Ultrasound Microbubbles-mediated MicroRNA-505 Regulates Cervical Cancer Cell Growth via AKT2

Leilei Xu (✉ LeileiXu3161@163.com)

the First Hospital of Huai'an Affiliated to Nanjing Medical University <https://orcid.org/0000-0001-7928-7881>

Qin Zhang

the First Hospital of Huai'an Affiliated to Nanjing Medical University

Changhua Li

the First Hospital of Huai'an Affiliated to Nanjing Medical University

Fu Hua

the First Hospital of Huai'an Affiliated to Nanjing Medical University

Xiaoping Liu

the First Hospital of Huai'an Affiliated to Nanjing Medical University

Research

Keywords: Cervical cancer, Microbubbles, Ultrasound, microRNA-505, AKT2

Posted Date: June 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-34897/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: The gene-loaded microbubbles (MBs) combined with ultrasound resulting in increased delivery efficiency, may be a novel method of gene delivery. We explored the effects of ultrasound and microbubbles (USMB)-mediated microRNA (miR)-505 on cervical cancer (CC) development.

Methods: miR-505 mediated by USMB was prepared. The effect of miR-505 on its transfection efficiency was studied by RT-qPCR. The effect of miR-505 on HeLa cell proliferation was evaluated by MTT and colony formation assays. Flow cytometry was used to study cell cycle changes, Hoechst was utilized to detect apoptosis. Through the wound healing and Transwell assay, the migration and invasion ability of HeLa cells were measured. The target gene of miR-505 was predicted, and its expression in CC was detected. The target relationship and the effect of the target gene on HeLa cells were further verified.

Results: USMB-miR-505 showed higher transfection efficiency than miR-505 alone. miR-505 inhibited HeLa cell malignant episodes, which were reinforced by USMB treatment. miR-505 targeted AKT2. AKT2 was highly expressed in CC, and overexpression of AKT2 significantly reversed the inhibitory effect of miR-505 mediated by USMB on HeLa cell malignant biological behaviors.

Conclusion: USMB-miR-505 inhibited HeLa cell malignant biological behaviors by targeting AKT2.

Introduction

Cervical cancer (CC) is an aggressive gynecological malignancy with a high risk of recurrence and death, mainly in women [1, 2]. It is the 4th most prevalent female cancer and causes approximately 265,700 annual deaths worldwide [3–5]. The incidence varies across the world, with the highest incidence rate in Eastern Africa and lowest incidence in Western Asia [6]. Over 70% of CC cases diagnosed in developing countries are locally invasive or metastatic, and the diagnosis of early stage CC is difficult, thus leading to high mortality [7]. Numerous efforts have been made to prevent CC that include the expansion of human papillomavirus vaccine coverage and primary screening [8], which eventually reduce the disease in many developed countries [9, 10]. Fortunately, radical hysterectomy has gained increasing popularity in the last twenty years and enjoyed broad acceptance across Europe and the Americas [11]. Although there is great progress in diagnostic and therapeutic strategies, the survival rate for CC patients remains poor [12]. Thus, this study aims to identify novel biomarkers and develop non-invasive therapies for the diagnosis and prognosis of CC.

microRNAs (miRs) participate in most biological processes like apoptosis and epithelial-to-mesenchymal transition (EMT) by regulating gene expression [13]. miRs and genes can serve as biomarkers for prognosis and treatment of CC [14]. miR-505 suppressed EMT and metastasis phenotypes in HK-1 cells and prevented macroscopic lung metastases [13]. But the role of miR-505 in CC is less studied. Microbubbles (MBs) were originally developed for ultrasound imaging, and now they are considered as ultrasound-assisted gene delivery tools to disturb cell membranes and accelerate gene entering into cells [15]. Behaviors of MBs under ultrasonic irradiation will lead to short-term membrane permeability of

surrounding cells, thus promoting targeted local administration without cell damage [16]. The combination of ultrasound and microbubbles (USMB) is an emerging approach for non-invasive enhancement of uptake of drugs and genes [17, 18]. Microbubbles promote ultrasound-mediated gene transfer efficiency in cell culture and tumor transplantation of hindlimb, and may selectively transfer therapeutic genes to disease sites [19]. USMB-mediated miR (USMB-miR) delivery has been considered as an effective tool for treatment of cancer and cardiovascular diseases [20, 21]. Interestingly, USMB-miR-133a prevented tumor growth and improved survival of breast cancer mice, and USMB-mediated miR-767 silencing offered a novel therapeutic strategy for non-small cell lung cancer treatment [22, 23]. However, the mechanism of USMB-miR-505 in CC remains unclear. We performed serials of molecular and histological experiments to evaluate the relevance of USMB-miR-505 in CC development.

Materials And Methods

Sample collection

From March 2018 to January 2019, cancer and adjacent normal tissues of 20 CC patients undergoing surgery in the First Hospital of Huai'an Affiliated to Nanjing Medical University were collected. The patients aged between 45 and 69 years old. They were free of any other malignant tumors and did not receive preoperative radiotherapy and chemotherapy. The tissues were immediately preserved at -80°C .

Cell culture

Hela cells at logarithm phase from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Gibco; Waltham, MA, USA) with 10% fetal bovine serum (FBS, Gemini Bio-products, West-Sacramento, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The media were renewed every 2 days. Cells were subcultured when cells reached 80% confluency.

Cell transfection and grouping

miR-505 mimic, overexpression (OE)-AKT2 and their respective negative controls (NC) were designed and synthesized by Genepharma (Shanghai, China), while miR-505 mediated by USMB was prepared in the laboratory. HeLa cells were assigned into NC mimic, miR-505 mimic, miR-505-MB, miR-505-MB + OE-NC, miR-505-MB + overexpression-AKT2 groups.

After 1 μg NC mimic or miR-505 mimic was mixed with 2 μL Lipofectamine 2000 (Invitrogen), cells were suspended again with 500 μL RPMI-1640. The mixed solution was put to the cells at 50 μL /well. Next, cells in the miR-505-MB group were treated with miR-505-MB at 50 μL /well and subjected to 10 Mhz ultrasound for 30 minutes. Based on the treatment in the miR-505-MB group, cells in the miR-505-MB + OE-NC or miR-505-MB + OE-AKT2 group were stably transfected with corresponding vectors using Lipofectamine 2000 after miR-505 expression was detected. After transfection and ultrasound exposure, cells were harvested for following experiments.

Preparation of USMB

The USMB was prepared as previously described [24]. MBs were synthesized by ultrasonic dispersion of 1 mg/mL polyethylene glycol-40 stearate, 2 mg/mL 1-bisstearyl phosphatidylcholine, 0.4 mg/mL 1,2-bisstearyl-3-trifluoromethylpropane and decafluorobutane (Avanti Polar Lipids Inc., Alabaster, AL, USA) in a water box. After that, the MBs were observed under an inverted fluorescence microscope (DM 4000B, Leica, Germany) and detected by a nanometer particle size analyzer (NS-90, OMEC Instruments Co., Ltd., Zhuhai, Guangdong, China). The MBs were filtered using a 1 μ m filtration membrane, and adjusted into $0.8-1.6 \times 10^9$ /mL. Thereafter, 1 μ g miR-505 mimic was blended with 50 μ L MBs suspension and cultured at 37 °C for 30 minutes. The unbound miR-505 was removed by 0.16 M phosphate buffer saline (PBS) to get miR-505-MB.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted by TRIzol (Invitrogen) and reversely transcribed into cDNA using a cDNA reverse transcription kit (TOYOBO, Japan). The expression of mRNAs and miR were quantified using a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with a SYBR green PCR Master Mix (TOYOBO, Japan), with U6 or GAPDH as references. The expression was calculated by the $2^{-\Delta\Delta C_t}$ method. The primers used were presented as follows: miR-505, 5'-GGAGCCAGGAAGTATTG-3' (forward), 5'-GAACATGTCTGCGTATCTC-3' (reverse); AKT2, 5'-CATCCTCATGGAAGAGATCCGC-3' (forward), 5'-GAGGAAGAACCTGTGCTCCATG-3' (reverse); E-cadherin, 5'-GCCTCCTGAAAAGAGAGTGGAAG-3' (forward), 5'-TGGCAGTGTCTCTCCAAATCCG-3' (reverse); N-cadherin, 5'-CCTCCAGAGTTTACTGCCATGAC-3' (forward), 5'-GTAGGATCTCCGCCACTGATTC-3' (reverse); Vimentin, 5'-AGGCAAAGCAGGAGTCCACTGA-3' (forward), 5'-ATCTGGCGTTCCAGGGACTCAT-3' (reverse); GAPDH, was 5'-GTCTCCTCTGACTTCAACAGCG-3' (forward), 5'-ACCACCCTGTTGCTGTAGCCAA-3' (reverse); U6, 5'-ATTGGAACGATACAGAGAAGATT-3' (forward), 5'-AGGAACGCTTCACGAATTTG-3' (reverse).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

An MTT kit (C0009, Beyotime, Shanghai, China) was employed to examine the vitality of cells. Each well was added with 10 μ L MTT solution, and cells were incubated for 4 hours. Then each well was added with 100 μ L formazan solution and mixed properly. After an incubation at 37 °C for 3–4 hours, the absorbance at 570 nm was determined with a microplate reader (Bio-Rad, Inc., Hercules, CA, USA).

Colony formation assay

After 24 hours of transfection, 500 cells were seeded into the 6-well plates and cultured for two weeks. The cell colonies were fixed for 5 minutes with methanol and stained for 15 minutes with 0.1% crystal violet. Thereafter, cell colonies were counted and photographed.

Flow cytometry

Flow cytometry was utilized to detect cell cycles. After 48 hours of transfection, the cell cycle was evaluated by PI staining. Then cells were detached with trypsin, washed twice with PBS, and fixed at 4 °C overnight in 70% cold ethanol. After twice washes with PBS, the cells were reacted with 50 µg/mL PI (Keygen) for 30 minutes. Cell cycle analysis was performed immediately using a FACS Calibur flow cytometer (BD, San Diego, USA). The proportion of cells in G1, S and G2 phases was detected.

Enzyme-linked immunosorbent assay (ELISA)

Human Bax (ab199080) and Bcl-2 (ab119506) was measured according to the ELISA kits (Abcam, Cambridge, MA, USA).

Hoechst 33258 staining

The transfected cells were seeded at 1×10^5 cells/mL (3 mL per well) into 6-well plates. After the cells were incubated at 37 °C with 5% CO₂ for 24 hours, the media were removed. Cells were then fixed with paraformaldehyde, washed with PBS, and stained with Hoechst 33258 (HY-15558, MedChemExpress, NJ, USA) in the dark. After 30 minutes, cells were observed under a fluorescence microscope, and 5 fields were randomly chosen from each slide.

Wound healing assay

Cells were plated into 6-well plates and cultured for 24 hours. Then, a 200 µL pipette tip was applied to scratch 3 parallel lines, and cells were washed twice in PBS. Then cells were cultivated at 37 °C, and photographed at 0 and 24 hours under a FSX100 microscope (Olympus) after wounding. Migration ratio was estimated by examining the change of scratch area.

Transwell assay

The invasion experiments were carried out in the Transwell chamber coated with Matrigel (BD Biosciences). Briefly, 500 µL DMEM with 10% FBS was supplemented to the basolateral chamber, while 2×10^5 cells were paved in the apical chamber. After 24 hours, cells passing through the Matrigel were fixed with methanol and stained by 0.1% crystal violet, otherwise removed by cotton swabs. Finally, cells were counted and the stained cells represented the invasiveness.

Western blot (WB)

Total protein was extracted using RIPA buffer (Solarbio Science & Technology Co., Ltd., Beijing, China) containing proteinase inhibitor. Then, the protein was quantified by a BCA kit (Thermo Fisher), and separated using SDS-PAGE, transferred onto PVDF membranes (Millipore, Billerica, MA, USA) and sealed with 5% skim milk. Subsequently, the membranes were incubated with rabbit anti-human AKT2 (1:1000, #2964, cell signaling technology) at 4 °C all night, with rabbit anti-human GAPDH (1:10000, ab181602, Abcam) as the control. Then membranes were probed with secondary antibody goat anti-rabbit IgG H&L (HRP, 1:25000, ab205718, Abcam) in the dark at 37 °C for 1 hour. Finally, the immunoblots were subjected to enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA).

Dual-luciferase reporter gene assay

The binding site between miR-505 and AKT2 was predicted from Starbase (<http://starbase.sysu.edu.cn/>), and amplified using PCR and cloned into pGL3 vector (Promega, Madison, WI, USA) to obtain AKT2 wild-type (WT). AKT2 mutant (MT) was obtained by mutating the binding site. These vectors were co-transfected with miR-505 mimic and its control into 293T cells (ATCC) by Lipofectamine 2000. After 48 hours of transfection, the luciferase activity was measured by the luciferase reporter system (Promega).

RNA immunoprecipitation (RIP)

RIP lysis buffer kit (Millipore) was used for RIP experiments. In short, HeLa cells were lysed in RIP lysis buffer, and RNA was precipitated with anti-AGO2 (Millipore) and anti-IgG (Millipore). TRIzol reagent was used to purify the immunoprecipitated RNA, and RT-qPCR was used to detect the gene expression.

Statistical analysis

SPSS 22.0 (IBM Corp. Armonk, NY, USA) was used for data analysis. The obtained data of 3 independent experiments were described as mean $\pm$ standard deviation. Comparisons between group pairs were analyzed using paired *t* test or unpaired *t* test, while comparisons among multi-groups were analyzed through one-way or two-way analysis of variance (ANOVA). Pairwise comparison after ANOVA analysis was conducted using Tukey's multiple comparisons test. *P* value shall be based on a two-sided test. *P* < 0.05 was considered as a statistical difference.

Results

USMB-miR-505 promoted miR-505 transfection efficiency in HeLa cells

According to a previous report [25], miR-505 is poorly expressed in CC, which can inhibit the development of CC. The poor expression of miR-505 in CC was confirmed by RT-qPCR in our collected CC tissues (Fig. 1A). The MBs were opalescent suspension, and the homogeneous parts were spherical and distributed uniformly (Fig. 1B). The average particle size was about 2.16–4.68 μ m (Fig. 1C).

RT-qPCR measured miR-505 expression in HeLa cells transfected with miR-505 mimic and miR-505-MB, which showed that miR-505 mimic significantly increased miR-505 expression, while the upregulation was more pronounced by USMB-miR-505 (Fig. 1D).

USMB-miR-505 suppressed proliferation and promoted apoptosis of HeLa cells

MTT assay and colony formation assays examined cell proliferation. Results demonstrated that miR-505 mimic inhibited cell proliferation, while miR-505-MB strengthened the inhibitory effects of miR-505 mimic (Fig. 2A/B). Flow cytometry detected cell cycle changes (Fig. 2C), and found that overexpression of miR-505 resulted in the increase of G1 phase and the decrease of S phase, especially in the miR-505-MB group. The expression of Bax and Bcl-2 was measured by ELISA kits (Fig. 2D). Compared with NC mimic

group, Bax expression in the other two groups increased, and Bcl-2 expression decreased significantly, and miR-505-MB group showed more powerful effect in regulating Bcl-2 and Bax expression. Hoechst staining detect the apoptosis rate of HeLa cells, and found that overexpression of miR-505 led to the increase of the apoptosis rate of HeLa cells, especially in the miR-505-MB group (Fig. 2E).

USMB-miR-505 inhibited migration, invasion and EMT of Hela cells

In HeLa cells transfected with NC mimic, miR-505 mimic and miR-505-MB, the expression of EMT-related factors E-cadherin, N-cadherin and Vimentin was measured by RT-qPCR. Overexpression of miR-505, especially miR-505-MB led to the increase of E-cadherin and decreases of N-cadherin and Vimentin (Fig. 3A).

After 24 hours of wound healing and Transwell assays, overexpression of miR-505 inhibited migration and invasion of HeLa cells, and the inhibitory effect of USMB-miR-505 was more pronounced (Fig. 3B/C).

miR-505 targets AKT2

Starbase (<http://starbase.sysu.edu.cn/>) predicted that miR-505 targeted AKT2 (Fig. 4A). The expression of AKT2 in CC and adjacent normal tissues was examined by RT-qPCR, and AKT2 was significantly increased in CC tissues (Fig. 4B). AKT2 expression was measured by WB in HeLa cells transfected with NC mimic, miR-505 mimic and miR-505-MB. Compared with NC mimic, AKT2 expression in the other two groups decreased significantly, especially in the miR-505-MB group (Fig. 4C).

AKT2-WT and AKT2-MT were transfected with NC mimic or miR-505 mimic into 293T cells. miR-505 mimic significantly reduced the luciferase activity of AKT2-WT luciferase vector, but had no significant effect on AKT2-MT luciferase vector (Fig. 4D). RIP experiment found that anti-AGO2 significantly enriched miR-505 and AKT2 (Fig. 4E) compared with anti-IgG, which proved that miR-505 targeted AKT2

Overexpression of AKT2 compromised the inhibitory effects of USMB-miR-505 on Hela cells

In the HeLa cells transfected with miR-505-MB + OE-NC, miR-505-MB + OE-AKT2, the effect of overexpression of AKT2 on the HeLa cells treated with miR-505-MB was studied.

MTT and colony formation assays showed that the vitality of HeLa cells treated with miR-505-MB increased after AKT2 overexpression (Fig. 5A), and the number of cell colonies increased (Fig. 5B). Flow cytometry showed that overexpression of AKT2 attenuated the effect of miR-505-MB on HeLa cell cycle arrest (Fig. 5C). RT-qPCR showed that overexpression of AKT2 resulted in the decrease of E-cadherin and Bax, and the increases of N-cadherin, vimentin and Bcl-2 in miR-505-MB treated cells (Fig. 5D). Wound healing and Transwell assays discovered that overexpression of AKT3 promoted migration and invasion of HeLa cells (Fig. 5E/F). ELISA found that overexpression of AKT2 resulted in the decrease of Bax and

the increase of Bcl-2 (Fig. 5G). Hoechst staining confirmed the inhibition of AKT2 on HeLa cell apoptosis (Fig. 5H).

Discussion

About 30%-35% of CC patients fail to completely recover from the treatment, including surgical resection and radiotherapy [26]. Additionally, the conventional clinical variables, such as parametrial involvement and lymph node metastasis, are not enough to predict curative effect or formulate supplementary therapy for CC patients after surgery [27]. The combination of drug-loaded USMB has been used in preclinical studies on drug and gene delivery to solid tumors, and ablation of blood vessels [28]. Based on these facts, we discussed the possible mechanism of USMB-miR-505 in the malignant episodes of CC cell. As expected, our results provided evidence that USMB-miR-505 further strengthened the inhibitory role of miR-505 in CC malignancy by targeting AKT2 (Fig. 6), which offered novel insights for CC treatment.

The abnormal expression of miRs is related to the pathological changes of human, including cancer, and some are regarded as potential prognostic markers in different tumors, such as CC [29]. In this study, we found poor miR-505 expression in CC. Consistently, miR-505 was poorly expressed in CC, and negatively correlated with tumor histology grade and lymph node metastasis [25, 30]. Additionally, miR-940 inhibited CC cell proliferation, and USMB-miR-940 showed better effects [31]. However, the role of USMB treatment on miR-505 expression was not yet studied. Thus, MBs were synthesized by ultrasonic dispersion, and HeLa cells were transfected with USMB-mediated miR-505. We discovered that USMB-miR-505 had the most significant effect on miR-505 expression.

miRs can intervene several aspects of CC, including proliferation, EMT and chemosensitivity [32]. A recent study unveiled that paclitaxel-miR-34a-USMBs are a promising anticancer strategy for treating CC [21]. In this study miR-505 blocked malignant episodes of Hela cells, which were reinforced by USMB treatment. EMT is a crucial mechanism of tumor cell invasion and cancer metastasis, by which epithelial cells obtain mesenchymal fibroblast-like properties [33]. Vimentin is a well-recognized metastasis marker [34], while E-cadherin could repress invasion and metastasis of epithelial cells [35]. EMT contributes to chemotherapy and radiotherapy resistance of CC cells, thus inhibiting EMT sensitizes CC cells sensitive to radiotherapy and drugs, and improves the survival rate of CC patients [32]. miR-505 mimic elevated N-cadherin expression but decreased E-cadherin in gastric cancer cells [36]. miR-505-5p was correlated with metastasis, and overexpression of miR-505-5p inhibited metastasis and EMT in CC cells [37]. In Ca-Ski and HeLa cells, miR-505 upregulation suppressed proliferation and tumorigenicity, indicating miR-505 may act as an inhibitor in CC [30]. Similarly, compared with liposome, MBs or ultrasound transfection, USMB-miR-940 further inhibited CC malignant episodes [31]. Our study may offer a novel approach for CC treatment from the miR-505 delivery by USMB treatment.

Furthermore, miR-505 targeted AKT2. There are three subtypes of AKT, namely AKT1, AKT2 and AKT3, among which AKT2 is responsible for tumor progression and metastasis through regulating EMT-related proteins [38]. Increased AKT2 expression was associated with the cervical lesion progression [39]. AKT2

was highly expressed in CC tissues, and AKT2 overexpression promoted the proliferation and colony formation ability in SiHa cells [40]. Overexpression of AKT2 compromised the inhibitory effects of USMB-miR-505 on Hela cells. It is known that activated Akt can promote the proliferation of cancer cells including CC [41]. AKT2 expression was required for EMT-like morphological changes [42]. Knockdown of AKT2 prevented cell proliferation and stimulated apoptosis in CC cells [43]. A recent study showed that AKT2 inhibition is potential for anti-cancer therapies for its function in EMT reversion, metastasis reduction and tumor recurrence prevention [44]. To sum up, AKT2 silencing is possible to prevent CC development.

Conclusion

In conclusion, our study provided substantial evidence that miR-505 overexpression repressed CC progression, and USMB mediated miR-505 reinforced the inhibitory effects of miR-505 in CC. These results indeed unveiled a promising approach for CC treatment. More works should be done in the future to identify the application value of our results. We will also make comprehensive investigation to figure out the downstream pathways involving in the beneficial roles of USMB-miR-505 in CC treatment.

Abbreviations

ANOVA, analysis of variance; CC, cervical cancer; DMEM, Dulbecco's modified Eagle medium; ELISA, Enzyme-linked immunosorbent assay; EMT, epithelial-to-mesenchymal transition; FBS, fetal bovine serum; MBs, microbubbles; miR, microRNA; MT, mutant; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NC, negative controls; OE, overexpression; RIP, RNA immunoprecipitation; RT-Qpcr, Reverse transcription quantitative polymerase chain reaction; USMB, ultrasound and microbubbles; USMB-miR, USMB-mediated miR; WB, Western blot; WT, wild-type.

Declarations

Ethics approval and consent to participate

The study followed the *Declaration of Helsinki* and was approved by the ethics committee of the First Hospital of Huai'an Affiliated to Nanjing Medical University. All patients signed the informed consent.

Consent for publication

Not applicable.

Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

LLX conceived the study and participated in its design and coordination; QZ, CHL and FH performed all experiments; XPL and LLX analyzed and interpreted the data; The draft was improved through discussion and editing by all the authors who read and approved the final manuscript.

Acknowledgments

Not applicable.

References

1. Ayen A, Jimenez Martinez Y, Boulaiz H. Targeted Gene Delivery Therapies for Cervical Cancer. *Cancers (Basel)*. 2020;12(5).
2. Peng X, Gao J, Cai C, Zhang Y. LncRNA LINC01503 aggravates the progression of cervical cancer through sponging miR-342-3p to mediate FXYD3 expression. *Biosci Rep*. 2020.
3. Chauhan SR, Bharadwaj M. Gearing up T-cell immunotherapy in cervical cancer. *Curr Probl Cancer*. 2018;42(2):175–88.
4. Okeah BO, Ridyard CH. Factors Influencing the Cost-Effectiveness Outcomes of HPV Vaccination and Screening Interventions in Low-to-Middle-Income Countries (LMICs): A Systematic Review. *Appl Health Econ Health Policy*. 2020.
5. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. 2015;65(2):87–108.
6. Shrestha AD, Neupane D, Vedsted P, Kallestrup P. Cervical Cancer Prevalence, Incidence and Mortality in Low and Middle Income Countries: A Systematic Review. *Asian Pac J Cancer Prev*. 2018;19(2):319–24.
7. Chen J, Gu W, Yang L, Chen C, Shao R, Xu K, et al. Nanotechnology in the management of cervical cancer. *Rev Med Virol*. 2015;25 Suppl 172 – 83.
8. Ouh YT, Lee JK. Proposal for cervical cancer screening in the era of HPV vaccination. *Obstet Gynecol Sci*. 2018;61(3):298–308.
9. Vaccarella S, Franceschi S, Engholm G, Lonnberg S, Khan S, Bray F. 50 years of screening in the Nordic countries: quantifying the effects on cervical cancer incidence. *Br J Cancer*. 2014;111(5):965–9.
10. Vaccarella S, Franceschi S, Zaridze D, Poljak M, Veerus P, Plummer M, et al. Preventable fractions of cervical cancer via effective screening in six Baltic, central, and eastern European countries 2017-40:

- a population-based study. *Lancet Oncol.* 2016;17(10):1445–52.
11. Kimmig R, Lind T. Minimally invasive surgery for cervical cancer: consequences for treatment after LACC Study. *J Gynecol Oncol.* 2018;29(4):e75.
 12. Huang L, Huang Z, Fan Y, He L, Ye M, Shi K, et al. FOXC1 promotes proliferation and epithelial-mesenchymal transition in cervical carcinoma through the PI3K-AKT signal pathway. *Am J Transl Res.* 2017;9(3):1297–306.
 13. Qin Z, He W, Tang J, Ye Q, Dang W, Lu Y, et al. MicroRNAs Provide Feedback Regulation of Epithelial-Mesenchymal Transition Induced by Growth Factors. *J Cell Physiol.* 2016;231(1):120–9.
 14. Chen S, Gao C, Wu Y, Huang Z. Identification of Prognostic miRNA Signature and Lymph Node Metastasis-Related Key Genes in Cervical Cancer. *Front Pharmacol.* 2020;11:544.
 15. Delalande A, Postema M, Mignet N, Midoux P, Pichon C. Ultrasound and microbubble-assisted gene delivery: recent advances and ongoing challenges. *Ther Deliv.* 2012;3(10):1199–215.
 16. Suzuki R, Oda Y, Utoguchi N, Maruyama K. Progress in the development of ultrasound-mediated gene delivery systems utilizing nano- and microbubbles. *J Control Release.* 2011;149(1):36–41.
 17. Espitalier F, Darrouzain F, Escoffre JM, Ternant D, Piver E, Bouakaz A, et al. Enhanced Amikacin Diffusion With Ultrasound and Microbubbles in a Mechanically Ventilated Condensed Lung Rabbit Model. *Front Pharmacol.* 2019;10:1562.
 18. Wei Y, Shang N, Jin H, He Y, Pan Y, Xiao N, et al. Penetration of different molecule sizes upon ultrasound combined with microbubbles in a superficial tumour model. *J Drug Target.* 2019;27(10):1068–75.
 19. Wang DS, Panje C, Pysz MA, Paulmurugan R, Rosenberg J, Gambhir SS, et al. Cationic versus neutral microbubbles for ultrasound-mediated gene delivery in cancer. *Radiology.* 2012;264(3):721–32.
 20. Kopechek JA, McTiernan CF, Chen X, Zhu J, Mburu M, Feroze R, et al. Ultrasound and Microbubble-targeted Delivery of a microRNA Inhibitor to the Heart Suppresses Cardiac Hypertrophy and Preserves Cardiac Function. *Theranostics.* 2019;9(23):7088–98.
 21. Yu J, Zhao Y, Liu C, Hu B, Zhao M, Ma Y, et al. Synergistic anti-tumor effect of paclitaxel and miR-34a combined with ultrasound microbubbles on cervical cancer in vivo and in vitro. *Clin Transl Oncol.* 2020;22(1):60–9.
 22. Ji Y, Han Z, Shao L, Zhao Y. Evaluation of in vivo antitumor effects of low-frequency ultrasound-mediated miRNA-133a microbubble delivery in breast cancer. *Cancer Med.* 2016;5(9):2534–43.
 23. Li X, Xu M, Lv W, Yang X. Ultrasound-targeted microbubble destruction-mediated miR-767 inhibition suppresses tumor progression of non-small cell lung cancer. *Exp Ther Med.* 2020;19(5):3391–7.
 24. Meng L, Yuan S, Zhu L, ShangGuan Z, Zhao R. Ultrasound-microbubbles-mediated microRNA-449a inhibits lung cancer cell growth via the regulation of Notch1. *Onco Targets Ther.* 2019;12:7437–7450.
 25. Feng S, Liu W, Bai X, Pan W, Jia Z, Zhang S, et al. LncRNA-CTS promotes metastasis and epithelial-to-mesenchymal transition through regulating miR-505/ZEB2 axis in cervical cancer. *Cancer Lett.* 2019;465:105–117.

26. Lin L, Liu Y, Zhao W, Sun B, Chen Q. Wnt5A expression is associated with the tumor metastasis and clinical survival in cervical cancer. *Int J Clin Exp Pathol*. 2014;7(9):6072–8.
27. Zhang L, Huang H, Zhang L, Hou T, Wu S, Huang Q, et al. URG4 overexpression is correlated with cervical cancer progression and poor prognosis in patients with early-stage cervical cancer. *BMC Cancer*. 2014;14885.
28. Khokhlova TD, Haider Y, Hwang JH. Therapeutic potential of ultrasound microbubbles in gastrointestinal oncology: recent advances and future prospects. *Therap Adv Gastroenterol*. 2015;8(6):384–94.
29. Ribeiro J, Sousa H. MicroRNAs as biomarkers of cervical cancer development: a literature review on miR-125b and miR-34a. *Mol Biol Rep*. 2014;41(3):1525–31.
30. Ma C, Xu B, Husaiyin S, Wang L, Wusainahong K, Ma J, et al. MicroRNA-505 predicts prognosis and acts as tumor inhibitor in cervical carcinoma with inverse association with FZD4. *Biomed Pharmacother*. 2017;92586–594.
31. Xiao X, Zhang Y, Lin Q, Zhong K. The better effects of microbubble ultrasound transfection of miR-940 on cell proliferation inhibition and apoptosis promotion in human cervical cancer cells. *Onco Targets Ther*. 2019;126813–6824.
32. Qureshi R, Arora H, Rizvi MA. EMT in cervical cancer: its role in tumour progression and response to therapy. *Cancer Lett*. 2015;356(2 Pt B):321–31.
33. Pal M, Bhattacharya S, Kalyan G, Hazra S. Cadherin profiling for therapeutic interventions in Epithelial Mesenchymal Transition (EMT) and tumorigenesis. *Exp Cell Res*. 2018;368(2):137–46.
34. Wang TH, Lin YS, Chen Y, Yeh CT, Huang YL, Hsieh TH, et al. Long non-coding RNA AOC4P suppresses hepatocellular carcinoma metastasis by enhancing vimentin degradation and inhibiting epithelial-mesenchymal transition. *Oncotarget*. 2015;6(27):23342–57.
35. Li J, Dai X, Zhang H, Zhang W, Sun S, Gao T, et al. Up-regulation of human cervical cancer proto-oncogene contributes to hepatitis B virus-induced malignant transformation of hepatocyte by down-regulating E-cadherin. *Oncotarget*. 2015;6(30):29196–208.
36. Dang SC, Wang F, Qian XB, Abdul M, Naseer QA, Jin W, et al. MicroRNA-505 suppresses gastric cancer cell proliferation and invasion by directly targeting Polo-like kinase-1. *Onco Targets Ther*. 2019;12795–803.
37. Kapora E, Feng S, Liu W, Sakhautdinova I, Gao B, Tan W. MicroRNA-505-5p functions as a tumor suppressor by targeting cyclin-dependent kinase 5 in cervical cancer. *Biosci Rep*. 2019;39(7).
38. Hinz N, Jucker M. Distinct functions of AKT isoforms in breast cancer: a comprehensive review. *Cell Commun Signal*. 2019;17(1):154.
39. Xu J, Xu S, Fang Y, Chen T, Xie X, Lu W. Cyclin-dependent kinase 9 promotes cervical cancer development via AKT2/p53 pathway. *IUBMB Life*. 2019;71(3):347–56.
40. Fu Y, Meng Y, Gu X, Tian S, Hou X, Ji M. miR-503 expression is downregulated in cervical cancer and suppresses tumor growth by targeting AKT2. *J Cell Biochem*. 2019.

41. Yang W, Tan W, Zheng J, Zhang B, Li H, Li X. MEHP promotes the proliferation of cervical cancer via GPER mediated activation of Akt. *Eur J Pharmacol.* 2018;82411–16.
42. Lan A, Qi Y, Du J. Akt2 mediates TGF-beta1-induced epithelial to mesenchymal transition by deactivating GSK3beta/snail signaling pathway in renal tubular epithelial cells. *Cell Physiol Biochem.* 2014;34(2):368–82.
43. Xu J, Wan X, Chen X, Fang Y, Cheng X, Xie X, et al. miR-2861 acts as a tumor suppressor via targeting EGFR/AKT2/CCND1 pathway in cervical cancer induced by human papillomavirus virus 16 E6. *Sci Rep.* 2016;628968.
44. Gener P, Rafael D, Seras-Franzoso J, Perez A, Pindado LA, Casas G, et al. Pivotal Role of AKT2 during Dynamic Phenotypic Change of Breast Cancer Stem Cells. *Cancers (Basel).* 2019;11(8).

Figures

Figure 1

USMB enhanced the transfection efficiency of miR-505 mimic. A. miR-505 expression in CC and adjacent normal tissues detected by RT-qPCR; B, the morphology of MBs observed under the inverted microscope; C, the particle size of the prepared MBs determined by nanoparticle size analyzer; D, the transfection efficiency measured by RT-qPCR. Paired t test for panel A; @@ $p < 0.01$ vs. the adjacent normal tissues. One-way ANOVA for panel D; ** $p < 0.01$ vs. NC mimic, # $p < 0.05$ vs. miR-505 mimic.

Figure 2

USMB-mediated miR-505 inhibited HeLa cell growth. A. HeLa cell viability detected by MTT assay. B. the ability of cell proliferation measured by colony formation assay. C, cell cycle detected by flow cytometry. D. Bax and Bcl-2 expression detected by ELISA; E, Hoechst staining detected the apoptosis rate. One-way ANOVA for data analysis in panels A/B/E; two-way ANOVA for data analysis in panels C/D; * $p < 0.05$ vs. NC mimic; # $p < 0.05$ vs. miR-505 mimic.

Figure 3

USMB-mediated miR-505 inhibited on HeLa cell migration and invasion. A, expression of EMT-related factors in HeLa cells measured by RT-qPCR; B, the migration ability of cells measured by wound healing; C, the invasion of cells examined by Transwell assay. One-way ANOVA for data analysis in panels B/C; two-way ANOVA for data analysis in panel A; * $p < 0.05$ vs. NC mimic; # $p < 0.05$ vs. miR-505 mimic.

Figure 4

miR-505 targeted AKT2. A, a binding site between miR-505 and AKT2. B, AKT2 expression in CC and adjacent normal tissues detected by RT-qPCR. C. WB detected AKT2 expression in HeLa cells; D, the activity of WT-AKT2 and MT-AKT2 luciferase detected by dual-luciferase assay; E, the binding relationship between miR-505 and AKT2 verified by RIP experiment. Paired t test for panel B, @@ $p < 0.01$ vs. the

adjacent normal tissues; one-way ANOVA for data analysis in panel C; two-way ANOVA for data analysis in panes D/E; * $p < 0.05$ vs. NC mimic; # $p < 0.05$ vs. miR-505 mimic; % $p < 0.01$ vs. Anti-IgG.

Figure 5

Overexpression of AKT2 reversed the effect of miR-505 mediated by USMB on HeLa cells. A. MTT measured HeLa cell viability; B. HeLa cell proliferation measured by colony formation assay; C. cell cycle changes measured by flow cytometry. D. expression of EMT-related factors measured by RT-qPCR. E, HeLa cell migration was measured by wound healing. F, HeLa cell invasion measured by Transwell assay, G. expression of apoptosis-related factors measured by ELISA. H. Hoechst staining detected the apoptosis rate. Paired t test for panels A/B/E/F/H, & $p < 0.05$ vs. the miR-505-MB + OE-NC group; two-way ANOVA for data analysis in panes C/D/G; & $p < 0.05$ vs. the miR-505-MB + OE-NC group.

Figure 6

Experimental mechanism diagram. USMB-mediated miR-505 improves the transfection efficiency of miR-505, and inhibits the growth and metastasis of HeLa cells by targeting AKT2.