The role and function of secretory protein MMP3 in cervical cancer

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Abstract

OBJECTIVE

Cervical cancer(CCA) is the second commonest malignancy among female all over the world, and present clinical treatments cannot solve the problems of high metastasis and chemotherapy-resistant in CCA. This study starts with RNA-seq analysis and aims to investigate the possibility of secretory protein MMP3 as a new diagnosis and therapeutic target in CCA.

METHODS

Through conjoint analysis of gene expression data as well as survival rate data, we explored the potential secretary proteins associated with CCA carcinogenesis and advance and verify the expression changes in serum of clinical patients. We knockdown or overexpress the secretory proteins then explore its influence on biological function of CCA cells. Cell viabilities and apoptosis levels are detected using CCK-8 kit and TUNEL staining assay respectively, the expression of apoptosis related proteins was verified by western blot.

RESULTS

By cross-analysis of The Cancer Genome Atlas (TCGA) database and MetazSecKB database, MMP3 gene was most significantly upregulated in CCA patients. Also, MMP3 protein was remarkably increased in the serum of clinical CCA 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 ($p < 0.05$). What's more, 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

The secreted protein MMP3 is significantly related to the development and progression of CCA in clinical, and MMP3 can inhibit apoptosis of CCA cells through regulating PI3K/Akt signal pathway. Therefore, this study suggests that MMP3 could be an underlying target for early diagnosis, together wo and treatment of CCA in the future.

Introduction

Cervical cancer (CCA), which caused 530,000 new cases and 275,000 deaths each year worldwide, is a malignant tumor occurred in the epithelium of the uterine cervix in female. The incidence and mortality rates of CCA are significantly higher than those of other gynecological tumors, and seriously threaten the

health of women worldwide [1]. The current treatments for CCA are including radiotherapy, surgical resection, chemotherapy, as well as molecular targeted treatment. Although the current treatments could partially suppress tumor growth, these therapy methods could not solve the characteristics of high metastasis and chemotherapy resistance of CCA [2]. Therefore, it is a greatly clinical implication to investigate new therapeutic approaches and CCA-related molecular mechanisms.

Matrix metalloproteinase3 (MMP3) is one of the major members of the MMPs family [3]. The human MMP3 gene is localized in the chromosome 11q22.3 region and is approximately 1.8 kb. Over the past 40 years, MMP3 has been recognized to play a vital role in the degradation of the extracellular matrix. In addition, MMP3 has shown a key regulated role in many important signaling pathways, such as cell migration, apoptosis, immunity as well as angiogenesis. Importantly, MMP3 is involved in the regulation of tumor cell invasion, immunosuppression, and metastasis during the development and progression of many cancers [4]. Although there are many studies about MMP3 and cancers, the specific functions and mechanisms involved in cervical carcinogenesis are not very clear.

Apoptosis is very important in tumorigenesis and development, exploring the mechanism of tumor cell apoptosis and potential methods of regulating apoptosis levels could bring new targets for tumor diagnosis and therapy [5]. 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 [6]. PI3K is an intracellular phosphatidylinositol kinase protein containing the catalyst subunit p110α and regulation subunit p85. The amplification and mutation of protooncogene PIK3CA could lead to the up-regulated expression of p110α and then enhance the catalytic activity of PI3K. The protein kinase B (PKB) also named Akt is the directly targeted protein of the downstream of PI3K pathway, 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 [7].

In this article, we focus on the function of the secretory protein MMP3 in CCA and explore the relationship between MMP3 and the PI3K/Akt signaling pathway in regulating CCA of cell growth as well as apoptosis. This work provides a possible blood biomarker for the clinical diagnosis of CCA and hints an underlying clinic therapeutic target for future treatments of CCA.

Methods

TCGA/MetazSecKB data analysis and KEGG pathway enrichments

Gene expression data associated with CCA patients was downloaded from the The Cancer Genome Atlas (TCGA) database, and the detectable secreted proteins in the peripheral blood of CCA patients were obtained from the MetazSecKB database. The healthy tissues (n = 3) and CCA 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, $p < 0.05$). CCA patients were dived into low expression group as well as high expression group based on the expression median of genes, then analyze to obtain t value of the whole genome, and 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, 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. A total of 100 patients aged 38–72 years with clinical stage I-IV CCA, 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 also 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 CCA 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°C with 5% $CO2$. The reagents and media were from Gibco, USA. The MMP3 siRNA was purchased from GenePharma company (Shanghai, China), and siRNA transfection was performed according to the manufacturer's recommended steps as follows: First, cells were passaged in 12-well plates, then the medium was changed to 10% FBS DMEM medium without Pen/Strep. Next, when the cell fusion rate reached 70%, the 100 pmol MMP3 siRNA or Ctrl siRNA mixed with 4 µL Lipofectamine 3000 (L3000001, Invitrogen, USA) was transiently transfected into cells. After 2 or 5 days of transfection, the cells were collected for subsequent experiments. The transfection methods of MMP3 and GFP overexpression plasmids were similar to siRNA transfection. After replacing the cell culture medium with the fusion rate of about 70% in the 12-well plate with the anti-free medium, 1 ug of MMP3 or GFP overexpression plasmid mixed with 1 µL of Lipofectamine 3000 was added to each well for transfection, and the cells were collected after 48 hours of transfection for subsequent experimental analysis. PI3K phosphorylation inhibitor wortmannin (W8030, Solarbio, China) powder was diluted into 40 µM using DMSO and added to the cell culture medium to reach a final concentration of 4 µM. Cells were treated with wortmannin for 48 h and collected for the following analysis.

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 °C. The OD values are detected at 450 nm and the concentrations of samples are 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	CTCCTTAATGTCACGCACGAT

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 °C 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 including GAPDH (ab8245. 1:10,000, Abcam, UK), MMP3 (sc-21732, 1:1000, Santa Cruz, USA), Bcl-2 (ab32124, 1:1000, Abcam, UK), Cyt-C (4272S, 1:3000, CST, USA), Caspase3 (ab32351, 1:1000, Abcam, UK), Cleaved-Caspase3 (ab32042, 1:500, Abcam, UK), PI3-kinase (4249S, 1:1000, CST, USA), phospho-PI3-kinase (4248S, 1:1000, CST, USA), AKT1 (ab179463, 1:1000, Abcam, UK) p-AKT1 (phospho S473) (ab81283, 1:1000, Abcam, UK) overnight at 4 °C. 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 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 SPSS Statistics, Chicago, IL, 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 different time points. The statistical significant was mean $p < 0.05$.

Results

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

To explore the protein molecules and potential targets related to the early development of CCA, the RNA-Seq raw data of CCA patients (n = 306) and normal tissues (n = 3) were first downloaded from the TCGA database, and the differential expression genes analysis find 619 genes were highly expressed in CCA patients ($p < 0.05$, |log2FC| > 1), TCGA database analysis zfind 811 genes were related to the survival rate of CCA patients ($p < 0.05$, HR > 1), and we also found 2594 secreted proteins could be detected in the human peripheral blood (Fig. 1A). 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 CCA 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 CCA sets showed the expression of MMP3 was higher in para-cancer tissues than normal tissues ($p < 0.05$, HR > 1) (Fig. 1C). Furthermore, the survival rate of CCA patients with high MMP3 was significantly lower than those who with low MMP3 expression (p < 0.05) (Fig. 1D). These findings indicated that MMP3 was highly expressed in CCA patients in the TCGA data, and the expression of MMP3 shows a negative correlation with the survival rate of patients.

Serum MMP3 concentration is highly associated with disease status

To further investigate the relatoinship between MMP3 and CCA in clinical, we examined the expression of MMP3 protein in the serum of CCA patients before and after surgery. The MMP3 protein concentrations in the serum of healthy volunteers were also detected through the MMP3 ELISA kit. The results showed that MMP3 expression was remarkably increased ($p < 0.01$) in the serum of CCA 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 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 that the survival rate of patients with high expression of MMP3 in serum before operation was significantly lower than that of patients with low expression of MMP3 in serum ($p < 0.05$) (Fig. 2D). In addition, we collect the clinical information of CCA 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)	56 (56.00%)
< 50	44 (44.00%)
≥50	
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)	

These results suggested that MMP3 was highly expressed in the serum of CCA patients compared to healthy people, and MMP3 was highly correlated with survival rates of patients with CCA.

MMP3 specifically regulates the proliferation of CCA cells

To investigate the role and function of MMP3 in CCA, we first explored the impact of MMP3 knockdown on CCA cell lines as well as other cancer cell lines through the CRISPR score of the Depmap database. The results showed that the CRISPR score after MMP3 knockdown in CCA cell lines was significantly lower compare with that of other background cell lines ($p < 0.05$), which hint that MMP3 might play a vital role in the CCA cells (Fig. 3A). To verify this hypothesis, we knocked down MMP3 using the siRNA in human CCA epithelial cell lines HT-3 and HeLa cell lines. QRT-PCR results showed that 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 that MMP3 siRNA could effectively knock down the expression of MMP3 in HT-3 and HeLa cells. Next, we examined the cell viability of HT-3 as well as HeLa cells with decreased MMP3 expression through CCK-8 assay. The results suggested 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 is more significant compared to HeLa cells (Fig. 3F). Thus, knockdown MMP3 could significantly affect the proliferation viability of tumor cells, and HT-3 cells show more sensitivity to the MMP3 expression.

Overexpression of MMP3 increases cell viability in HT-3 cells

Since MMP3 might be related to the proliferation of CCA cells, and knockdown MMP3 could lead to decreased cell viability in HT-3 as well as HeLa cells. Thus, we speculated that overexpressing MMP3 could enhance the proliferation of cervical cancer cells. To verify this hypothesis, we construct the human MMP3 gene into the PRK5 vector and transfect the PRK5-MMP3 plasmids into HT-3 cells. Western blotting results show that after 2 days of transfection, the MMP3 expression level of the oe-MMP3 group is highly significantly increased ($p < 0.001$) (Fig. 4A-B), also the cell viability and BrdU positive cells is greatly elevated in oe-MMP3 group ($p < 0.001$) (Fig. 4C-D). Considered the MMP3 is a secreted protein which may highly expressed in culture medium, thus the ELISA assay is performed on the supernatant of the cell culture medium after 48 hours of transfection in the oe-MMP3 and oe-GFP groups respectively, and the results show MMP3 is higher in the supernatant of the culture medium in the oe-MMP3 group compare with the oe-GFP group (p less than 0.01) (Fig. 4E). Then, we collect the culture medium supernatant of oe-GFP and oe-MMP3 groups after 48 hours of transfection and transfer the culture medium into new passaged HT-3 cells. We find the cell viability (Fig. 4F) as well as the number of BrdU-positive cells (Fig. 4G) of HT-3 cells in the oe-MMP3-Sup group were higher than the oe-GFP-Sup group (p less than 0.01, p less than 0.01). Therefore, the findings hint that high intracellular and extracellular expression of MMP3 could promote cervical cancer cell viability and proliferation.

Knockdown MMP3 increases apoptosis in HT-3 cells

Recent publications reported MMP3 is highly correlated with the apoptotic process of cancer cells, thus we speculate MMP3 may also regulate cellular apoptosis and influence cell viability and proliferation. To prove this suppose, we knock down MMP3 expression using siRNA in HT-3 cells and find that TUNEL-positive cells are significantly increased in the si-MMP3 group than si-Ctrl group (p less than 0.01) (Fig. 5A). What's more, the western blot showed the apoptosis-related proteins Bcl-2 are significantly decreased (p less than 0.05), Cyt-C (p less than 0.05) and Cleaved-Caspase 3 (p less than 0.05) 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.

MMP3 inhibits cell apoptosis through PI3K/Akt signaling pathway

To investigate the potential mechanism of secretory protein MMP3 regulating cervical carcinogenesis and progression, we perform the pathway enrichment analysis of DEGs between low-MMP3 expression and high-MMP3 expression patients from the TCGA database, the results show PI3K/Akt pathway is up-regulated in high-MMP3 expression group (Fig. 6A). Therefore, we explore the PI3K/Akt pathway-

associated proteins in MMP overexpression cells. Western blotting results show that MMP3 protein is decreased in the supernatant of the culture medium of si-MMP3 group (p less than 0.01) (Fig. 6B), the PI3K and Akt proteins are not significantly changed between si-MMP3 and si-Ctrl group (p more than 0.05, p more than 0.05), but the phosphorylation levels of PI3K and Akt present decreased in si-MMP3 group (p less than 0.05, p less than 0.05) (Fig. 6C-D). Then, we add the PI3K phosphorylation inhibitor wortmannin (4 nm) into MMP3 overexpressed HT-3 cells, and find although MMP3 protein is highly expressed in oe-MMP3 groups' supernatant of culture medium ($p < 0.01$) (Fig. 6E), the p-PI3K and p-Akt are greatly decreased in oe-MMP3/wortmannin group compared to oe-MMP3/DMSO group (p less than 0.01, p less than 0.01) (Fig. 6F-G). And the TUNEL positive cells are increased in oe-MMP3/wortmannin group (p less than 0.01) (Fig. 6H). These results suggest that MMP3 may inhibit the apoptosis of cervical cancer cells by promoting the phosphorylation levels of PI3K and AKT, then down-regulating the apoptosis-related pathways and regulating the cell viability of cancer cells.

In summary, the present study explored the association of secretory protein MMP3 and cervical cancer through analysis of the ATGC database, and clinical data show MMP3 was highly presented in the peripheral blood of CCA patients. Importantly, we found MMP3 could suppress cellular apoptosis and enhance cell viability and proliferation by elevating the phosphorylation of PI3K as well as Akt proteins then activating PI3K/Akt downstream targets (Fig. 7).

Discussion

Here, combining bioinformatics and life sciences, we first downloaded the gene expression, also with survival data related to cervical cancer patients through TCGA database, and also obtained the information of detectable secreted proteins in peripheral blood through MetazSecKB database, and obtained the related targets and signaling pathways of cervical cancer development and progression through combined analysis of difference and survival data, and detected by ELISA. The relationship between MMP3 and cervical cancer was verified by detecting the expression of MMP3 in the serum of CCA patients and normal controls, as well as pre- and postoperative cervical cancer patients. By transfecting HT-3 cells with MMP3 overexpression plasmid in vitro and inhibiting PI3K/Akt signaling pathway using wortmannin, followed by detecting apoptosis levels by TUNEL staining and expression of apoptosis-related proteins (Bcl-2, Cyt-c, Casepase3) by Western blot, to further study the relationship between MMP3- PI3K/Akt and apoptosis in CCA cells. It was found that 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 that 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, which in turn inhibited the PI3K/Akt signaling pathway, significantly attenuated the inhibitory impact of MMP3 on cervical cancer cell apoptosis. The results suggested that MMP3 inhibits apoptosis through the PI3K/Akt signaling pathway and it was very vital in the development and progression of cervical canceit was r.

MMP3 is a member of the MMPs family of proteins, a group of proteasomes that require Zn2+, Ca2+, together with other metal ions for activation. MMPs proteins can be divided into four subclasses according to their structural domains, and they have different kinds of biological functions in animals like cell autophagy, proliferation, together with angiogenesis [8]. The proteins of MMPs in tumor tissues is consistent with the expression of different MMPs proteins. The proteins of MMPs in tumor tissues 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 [9–10]. Stromelysin-3 as well as MMP13 is predominantly expressed in stromal cells in breast cancer[11], and MMP2 is expressed in malignant tumors [12]. MMP9 is expressed in brain malignant tumor cells [13]. In particular, a recent paper analyzed the expression of 24 MMPs based on patient data from 15 different cancers in the TCGA database (the Cancer Genome Atlas) and showed that MMP3 was remarkably upregulated in seven different cancer tissues, and MMP3 expression was strongly related to tumor progression [14]. In fact, positive correlations between MMP3 expression and tumor infiltration and metastasis probability have been reported in breast, colorectal, lung, and bone cancers [15–18]. Ectopic expression of MMP3 lead to extracellular matrix rearrangement (ECMR) and induce carcinogenesis of epithelia cells, which suggests the disrupted balance of signal factors could result in carcinogenesis in epithelia cells [19]. In addition, overexpression of MMP3 in breast epithelial cells causes shearing of E-cadherin and converts epithelial cells to mesenchymal cells [20–21]. Similarly, in the present study, ELISA of MMP3 in plasma of CCA patients as well as healthy volunteers showed a significant increase in MMP3 expression in the blood of CCA patients, while comparing MMP3 levels in plasma of patients before and after excisional surgery. The ELISA results showed a remarkably decrease in MMP3 expression after surgery. In addition, the results of survival curve analysis also showed that patients with MMP3 expression had higher 5-year and 10-year survival rates than those with high plasma expression.

MMP3 protein has several important biological functions, like cell growth, angiogenesis as well as cell invasion. It has been shown that MMP3 upregulates the expression of Rac1b, a homodimer of small GTPase Rac1 protein, in breast, lung, and pancreatic cancer cells, and further induces epithelial mesenchymal transition and genomic instability [22–23]. In addition, knockdown of MMP3 expression in MC-38 mouse colorectal cancer cells inhibited cell proliferation and infiltration by suppressing the Erk/κB pathway, and significantly reduced tumorigenic capacity and lung metastasis in a nude mouse model [24]. As a type of secreted protein, it was found that MMP3 in Extracellular vesicles (EVs) could enter the nucleus of recipient cells and interact with It has been found that MMP3 in EVs can enter the nucleus of recipient cells and bind to DNA and heterochromatin proteins HP1/CBXs to transactivate the Connective tissue growth factor (CTGF) gene CCN2, which causes the transformation of cells from normal to cancerous state [25–26]. EVs with high expression of MMP3 can transactivate the promoter of CCN2 gene, while those with knockdown of MMP3 in EVs can transactivate the promoter of CCN2 gene. knockdown of MMP3 expression in EVs inhibits this transactivation effect. In addition, CCN2 regulates the cell cycle by upregulating cyclin A. In malignant glioma cells, CTGF upregulates the expression of anti-apoptotic factors such as survivin, Bcl-xl and Flip [27–28]. The MMP3 protein in the nucleus also

transactivates the HSP gene, which encodes a cytoprotective factor, by interacting with HP1/CBXs. Thus, MMP3 proteins in the nucleus can regulate a wide range of nuclear proteins and genes related to cell transformation. In fact, the functions of MMPs are not limited to degradation or inhibition of matrix proteins through proteasomal hydrolysis, but MMPs can regulate or increase the functions of certain matrix proteins [29]. MMP3 in EVs can activate the extracellular transduction signal TGF-β by shearing precursor proteins or degrading inhibitory factors. Many proteins can be activated by MMP3 hydrolysis, such as CCN2/ CTGF, Insulin-like growth factor binding proteins (IGFBPs), Fibroblast growth factor receptor (FGF-R), Heparin-binding epidermal growth factor (HB-EGF) and Fibroblast growth factor receptor (FGFR). Tumor necrosis factor-alpha (TNF-alpha), Fibroblast growth factor receptor 1 (FGGR1) and interleukin-1β (IL-1β) [30]. In the present study, MMP3 could affect the phosphorylation level of PI3K/Akt molecules by regulating 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 that PI3K-Akt is aberrantly activated in prostate cancer cells and tissues, and that exogenous downregulation of PI3K-Akt axis expression or inhibition of PI3K-Akt axis activation can exert significant anti-tumor and anti-metastatic effects on prostate cancer [31]. *Li* et al. found that the PI3K/Akt pathway is closely associated with the migration and invasion of ovarian cancer cells [32]. PI3K/Akt is activated in ovarian cancer cell while inhibition of it could effectively inhibit ovarian cancer cell proliferation and facilitate tumor cell senescence and apoptosis. The expression of p-mTOR as well as p-PI3K/p-Akt was remarkably elevated in tissues and cells of cervical cancer, suggesting that PI3K-Akt is abnormally activated in cervical cancer, and 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 [33]. The above studies illustrate that PI3K/Akt signaling pathway has closely relationship with tumor cell genesis and progression, and how to effectively inhibit PI3K/Akt signaling pathway can provide an effective strategy for the prevention and therapy of cervical carcinoma. In the present study, MMP3 could influence the PI3K/Akt signaling pathway with regulating the phosphorylation levels of PI3K and Akt proteins, thus regulating the level of apoptosis and affecting the proliferation of CCA cells.

Conclusion

Here, we analyzed the gene expression data as well as survival data of CCA from the TCGA database and found the secretory protein MMP3 could be recognized as a biomarker closely associated with cervical carcinogenesis, progression, also with apoptosis in CCA cells. Furthermore, the concentration of MMP3 in patients' blood was found to be closely related to the development of CCA and also significantly related to the survival rate of CCA patients. Notably, we found MMP3 could increase the cell viability of CCA cells and decrease apoptosis by inhibiting the expression of apoptosis-related proteins through enhance phosphorylation of PI3K and Akt proteins. The work in this study suggests that MMP3 has potential applications in the early diagnosis, early screening and therapy of CCA.

Declarations

Ethical approval and consent to participate

This study was performed in accordance to the principles of the Helsinki. All experiments were proved and approved by the medical ethics committee of the Second Affiliated Hospital of Qiqihar Medical University hospital, and all patients and healthy volunteers included in the study were informed of the details of all trials and signed the informed consent before the trial.

Availability of data and materials

Patients with CCA related gene expression data downloaded from TCGA database (https://portal.gdc.cancer.gov/), Serum secreted protein data of CCA patients were downloaded from MetazSecKB database(http://proteomics.ysu.edu/secretomes/animal/index.php). All data generated or analysed during this study are included in this published article and its supplementary information files.

Consent to publish

Not applicable.

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Conflicts of Interest

The authors have stated that they have no conflict of interest.

Author contributions

L Shao, X Wang: Conception and Design; W Liu, X Yu: Administrative support; C Zhang, J Han: Provided research materials or patients; W Ma, L Shao, W Liu: Data collection and Summary; L Shao, X Wang, J Han: Data Analysis and Interpretation. All author read and approved the final manuscript.

Acknowledgement

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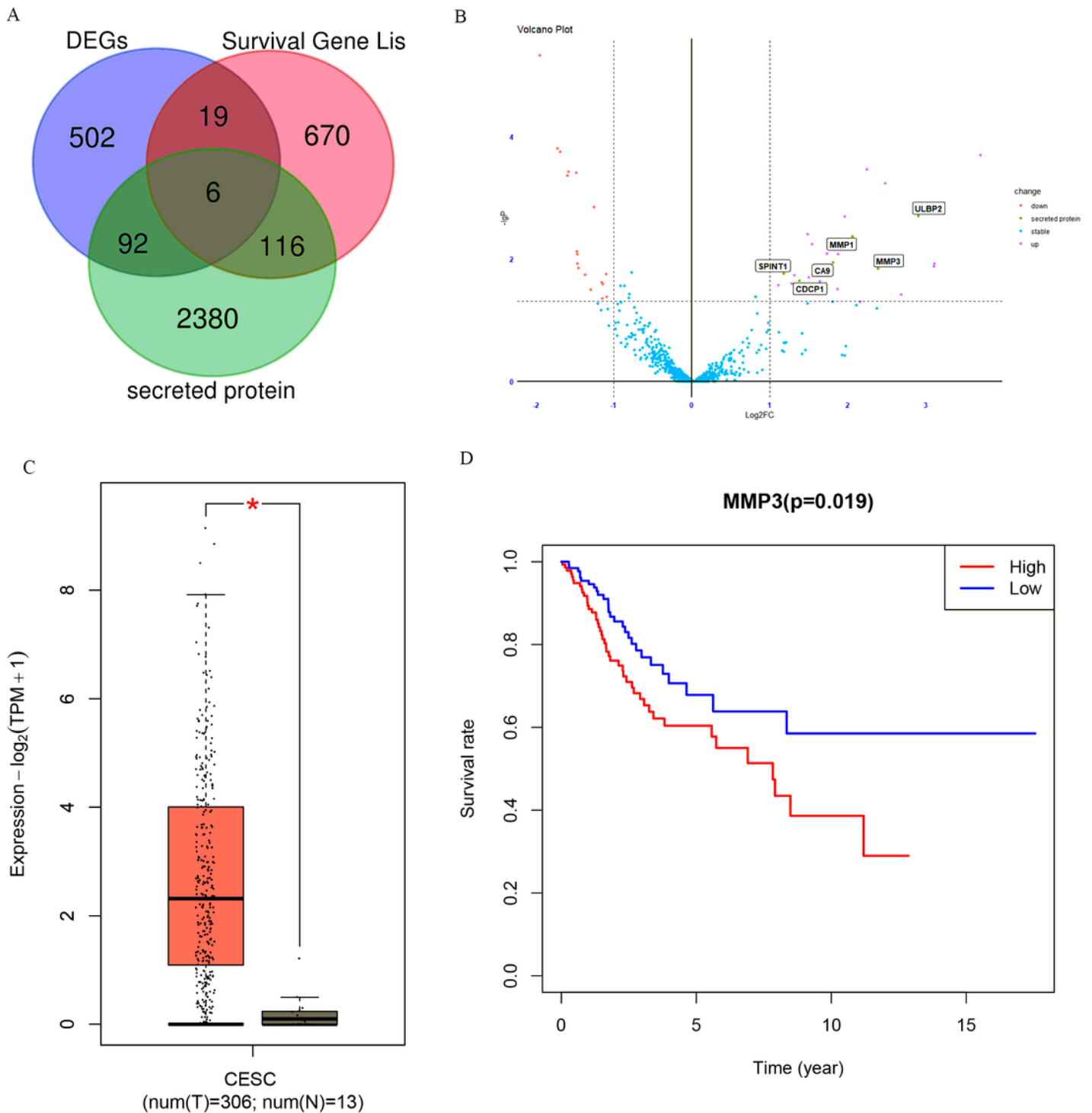
Figures

Figure 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 as well as normal tissues of CCA patients. D: Kaplan-Meier survival analysis of the two groups of high as well as low MMP3 expression in CCA patients. * indicates *p* < 0.05.
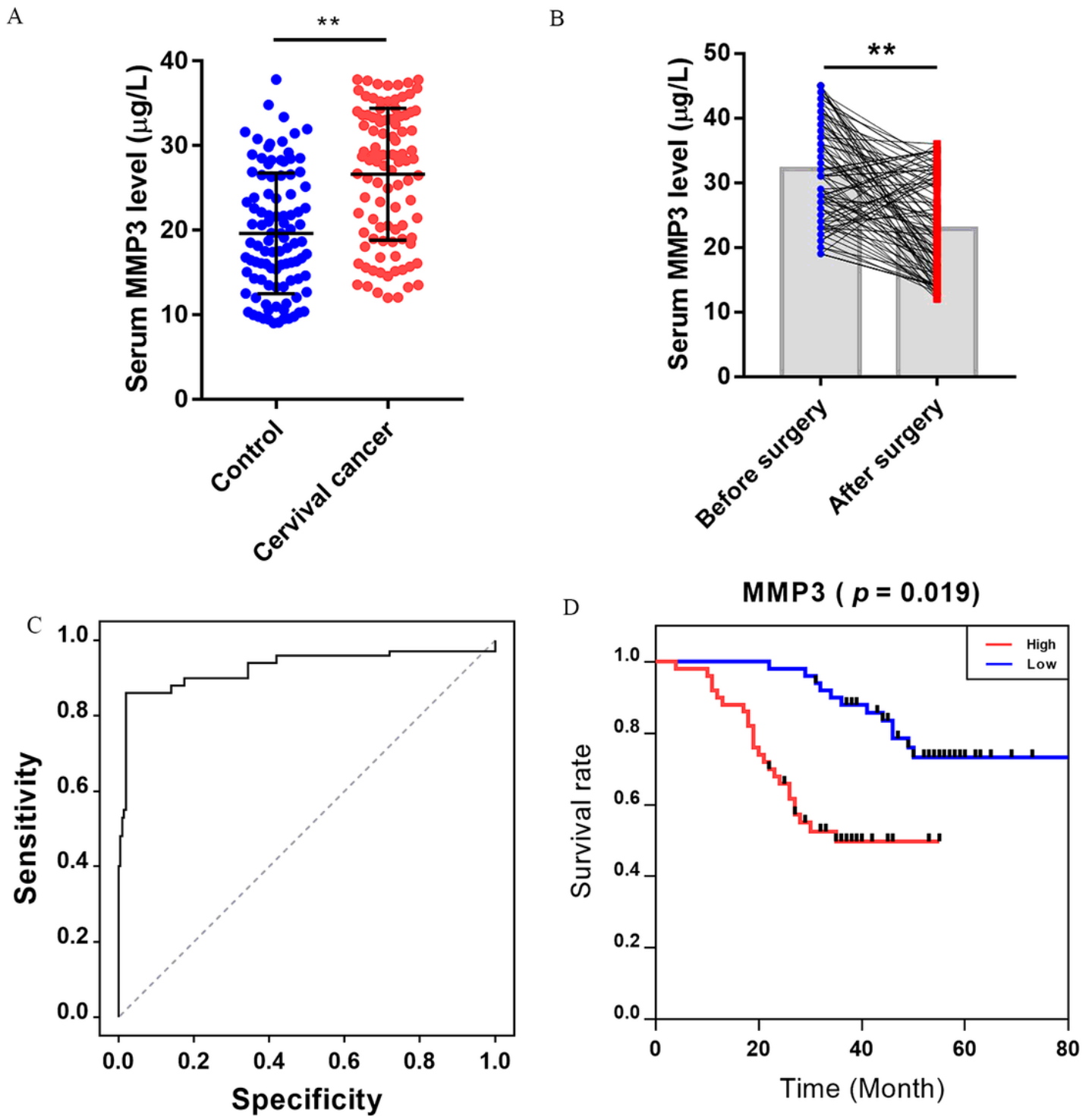
Figure 2

Expression of MMP3 in the serum of CCA patients

A: ELISA results of MMP3 expression in serum of CCA patients and normal controls. B: The serum MMP3 expression levels in CCA patients before and after surgery. C: ROC analysis of serum MMP3 concentration and cervical caner status. D: Survival analysis of patients with high MMP3 expression and low MMP3 expression. ** indicates $p < 0.01$. Data in the graphs were measured and expressed as mean ± standard deviation, and one-way ANOVA with Tukey's post hoc was used for comparison between multiple groups, and the experiment was repeated five times
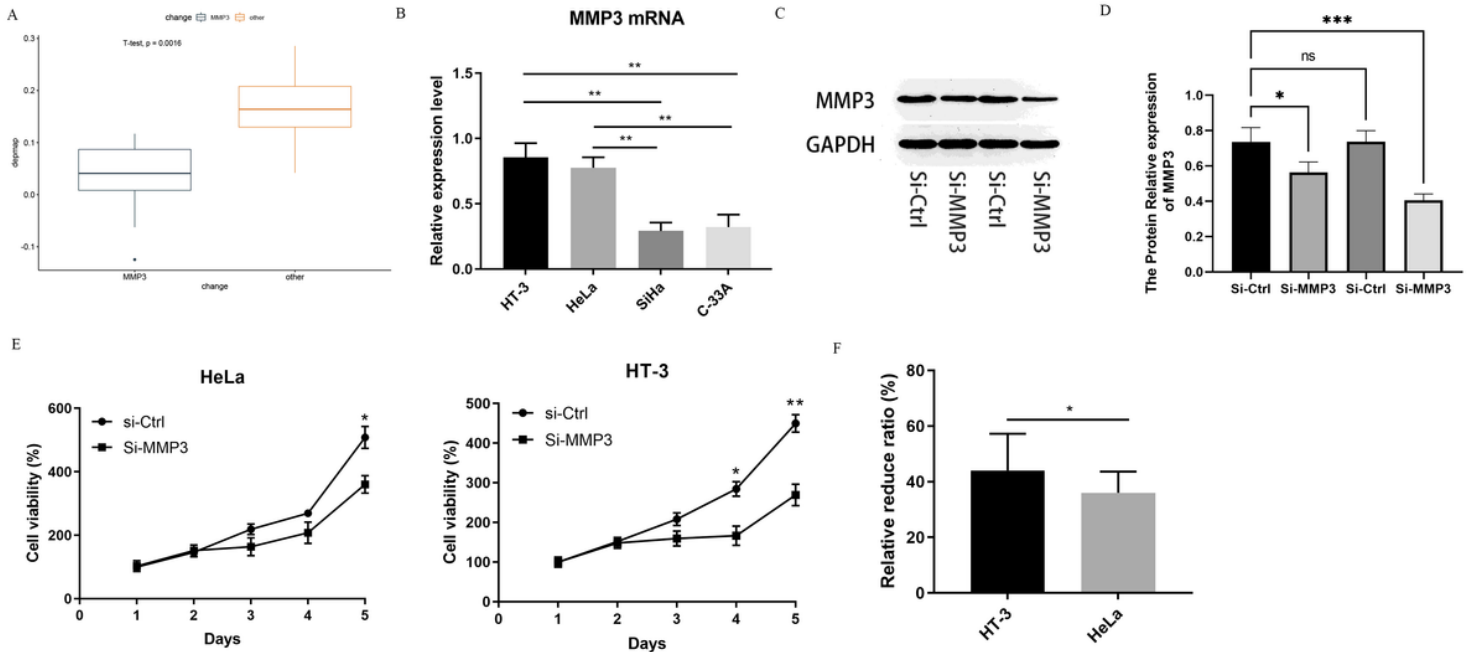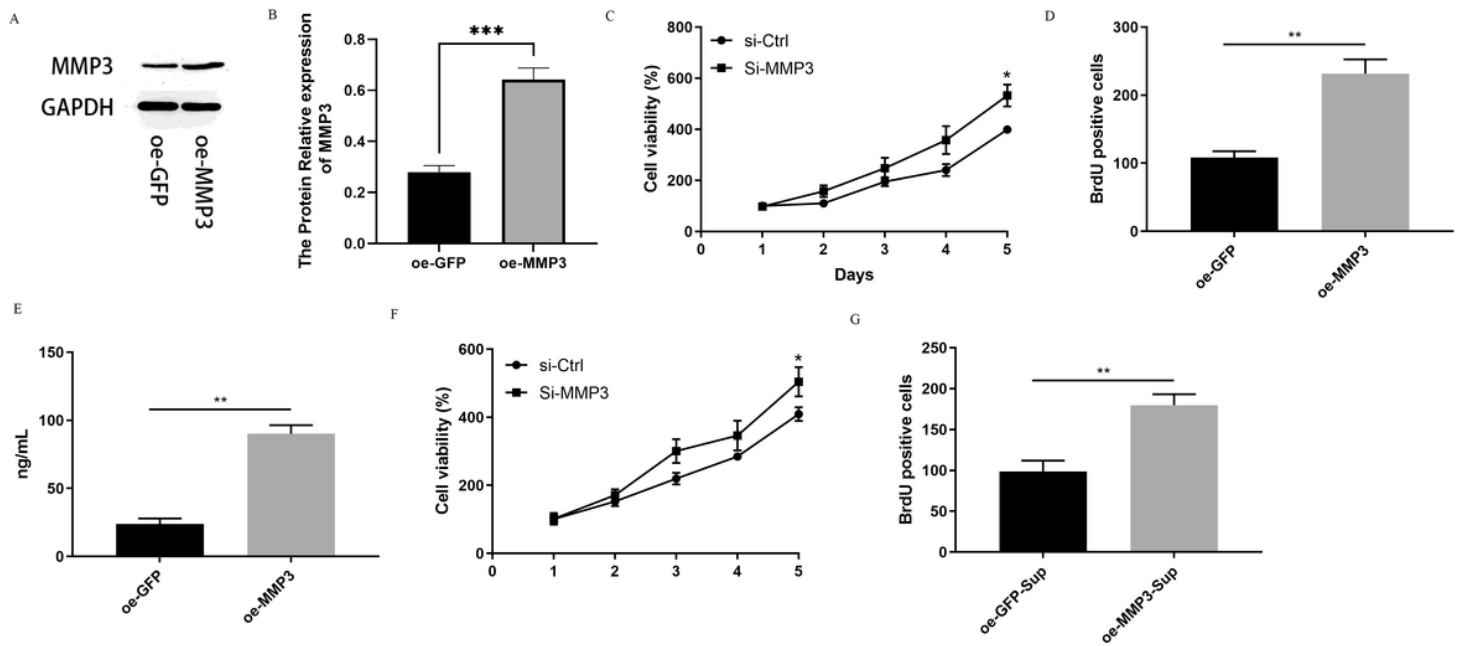

Figure 3

The role of MMP3 in cervical cancer

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, data in the graphs were measured and expressed as mean ± standard deviation, and one-way ANOVA with Tukey's post hoc was used for comparison between multiple groups, and the experiment was repeated five times

Figure 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 are detected through ELISA kit. F: CCK-8 assay detected the cell viability of two groups. G: Number of BrdU positive cells in each group. *$p$less than 0.05, ** p less than 0.01, ***p less than 0.001. The data in the graphs were measured and expressed as mean ± standard deviation, and one-way ANOVA with Tukey's post hoc was used for comparison between multiple groups, the experiment was repeated five times.
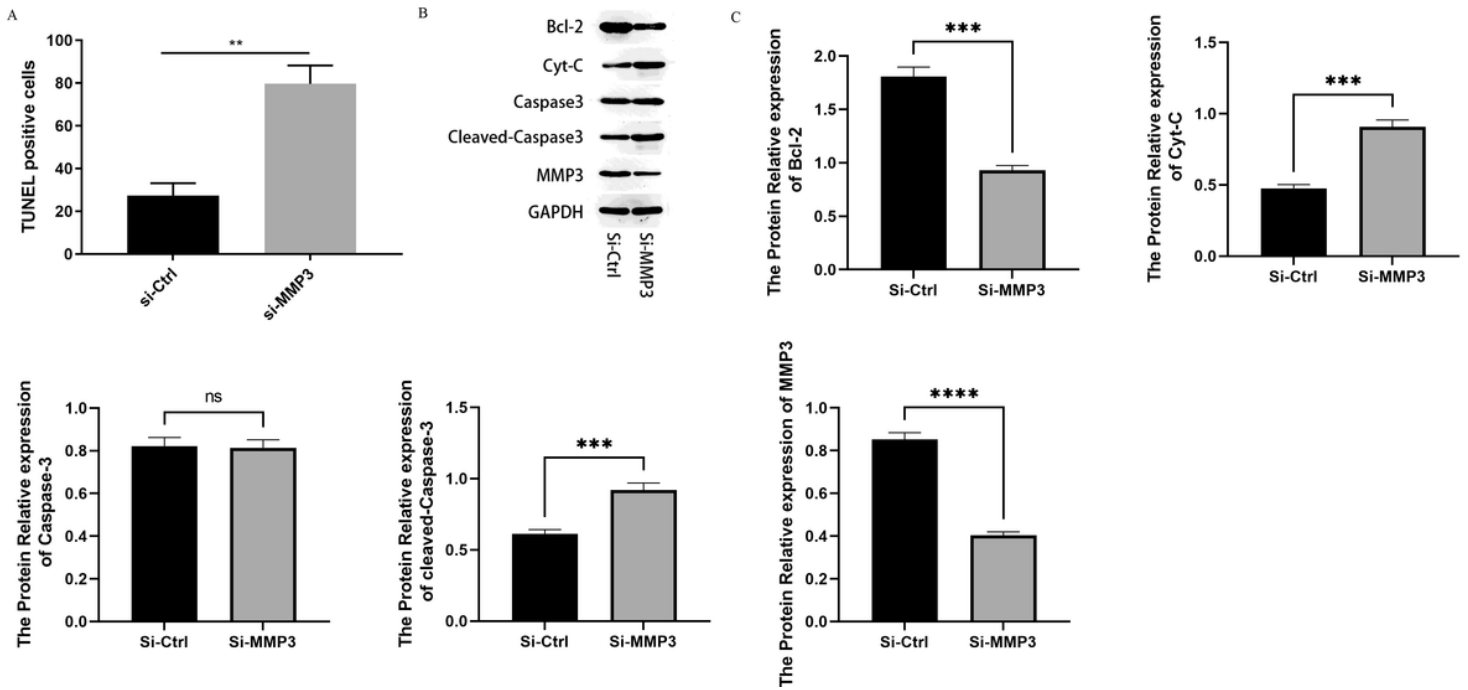
Figure 5

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. **$*p$ less than 0.01,***p less than 0.001,****p less than 0.0001,ns indicated not significant. The data in the graphs were measured and expressed as mean ± standard deviation, and one-way ANOVA with Tukey's post hoc was used for comparison between multiple groups, the experiment is repeated five times.
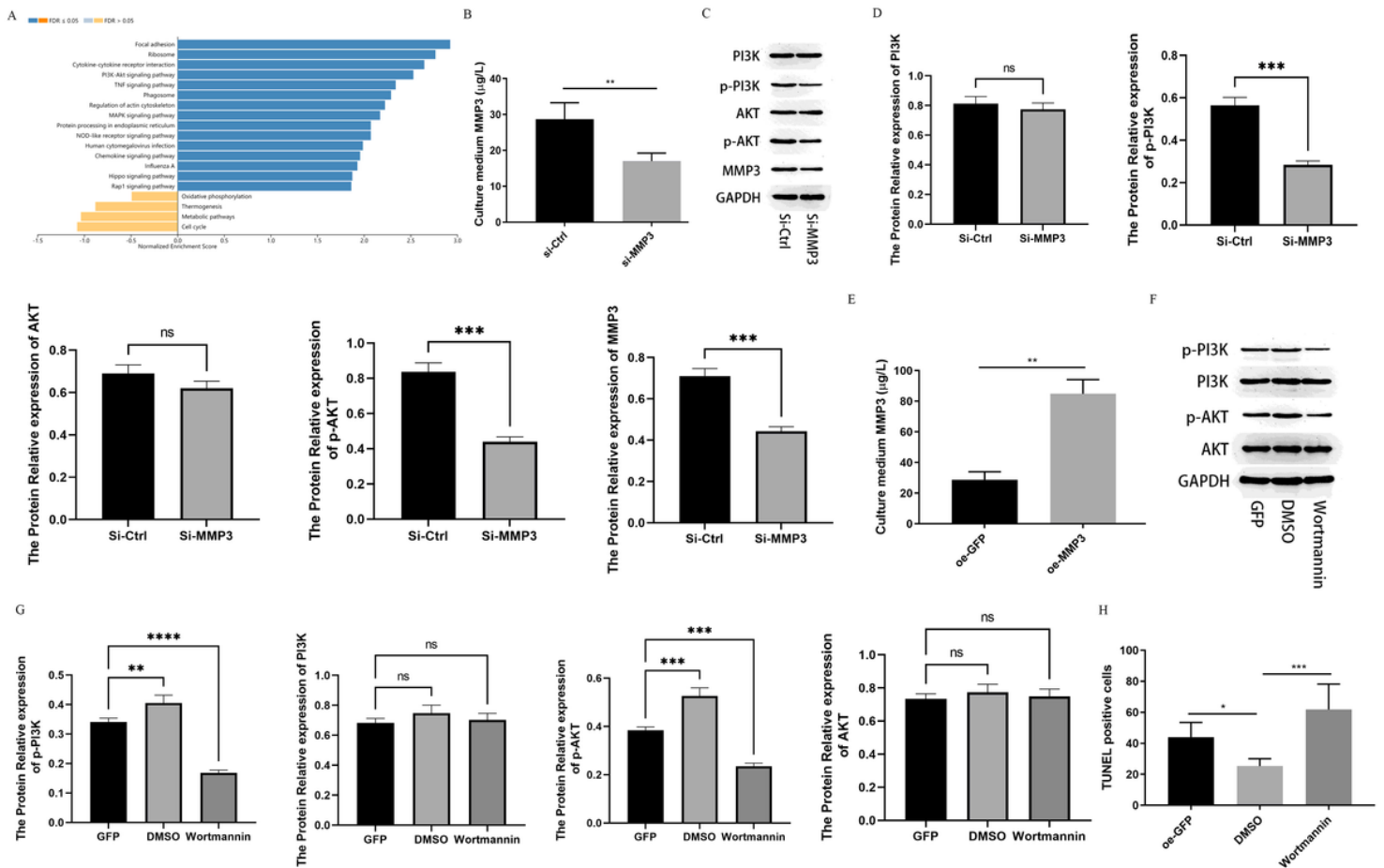
Figure 6

The relationship between MMP3 and PI3K/Akt pathway

A: The KEGG pathways enrichment analysis shows PI3K/Akt pathway is up-regulated in the high-MMP3 expression group; B: ELISA detect 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 detect the PI3K/Akt pathway associated proteins expression in each group; H: TUNEL assay results of three groups. *p less than 0.05, **p less than 0.01, ***p less than 0.001, ns indicates not significant. The data in the graphs were measured and expressed as mean ± standard deviation, and one-way ANOVA with Tukey's post hoc was used for comparison between multiple groups, the experiment was repeated five times.
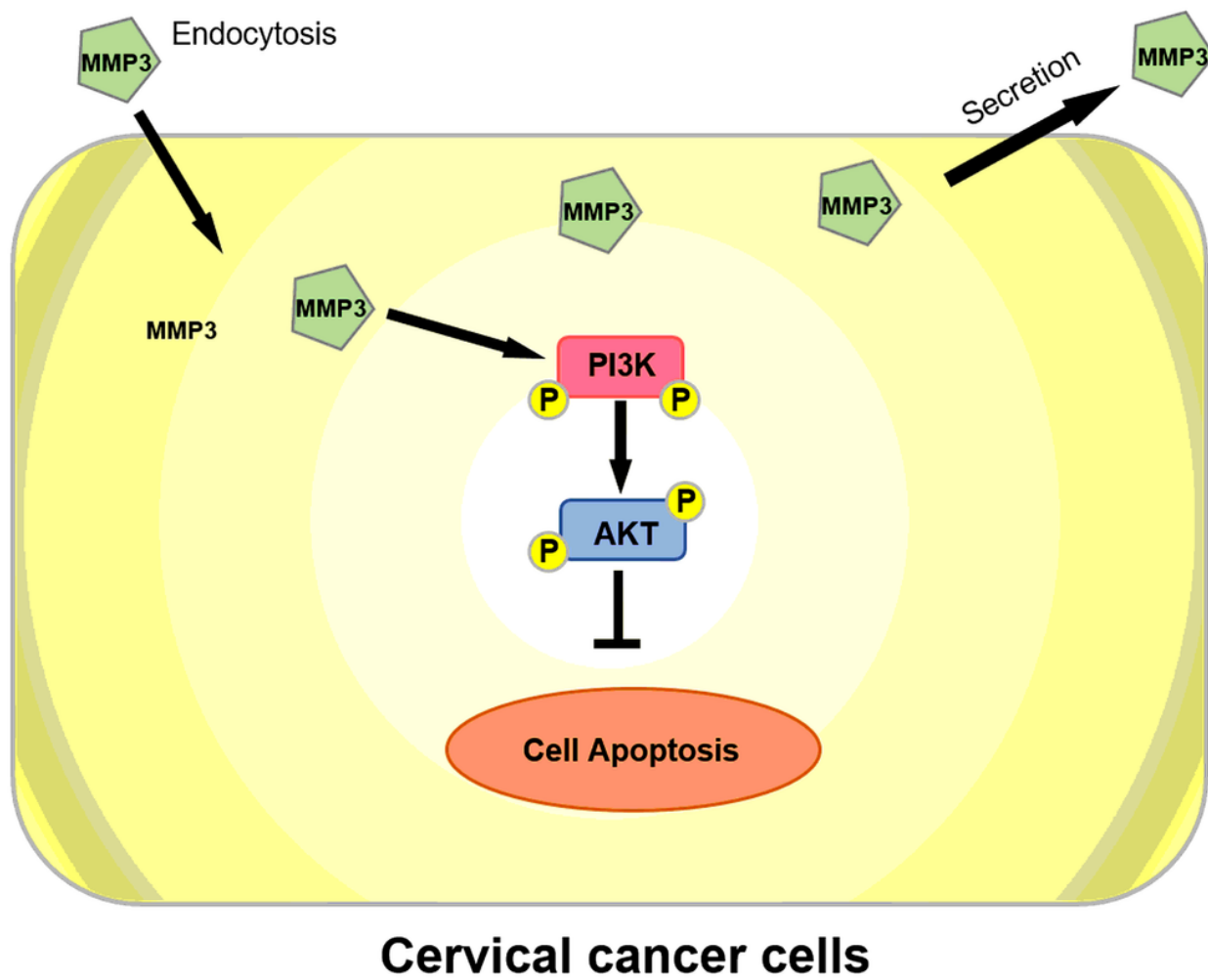
Figure 7

MMP3 inhibits apoptosis through PI3K/AKT signaling pathway in cervical cancer cells

Supplementary Files

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- Fig3CGAPDH1.tif
- Fig3CMMP31.tif
- Fig4AGAPDH1.tif
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- Fig5BCytC1.tif

- Fig5BGAPDH1.tif
- Fig6CGAPDH1.tif
- Fig6CMMP31.tif
- Fig6CpAKT12.tif
- Fig6CpAKT1.tif
- Fig6CPI3K1.tif
- Fig6CpPI3K1.tif
- Fig6FpAKT1.tif
- Fig6FPI3K1.tif
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