and metastasis probability (14-17). And Ectopic expression of *MMP3* lead to extracellular matrix rearrangement (ECMR) and induce carcinogenesis of epithelia cells, which suggested the disrupted balance of signal factors could result in carcinogenesis in epithelia cells (18). In addition, overexpression of *MMP3* in breast epithelial cells causes shearing of E-cadherin and converts epithelial cells to mesenchymal cells (19,20). In fact, MMPs also can regulate or increase the functions of certain matrix proteins (21). Many proteins can be activated by *MMP3* hydrolysis, such as CCN2/ CTGF (22). In the present study, *MMP3* could affect the cell proliferation ability of HT-3 cells by regulating the phosphorylation of PI3K/Akt molecules, which provided a new perspective on the molecular mechanism of *MMP3* regulation of cancer cell proliferation process.

Recent studies have revealed exogenous down-regulation of PI3K-Akt axis expression or inhibition of PI3K-Akt axis activation can exert significant anti-tumor and anti-metastatic effects on prostate cancer (23, 24). Li et al. found the PI3K/Akt pathway is closely associated with the migration and invasion of ovarian cancer cells (25). Exogenous inhibition of p-mTOR as well as p-PI3K/p-Akt can induce apoptosis through the mitochondrial pathway and have a significant inhibitory effect on the progression of cervical cancer (26).

The above studies illustrate that PI3K/Akt signaling pathway has closely relationship with tumor cell genesis and progression. Besides, they show how to effective inhibition of PI3K/Akt signaling pathway could provide an effective strategy for the prevention and therapy of cervical carcinoma.

Conclusion

MMP3 has potential applications in the early diagnosis, early screening and therapy of cervical cancer.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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