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Original Article

The exosome-mediated PI3k/Akt/mTOR signaling pathway in cervical cancer

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Abstract: Background: Cervical cancer is the second most common cancer and one of the leading causes of cancer deaths among women worldwide. Objective: To evaluate the clinical significance of the PI3k/Akt/mTOR signaling pathway in cancer tissues and exosomes extracted from vaginal secretions. Methods: Immunohistochemical staining was used to detect the protein expression of PI3k, Akt, and mTOR in tissue samples from the control group, the CIN (cervical intraepithelial neoplasia) group, and the cervical cancer group. qPCR (quantitative PCR) was used to detect the expressions of PI3k, Akt, and mTOR in cervical cancer tissues, the corresponding adjacent tissues, and exosomes extracted from vaginal secretions. Results: Compared with those of healthy people and CIN, the PI3k/Akt/mTOR protein levels in extracts from tissues were higher in the cervical cancer patients. The PI3k/Akt/mTOR gene and protein levels increased in the cervical cancer tissues with the increase in the degree of malignancy of the cancer. There was no significant difference in PI3k/Akt/mTOR gene expression between the cervical cancer tissues and the exosomes extracted from vaginal secretions, but both were significantly higher than the expressions of the corresponding adjacent tissues. Conclusions: The PI3k/Akt/mTOR signaling pathway mediated by exosomes extracted from vaginal secretions may provide candidate diagnostic biomarkers or potential therapeutic targets.

Keywords: PI3k, Akt, mTOR, cervical cancer, exosome

Introduction

Cervical cancer, as the second most common cancer, is a malignant tumor originating from the squamocolumnar junction of the cervix. It is one of the leading causes of cancer deaths in women, especially among cancers that are not detected at an early stage [1, 2]. Accurate and effective early screening methods are capable of providing early detection and prevention of cervical cancer and of improving the survival of patients [3]. Its pathogenesis may be related to proto-oncogene activation, anti-oncogene inactivation, and the regulation of related gene signaling pathways [4]. Therefore, strengthening the study of the molecular pathology of cervical cancer not only contributes to clarifying the mechanism of its occurrence and development but is also conducive to early diagnosis and early intervention.

PI3k, as a family of lipid kinases in cells, can be activated by several receptor tyrosine kinases, such as epidermal growth factor (EGF), insulin growth factor (IGF), and hepatocyte growth factor activate receptor tyrosine kinases (RTKs), and be converted to PIP3 (3, 4, 5, phosphatidylinositol triphosphate) [5, 6]. PIP3, as a second messenger, binds to the PH domain of Akt and PDK1 (phosphoinositol dependent kinase 1). PIP3 binds to the signal protein Akt and phosphoinositol-dependent kinase (PDK1), which contain PH domains in cells [7]. The protein structure of Akt changes and causes aggregation of Akt at the membrane. This results in protein phosphorylation of PDK1 and PDK2 on the membrane. Akt is activated by the phosphorylation of PDK1 [8, 9]. Activated PI3k-Akt complex further activates its downstream molecule mTOR [10]. PI3k regulates the cell cycle, cellular growth, differentiation, survival, apoptosis, metabolism, angiogenesis, and migration through Akt and mTOR [11-13]. It is linked to the development and progression of many malignant tumors, such as cervical cancer [14, 15], colorectal cancer [16], breast cancer [17], and lung

Table 1. Characteristics of the study participants

		Healthy volunteers	CIN	Cervical cancer	F/X^2 value	*p* value
Enrollment numbers (n)	-	30	68	174	-	-
Age (n, year)	-	55.30±10.89	53.21±11.16	54.87±10.62	0.676	0.562
HPV infection (Positive)	-	18	41	111	0.35	0.841
CIN subgroups	Low-grade	-	37	-	-	-
	High-grade	-	31	-		
Cervical cancer Pathological stages	I	-	-	58	-	-
	II	-	-	58		
	III	-	-	36		
	IV	-	-	22		

Overall, 30 healthy volunteers, 68 cases of CIN and 174 cases of cervical cancer were recruited in the present study. There are no significant differences among the three groups in age and HPV infection rate. An SNK test was used to analyze the data of pairwise comparison among the groups.

cancer [18]. Many reagents, such as L-securinine, curcumin and Brusatol, may also play an antitumor role involving this signaling pathway [19-21]. Exosomes are a kind of extracellular vesicles that exist in many kinds of body fluids, including blood, urine, saliva, pleural effusion and bronchoalveolar lavage fluid (BALF) [22, 23], They are round or elliptical, with a diameter of 40~100 nm and show a complete membrane structure when viewed under a transmission electron microscope [24]. Exosomes contain many proteins, lipids, and nucleic acids and mediate material transduction and signal transduction between cells [25, 26]. They can participate in the process of blood coagulation, inflammation, cell migration, and differentiation [27-29]. Exosomes are mainly involved in intercellular communication and are closely related to tumor formation, development, metastasis, invasion, and drug resistance [30, 31]. They can induce the occurrence of leukemia by influencing cell proliferation, apoptosis, and autophagy and by regulating the bone marrow microenvironment [32-34].

Exosomes can be extracted from various body fluids, such as blood, vaginal lavage fluid, uterine cavity lavage fluid, etc., in patients with ovarian cancer, cervical cancer, endometrial cancer, or other common gynecologic malignancies [35, 36]. The extraction method is noninvasive, and the exosomes are specific to tumor cells; thus, some genes in exosomes can also be used as cancer biomarkers for early screening, early diagnosis, the monitoring of therapeutic effects, and the detection of drug resistance [37, 38]. In the present study, we analyzed and compared the protein expression of PI3k/Akt/mTOR among cervical tissue samples from a healthy control group, a CIN group, and a cervical cancer group. We also detected and analyzed the gene expression of PI3k/Akt/mTOR extracted from the tissues and exosomes of cervical cancer patients at different pathological stages. We found that PI3k/Akt/mTOR genes in exosomes may act as potential biomarkers for the diagnosis and treatment of cervical cancer.

Materials and methods

Inclusion criteria and exclusion criteria

Overall, 30 healthy volunteers, 68 cases of CIN and 174 cases of cervical cancer in Huzhou Central Hospital from March 2016 to December 2018 were recruited in the present study. The characteristics of the participants are shown in **Table 1**. There are no significant differences among the three groups in age or HPV infection rate. The clinical trials involving the patients and the informed consents were approved by the Ethics Committee of Huzhou Central Hospital (no. 201512028). The inclusion criteria were as follows: ① Diagnoses of CIN and cervical cancer were made based on pathological examination. ② At the time of enrollment, all participants signed informed consents under guidelines approved by the Ethics Committee of Huzhou Central Hospital at enrollment. ③ All participants did not have sex within 1 week. ④ All participants had completed their

menstrual cycle for more than 1 week. The exclusion criteria were as follows: ① Pregnant or lactating women. ② Patients suffering from pelvic infection, rheumatic disease, diabetes, or other malignant tumors. ③ Patients who had used a drug, such as a contraceptive, vaginal suppository, vaginal lotion, etc. within the last 3 months.

Collection of clinical data and samples

After securing the informed consent from each patient, basic information and clinical serological indicators were obtained from the medical record management system, and tissue specimens and microscopic images were obtained from the Department of Pathology in Huzhou Central Hospital. The vaginal secretion samples were collected from the participants and then centrifuged at 3000 rpm for 5 minutes. The supernatant was collected and stored in an ultralow temperature freezer.

Immunohistochemical staining

Streptomycin biophile protein/peroxidase binding assay (SP method) was used to detect the protein expression of PI3k, Akt, and mTOR. HE staining and immunohistochemical staining (IHC) were performed after 10% formaldehyde fixation, paraffin embedding, and sectioning (4 μm). The IHC kit and PI3k (1:200), Akt (1:100) and mTOR (1:100) rabbit monoclonal antibodies were provided by Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China) and Maixin Biotechnologies Co., Ltd. (Fujian, China), respectively. The steps were as follows: Dewaxing for 20 min in 60°C constant temperature box, soaking with xylene for 25 min. Hydration with different concentration gradients of alcohol, washing with PBS, Antigen repairing by 0.01 mmol/L citrate buffer (PH 6.0) at 95°C for 15 to 20 min, Sealing with goat serum for 20 minutes, Adding 50 ul antibodies I at 37°C for 1 h, washing with PBS, Adding 50 ul antibodies II at 37°C for 1 h, washing with PBS, adding streptavidin-peroxidase at 37°C for 1 h, washing with PBS, coloration by diaminobenzidine (DAB), washing with water, dyeing 10 min with hematoxylin, diluting 30 s with hydrochloric acid, rinsing 5 min, dehydration, sealing, and microscopic examination. PBS, instead of the first antibody, was used as a negative control, and known positive cervical cancer was used as a positive control under the same staining conditions. Five representative microscope visual fields (20*10 times) were selected for observation and counting. The score was based on the degree of staining and the percentage of stained cells: no staining was 0, light yellow was 1, yellow was 2, and brown was 3. The percentage of stained cells ≤ 5% was 0, the percentage of stained cells from 6% to 25% was 1, from 26% to 50% was 2, and ≥ 51% was 3. The staining degree score of each section was multiplied by the percentage of stained cells score, and a score ≤ 1 was negative (-), a score from 2 to 3 was weakly positive (+), a score from 5 to 6 was moderately positive (++) and a score ≥ 6 was strong positive (+++). All sections were read by two pathologists using the double-blind method.

Extraction and identification of exosomes

The supernatant of vaginal secretion samples was placed on ice until we were ready to perform the isolation. First, 1 ml of vaginal secretion was mixed with 200 μL Total Exosome Isolation reagent (GS0301; Guangzhou Geneseed Biotech Co., Guangzhou, China) by vortexing until the solution was homogenous. Then, the sample was incubated at 2°C for 30 minutes and then centrifuged at 10000 g for 10 minutes at room temperature. The exosomes were located in the pellet at the bottom of the tube. The exosomes were resuspended. An electron microscope (JEM 1011 transmission electron microscope; JEOL, Peabody, MA, USA) was used to observe the exosome ultrastructure to identify the extracted exosomes. Specific steps refer to previously published papers [39].

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR assays were performed to evaluate the expression of the PI3k, Akt, and mTOR genes. Total RNA from the tissues and exosomes was isolated with TRIzol reagent (15596-026; Invitrogen, Carlsbad, CA, USA). The First-Strand cDNA Synthesis kit (PC-0002; Fermentas, Vilnius, Lithuania) was used to synthesize first-strand cDNA. PCR was performed using 20 μL of a PCR reaction mixture (2 μl of the primer mixture (forward and reverse; 10 μmol), 10 μl of SYBR-Green, 7 μl of DEPC water and 1 μl of diluted cDNA). The thermal profile was as follows: 95°C for 5 min, followed by 40 cycles of 94°C for 15 sec, 60°C for 20

sec, and 72°C for 40 sec. The experiments were repeated three times. Real-time fluorescence quantitative PCR (ABI StepOnePlus Real-time PCR system, Applied Biosciences, USA) was used to detect the transcripts. The sequences of the primers used were as follows: GAPDH forward, 5'-TGTTGCCATCAATGACCCCTT-3' and reverse, 5'-CTCCACGACGTACTCAGCG-3'; PI3K forward, 5'-GGGGATGATTTACGGCAAGA-TA-3' and reverse, 5'-CACCACCTCAATAAGTCC-CACA-3'; AKT1 forward, 5'-GCAGCACGTGTACG-AGAAGA-3' and reverse, 5'-GGTGTCAGTCTCCG-ACGTG-3'; mTOR forward, 5'-ATT TGATCAGGTG-TGCCAGT-3' and reverse, 5'-GCTTAGGACATGGT-TCATGG-3'. The expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the housekeeping gene and the minimum value in the corresponding adjacent tissues of cervical cancers as the baseline, and the results were expressed as fold changes.

Statistical analysis

The statistical analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL) for Windows. Data are expressed as the mean ± standard deviation (SD). A chi-squared-test was used to calculate the categorical variables, and Student's *t*-test and SNK test were used to analyze the measurement data. The Spearman rank correlation test was used to analyze the degree and correlation of the findings. A value of $P < 0.05$ was considered statistically significant.

Results

PI3k/Akt/mTOR protein expression in cervical cancer

As shown in **Figure 1**, the protein expression of PI3k, Akt, and mTOR in tissue samples from all the participants were assayed by immunohistochemical staining. Panels A1-3 and B1-3 in **Figure 1** represented negative and positive expression of the PI3k, Akt, and mTOR protein from tissues of CIN, respectively. Panels C1-3 and D1-3 represent negative and positive expression of the PI3k, Akt, and mTOR protein, respectively, from cervical cancer tissues. The positive groups were all stained with yellow granules in the cytoplasms. This indicated that the PI3k, Akt, and mTOR proteins were mainly expressed in the cytoplasms in the CIN and cervical cancer tissues. CIN is a precancerous lesion of cervical cancer. The results of the difference of the PI3k, Akt, and mTOR protein expression in CIN and cervical cancer are shown in **Table 2**. The positive group includes strong positive, moderately positive, and weak positive. The results suggested that the PI3k, Akt, and mTOR protein levels were higher in patients with CIN and markedly higher in cervical cancer compared with the levels in the control group.

PI3k/Akt/mTOR expression in cervical cancer with different malignancy degrees

The protein and gene expressions of PI3k/Akt/mTOR in the cervical cancer tissues with different malignancy degrees were analyzed to further clarify the relationship between tumor biological behavior and the PI3k/Akt/mTOR signaling pathway. Ki-67 indicates the activity of cell proliferation, and the Ki-67 index level is related to the degree of differentiation, invasion, metastasis and prognosis of cancers [40, 41].

Therefore, we selected pathological grade and percentage of Ki-67 as indicators to evaluate the biological behavior of cervical cancer. As shown in **Figure 2**, panels A1-3 and B1-3 represented the PI3k/Akt/mTOR protein expression in the cervical cancer tissues with different pathological stages and percentages of Ki-67, respectively. These results suggested that the higher the pathological grade or the higher the percentage of Ki-67, the higher the PI3k/Akt/mTOR protein expression. Panels A and B in **Figure 3** show the PI3k/Akt/mTOR gene expression in the cervical cancer tissues with different pathological stages and percentages of Ki-67, respectively. These results suggest that the PI3k/Akt/mTOR gene expression levels were positively correlated with the malignancy degree in the cervical cancer tissues.

PI3k/Akt/mTOR gene expression of tissues and exosomes

Using the adjacent tissues as the control group, the PI3k/Akt/mTOR gene expression between the cancer tissues and the exosomes extracted from the vaginal secretions was determined. **Figure 4** shows the electron microscopic image of the exosomes that were successfully extracted from the vaginal secretion samples. The gene expressions of PI3k, Akt, and mTOR in the cervical cancer tissues, corresponding adjacent tissues, and exosomes were mea-

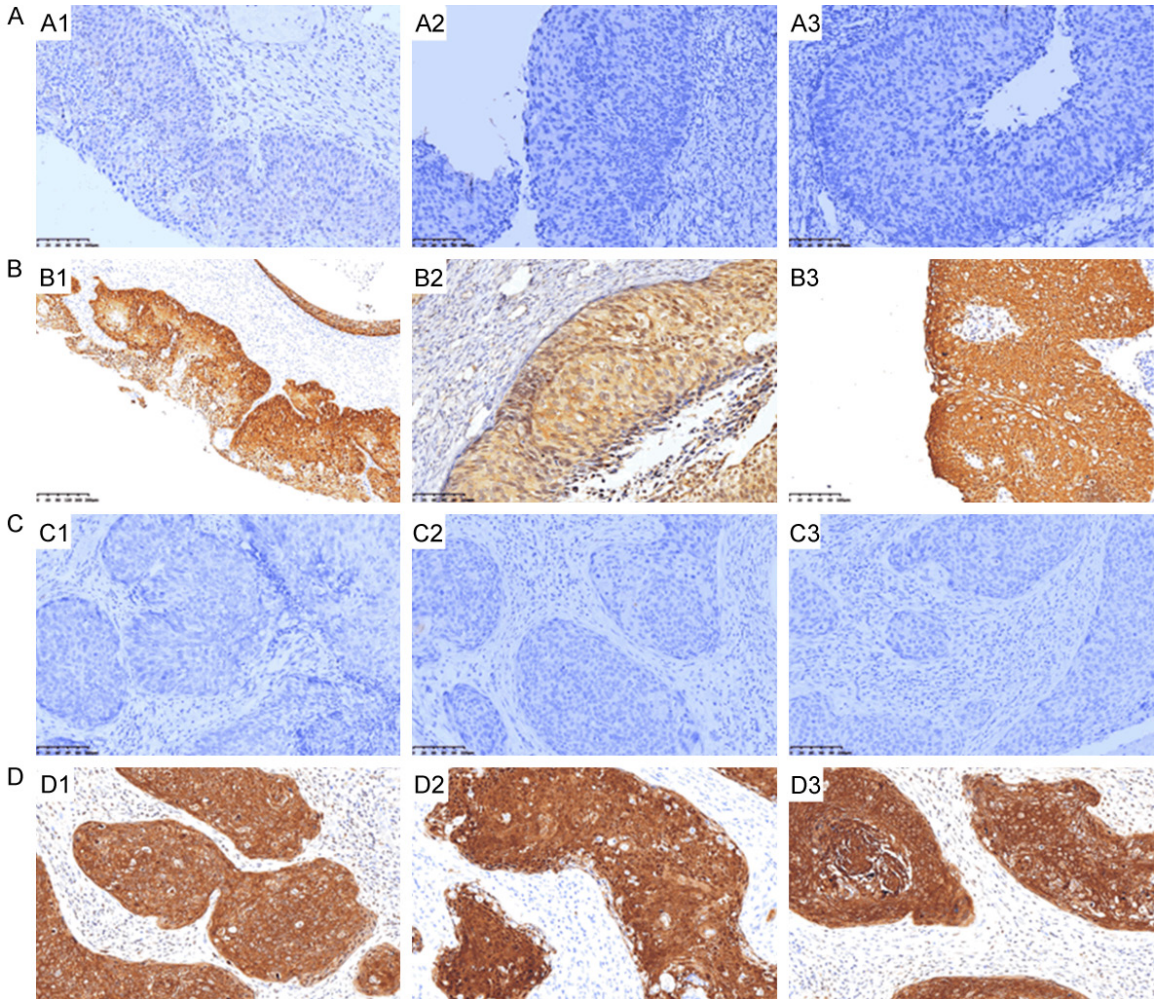

Figure 1. PI3k/Akt/mTOR protein expression in cervical cancer. The protein expression of PI3k, Akt, and mTOR in the tissue samples from all the participants was assayed by immunohistochemical staining. (A1-3) and (B1-3) represent negative and positive expressions of PI3k, Akt, and mTOR proteins from tissues of CIN, respectively. (C1-3) and (D1-3) represent negative and positive expression of PI3k, Akt, and mTOR proteins from cervical cancer tissues, respectively. The positive group were all stained with yellow granules in the cytoplasm.

Table 2. The difference of PI3k, Akt, and mTOR protein expressions

	PI3k		Akt		mTOR	
	Positive	Negative	Positive	Negative	Positive	Negative
Healthy Control	3	27	2	28	4	26
CIN	22	46	30	38	35	33
Cervical cancer	114	60	123	51	104	70
X^2	38.14		48.91		22.17	
p value	< 0.001		< 0.001		< 0.001	

The table shows the differences in the PI3k, Akt, and mTOR protein expressions in CIN and cervical cancer. The positive group includes strong positive, moderately positive, and weak positive. This suggests that the PI3k, Akt, and mTOR protein levels were higher in patients with CIN and markedly higher in cervical cancer.

sured. As shown in **Figure 5**, panels A, B and C represented the PI3k, Akt, and mTOR gene expressions, respectively. There was no significant difference in the PI3k/Akt/mTOR gene expression between the cervical cancer tissues and the exosomes, but both were significantly higher than the expressions of the corresponding adjacent tissues.

Discussion

The PI3k/Akt/mTOR signaling pathway can be activated by multiple cellular stimuli to regulate various physiological functions, such as cell survival, growth and proliferation, in cancers [42].

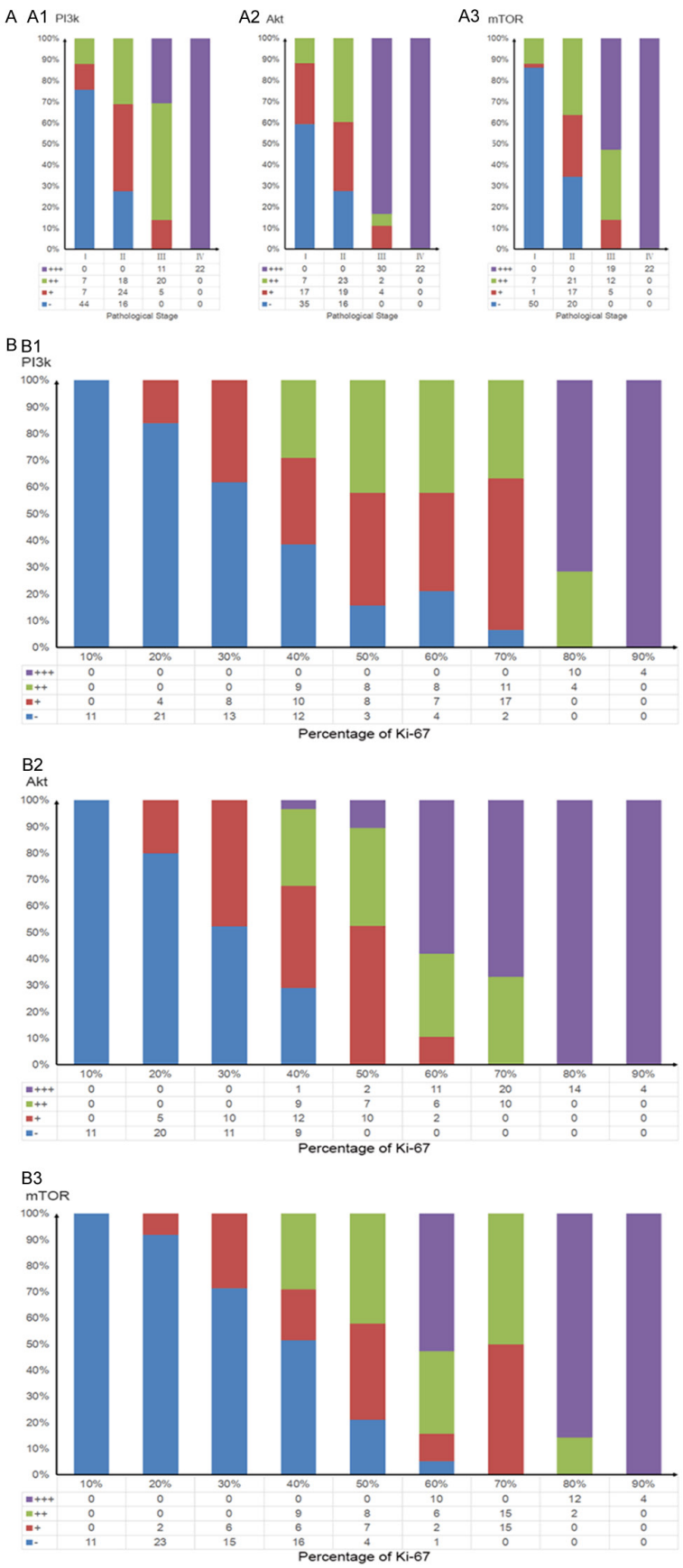

Figure 2. PI3k/Akt/mTOR protein expression in cervical cancer with different malignancy degrees. (A1-3) and (B1-3) represent the PI3k/Akt/mTOR protein expressions in the cervical cancer tissues with different pathological stages and percentages of Ki-67, respectively. The results suggest that the higher the pathological grade or the higher the percentage of Ki-67, the higher the positive rate and grade of PI3k/Akt/mTOR protein expression. The difference is statistically significant as determined by a chi-square test ($P < 0.001$).

Some in vitro studies have shown that some miRNA, such as miR-338 and miR-149, can regulate the genesis and development of cervical cancer through this signaling pathway [15, 43]. The heterogeneity of tumors necessitates that the study of cell lines in vitro is far from being a substitute for the study of specimens in vivo. Although this signaling pathway is widely studied in a variety of tumors, the study of this signaling pathway in cervical cancer has been relatively insufficient. In the present study, we found that the PI3k/Akt/mTOR protein levels were higher in cervical cancer patients by comparing them with those of healthy people and CIN. As the malignancy degree of the cancer increases, so do the levels of the PI3k/Akt/mTOR gene and protein expressions in the cervical cancer tissues.

Important progress in molecular classification for the treatment and prognosis of colorectal cancer and breast cancer has been made [44, 45]. The molecular classification is an innovation that will provide guidance for the diagnosis and treatment of cancers. However, the molecular classification of cervical cancer is not yet on

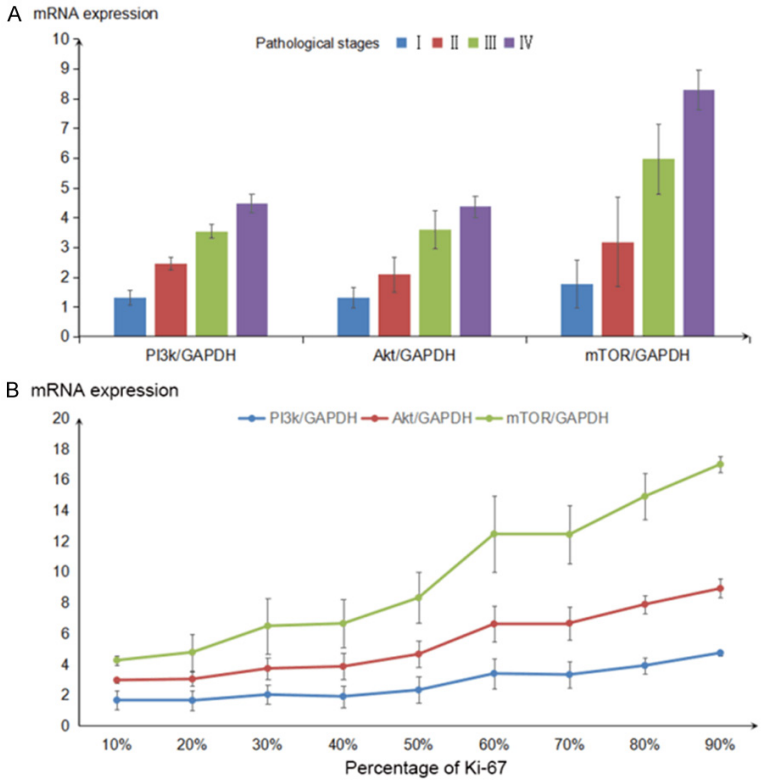

Figure 3. PI3k/Akt/mTOR gene expression in cervical cancer with different malignancy degrees. A and B. Show the PI3k/Akt/mTOR gene expression in the tissues of cervical cancer with different pathological stages and percentage of Ki-67, respectively. The SNK test was used to analyze the data of the pairwise comparisons among the groups, and all of the differences are statistically significant ($P < 0.001$). The Spearman rank correlation test was used to analyze the correlation, and all of the differences are statistically significant ($P < 0.001$). This suggests that the PI3k/Akt/mTOR gene expressions are positively correlated with the malignancy degree in cervical cancer tissues.

the agenda. Our study suggested that the PI3k/Akt/mTOR signaling pathway is associated with the tumorigenesis and malignancy of cervical cancer. A number of key proteins in the PI3K/Akt/mTOR signaling pathway are also hot drug design targets, and the development of small molecular inhibitors has made great progress. Many candidate drugs have entered into clinical studies, including pan-PI3K inhibitors, dual PI3K/mTOR inhibitors, isoform-specific PI3K inhibitors, AKT inhibitors, etc. [46]. Whether these drugs have the greatest antitumor effect or not, the selection of inhibitors, combination types and use methods need to be determined according to the molecular classification. Thus, our findings may provide a reference for the molecular classification of cervical cancer.

Histopathological diagnosis is the gold standard for cancer diagnosis, but the process of obtaining pathological tissue is invasive and has many negative effects, such as bleeding and infection. A vaginal secretion is easy to obtain, and the process of obtaining it is noninvasive. It can be used as a valuable humoral detection substance for gynecological diseases. Some studies have found that some genes in exosomes can be used as diagnostic targets for cervical cancer [47, 48]. In the present study, we found that there was no significant difference in the PI3k/Akt/mTOR gene expression between cervical cancer tissues and the exosomes extracted from vaginal secretions, but both were significantly higher than the expressions of the corresponding adjacent tissues. PI3k/Akt/mTOR in exosomes may be used as a potential clinical diagnostic marker for cervical cancer. As a novel discovered suborganelle structure, most of the studies on exosomes have focused on the role of exosomes in the development and metastasis of cancer by carrying proteins and genes [31, 49, 50]. Unfortunately, no breathrough has been made so far. However, as a diagnostic tool, exosomes in vaginal secretions can be used in the diagnosis of cervical cancer. This highlights the clinical value of exosomes from another angle.

At present, the role of the PI3k/Akt/mTOR signaling pathway and its regulatory mechanism are not fully understood, and there are still many problems to be further studied. For example, what is the connection between the PI3k/Akt/mTOR signaling pathway and other signaling pathways? How does the PI3k/Akt/mTOR pathway drive the development of tumors? We found the aggregation of genes related to the PI3k/Akt/mTOR signaling pathway in the exosomes of vaginal secretions. This will provide a novel idea and direction for the PI3K/Akt/mTOR signaling pathway mediated by exosomes to participate in the occurrence and development

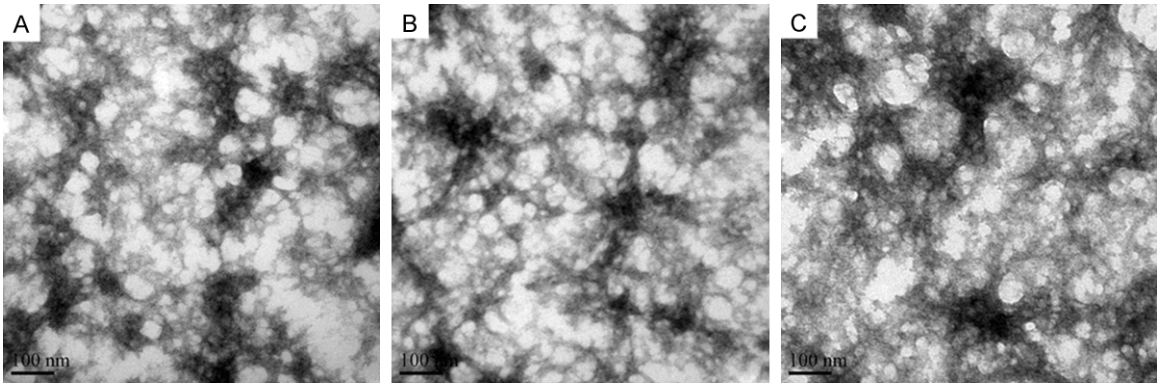

Figure 4. Ultrastructure of the exosomes. Transmission electron microscopy was used to observe the ultrastructural characteristics of the exosomes extracted from the vaginal secretion samples. A-C. Represent electron microscopic images of the exosomes that were successfully extracted from the vaginal secretion samples in patients with cervical cancer. The exosomes had a circular or elliptical shape, a size of approximately 40-150 nm in diameter and an integrated membrane.

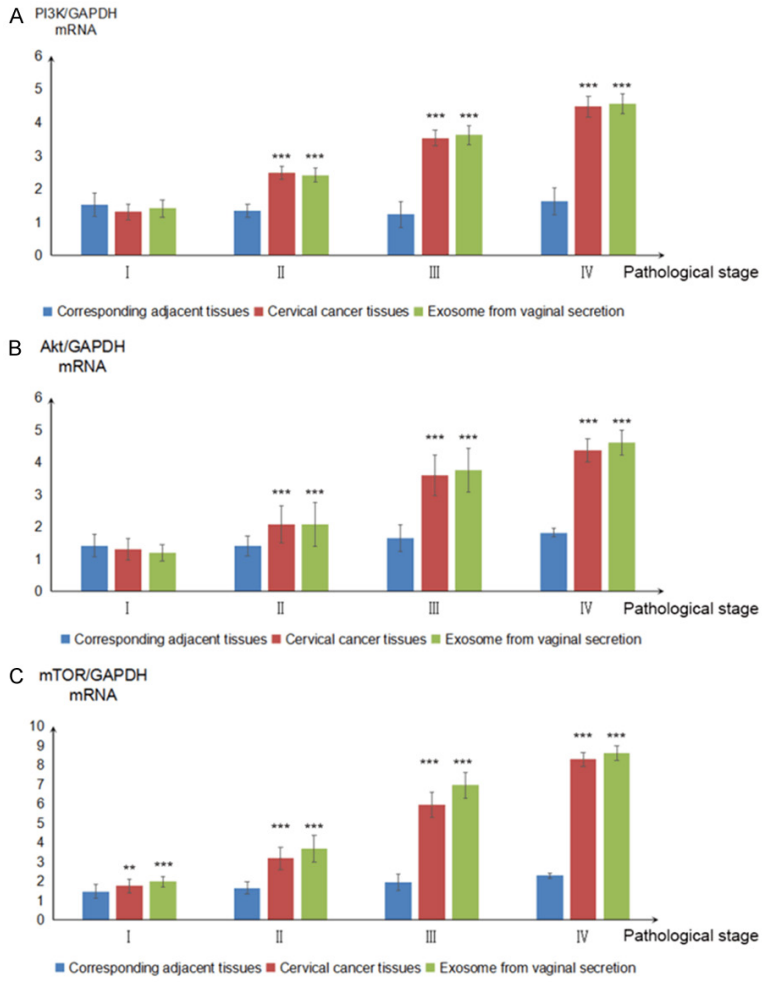

Figure 5. PI3k/Akt/mTOR gene expression of tissues and exosomes. The mRNA expression level of PI3k, Akt, and mTOR in cervical cancer tissues, corresponding adjacent tissues, and exosomes was determined by qPCR and expressed as a fold change relative to the minimum value in the control group. A-C. Represent the PI3k, Akt, and mTOR gene expressions, respectively. There was no significant difference in the PI3k/Akt/mTOR gene expression between the cervical cancer tissues and the exosomes, but both were significantly higher than the expressions of the corresponding adjacent tissues. Each column is shown as the mean of three separate experiments. An SNK test was used to analyze the data of the pairwise comparisons among the groups. **P < 0.01. ***P < 0.001.

of cervical cancer. Further molecular biology research in vitro will provide more useful information to solve the mystery of cervical cancer.

There are also limitations in the research findings, such as the lack of an in-depth study of pathogenesis, the small size of the clinical sample, the lack of research from multiple regions and multiple centers, and no detection of downstream genes from the PI3k/Akt/mTOR pathway. Moreover, follow-up visits should be performed, and more molecular experimentation should be done to make explicit the role of the exosome-mediated PI3k/Akt/mTOR signaling pathway in the outcomes of cervical cancer.

Conclusion

The PI3k/Akt/mTOR signaling pathway mediated by exosomes extracted from vaginal secretions may provide candidate diagnostic biomarkers or potential therapeutic targets.

Acknowledgements

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The clinical trials involving the patients and the informed consents were approved by the Ethics Committee of Huzhou Central Hospital (no. 201512028). Written informed consent was obtained from the patients for the publication of this paper and the accompanying images.

Disclosure of conflict of interest

None.

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