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Role and mechanism of leukemia inhibitory factor receptor in cervical cancer invasion and metastasis

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Abstract

Objective: To investigate the relationships of leukemia inhibitory factor receptor (LIFR) with cervical cancer invasion and metastasis.

Methods: From January 2021 to December 2022, 45 patients treated for cervical cancer and lung metastases were identified. Western blotting was used to determine the expression of Hippo–YAP signaling pathway-related proteins. Meanwhile, 40 healthy Sprague-Dawley nude mice were used and evenly randomized into two groups, which were injected with LIFR-overexpressing (study group) or normal cervical cancer cells (control group). The lung tissue of nude mice was removed for hematoxylin–eosin staining, and the number of lung cell metastases in nude mice was counted.

Results: The highest LIFR mRNA expression was found in paracancerous tissue, followed by cervix cancer tissue and metastatic lesions. The study group exhibited higher LIFR, P-YAP, and P-TAZ protein expression and lower YAP and TAZ protein expression than the control group. The study group had a lower number of lung metastases than the control group.

Conclusion: Decreased expression of LIFR and decreased phosphorylation of Hippo–YAP signaling pathway-related proteins might be the underlying mechanisms that promote lung metastasis of cervical cancer.

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Introduction

Lymph node metastasis is a defining feature of stage IIIC cancer. In particular, metastasis to only pelvic lymph nodes denotes stage IIIC1 cancer, whereas metastasis to para-aortic lymph nodes denotes stage IIIC2 cancer.[1] Current research has found that that leukemia inhibitory factor receptor (LIFR) participates in the relapse and metastasis of various malignant tumors.[2] It is currently believed that the increased expression of the Hippo–YAP signaling pathway-related proteins YAP and TAZ is involved in tumor metastasis.[3,4] The Hippo–YAP/TAZ pathway, originally identified in *Drosophila* as a major developmental pathway controlling organ size, was later found in mammals, in which the pathway consists of a conserved set of kinases. In mammals, membrane protein receptors upstream of Hippo signaling sense growth-inhibitory signals from the extracellular environment and undergo a series of kinase reactions that ultimately target the downstream effectors YAP and TAZ. YAP and TAZ then interact with cytoskeletal proteins, trapping them in the cytoplasm and thereby regulating organ size and volume.[5–7] The Hippo–YAP/TAZ pathway is perturbed in multiple types of human tumors and is associated with tumor growth, evasion, metastasis, and drug resistance.[8]

Leukemia inhibitory factor receptor (LIFR) is a member of the type I cytokine receptor family that promotes stem cell pluripotency and regulates cell proliferation and differentiation.[9] A large body of evidence suggests that LIFR is a marker of poor prognosis, and it is highly expressed in several tumors, such as melanoma, nasopharyngeal carcinoma, and prostate cancer.[10–12] LIFR has been found to enhance tumor growth in nasopharyngeal carcinoma by responding to its extracellular ligand leukemia inhibitory factor (LIF), thereby inhibiting DNA damage responses and promoting radioresistance. In prostate cancer, LIFR promotes epithelial–mesenchymal transition (EMT), leading to metastasis by triggering the PI3K–Akt pathway.[13] There is already evidence that the binding of LIF to LIFR activates the phosphorylation of Hippo–YAP signaling pathway-related proteins and increases the phosphorylation level of related proteins.[14] LIFR suppression can inhibit this process, thereby increasing the expression of related proteins, reducing their phosphorylation level, and regulating downstream gene signaling pathways. Therefore, the Hippo–YAP signaling pathway might be regulated by the expression of LIFR, which inhibits the metastasis of cervical cancer cells.[15]

In this study, patients treated for cervical cancer in our hospital were enrolled, the protein and RNA expression of relevant proteins of the cancer and paracancerous tissue were compared, and the mechanism of LIFR in the invasion and metastasis of cervical cancer was analyzed.

Materials and methods

General information

Patients treated for lung metastasis of cervical cancer from January 2021 to December 2021 were identified as the research subjects.

All patients were treated according to the latest guidelines, with adjustments based on individual differences. The inclusion criteria were as follows: (1) pathological diagnosis of cervical cancer for the first time; (2) clear lung metastases detected by X-ray/CT, surgery, or pathology; and (3) provision of informed consent. Meanwhile, the exclusion criteria were contraindications to thoracic or pelvic surgery and current participation in other studies. Intraoperative tumor tissue, adjacent tissue, and lung metastases were collected to determine the expression level of LIFR mRNA.

All methods were performed in accordance with the ethical standards in the Declaration of Helsinki and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of Hunan Environment Biological Polytechnic (Approval No. [2020]264; Approval date: 12 June 2020).

Clinical sample preparation. Written informed consent was obtained from patients or their legal representatives prior to sample collection. Tissue samples were obtained by biopsy or surgery. In the case of biopsy, a small piece of lung tissue was removed using a needle, whereas a larger portion of lung tissue was obtained by surgery. After tissue collection, it was immediately placed in a sterile container to prevent contamination. Samples were be stored in a −80°C freezer until use.

Primary reagents. Rabbit anti-human antibodies against LIFR (Cat. No. 17027T), GAPDH (Cat. No. 5174), P-YAP (Cat. No. 13008), and P-TAZ (Cat. No. 52420) were obtained from Cell Signaling Technology (Danvers, MA, USA). Goat anti-rabbit secondary antibodies (containing horseradish peroxidase) were provided by Wuhan Boster (Wuhan, China). Lipofectamine 2000 and TRIzol reagent were provided by Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). An RNA extraction kit was purchased from Applied Biosystems (Thermo Fisher Scientific). A TaqMan reverse transcription kit was obtained from Life Technologies (Thermo Fisher Scientific). An LIFR-encoding recombinant lentiviral plasmid and negative control vector were provided by GeneCopoeia (Rockville, MD, USA). An ECL enhanced chemiluminescence kit and BCA Protein Quantitative Detection Kit were provided by Thermo Fisher Scientific.

Detection of LIFR mRNA expression. Fluorescence quantitative PCR (qPCR) was used to measure LIFR mRNA expression in different tissues. Total RNA was extracted according to the kit instructions, and cDNA was obtained by reverse transcription. PCR was performed using the cDNA as a template, and GAPDH was used as an internal reference. The PCR program was as follows: 95°C for 3 minutes; 38 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and a final step at 72°C for 5 minutes. The primers were as follows: upstream primer, 5′-CAGCATCACTGAATCACAG AGC-3′; and downstream primer, 5′-AG TATGAAACATCCCCACAGGG-3′. The amplified fragment length was 560 bp. Finally, the $2^{-\Delta\Delta CT}$ method was adopted to calculate the mRNA expression of LIFR in the three tissues.

Establishment of SiHa cell models. SiHa cells were bought from American Type Culture Collection (Bethesda, MD, USA) and cultured in DMEM (iCell Bioscience Inc., Shanghai, China) containing 10% fetal bovine serum (LMAI Bio, Shanghai, China). In total, 2 mL of SiHa cells (1×10^5 cells/mL) were added to each well of a six-well plate. After the cell confluence reached approximately 80%, the cells were divided into three wells and transfected with the LIFR lentivirus plasmid or empty vector plasmid. Cells were added to sterile

tubes containing Lipofectamine 2000. After mixing, the cells were incubated for 20 minutes at room temperature, transferred to serum-free medium, mixed well, and then added to six-well plates for transfection. The cells were cultured for 6 hours in an incubator, and then the medium was replaced with conventional medium for culture. After 3 days, the fluorescence intensity was measured in, and then 2.0 μg/mL puromycin was added to the culture until all cells exhibited fluorescence.

Animal model and treatment. Forty BALB/c nude mice (4-week-old females) weighing 6 to 8 g with an average daily feed consumption of 5 g/100 g and average daily drinking water consumption of 6 to 7 mL/100 g were used. The mice were randomly divided into two groups of 20 each and transfected with LIFR-overexpressing (study group) or control SiHa cells (control group). Mice were euthanized via cervical dislocation.

Detection of the expression of proteins related to Hippo–YAP signaling by western blotting. Total protein was extracted from LIFR-overexpressing and control cells. After washing twice with PBS, the liquid was aspirated and placed in a Petri dish, and 1 mL of RIPA protein lysate was added. The cells were scraped and transferred to 1.5-mL centrifuge tubes, which were then placed on an ice shaker and shaken slowly for 30 minutes, followed by centrifugation at $1500 \times g$ for 5 minutes. The upper serum was collected to obtain the total cell protein. The BCA method was used to detect protein expression. In this assay, 30 μg of protein were subjected to gel electrophoresis and then transferred to a PVDF membrane (Sigma-Aldrich, St. Louis, MO, USA). The membrane was blocked with 5% BSA at room temperature for 1 to 2 hours and incubated overnight with 1:5000 rabbit anti-human antibody at 4°C. Then, the membrane was rinsed thrice with TBST (10 minutes/wash) and incubated with goat anti-rabbit secondary antibody at room temperature for 1 hour. The membrane was then rinsed thrice with TBST for 10 min each and exposed to ECL luminescent agent for X-ray exposure, development, and positioning. The band images were obtained and analyzed using Quantity One (Bio-Rad, Hercules, CA, USA). Protein expression was indicated as the ratio of the density of the target protein band to that of the GAPDH protein band.

Detection of lung metastases. A cell suspension (100 μL) was injected into the caudal vein of each mouse, and the morphological observation of a tumor diameter of ≥ 5 mm was regarded as the successful establishment of a disease model. All nude mice were sacrificed after 4 weeks, and the lung tissue was analyzed by hematoxylin–eosin staining to detect metastases, which were indicated by yellow or brown staining. The number of lung metastases lesions was measured in both groups.

Statistical processing. PASW Statistics for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA) was used for data analyses. Measurement data were expressed as the mean ± standard deviation and analyzed using the *t*-test. Numerical data were expressed as percentages and analyzed using the chi-squared test. Analysis of variance was used to compare differences between groups. $P < 0.05$ was considered statistically significant.

Results

LIFR mRNA expression levels

LIFR mRNA levels were higher in paracancerous tissue (1.06 ± 0.32) than in cervical cancer tissue (0.85 ± 0.22) and lung metastases (0.68 ± 0.19, $F = 26.17$, $P < 0.001$; Figure 1).

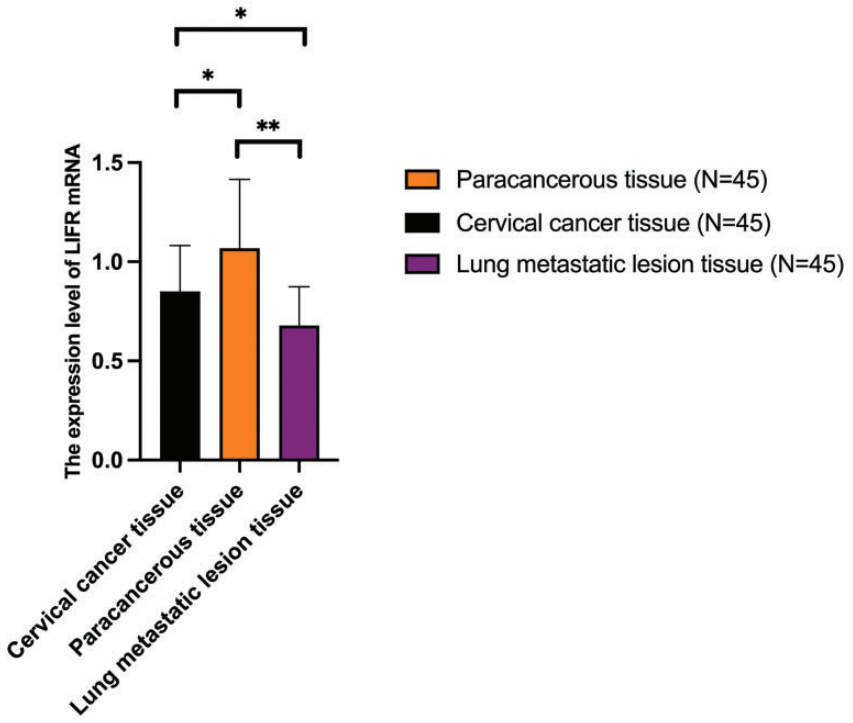

Figure 1. LIFR mRNA expression in cervical cancer tissue, paracancerous tissue, and lung metastases. LIFR mRNA expression was higher in paracancerous tissue was higher than that in cervical cancer tissue and lung metastases ($P < 0.05$). LIFR mRNA expression was higher in cervical cancer tissue than that in lung metastases ($P < 0.05$). LIFR, leukemia inhibitory factor receptor.

Table 1. Comparison of the expression of Hippo–YAP signaling pathway-related proteins in the two groups of cells.

Group	*N*	LIFR	YAP	TAZ	P-YAP	P-TAZ
Study group	45	1.75 ± 0.26	0.78 ± 0.06	0.81 ± 0.08	1.03 ± 0.26	1.02 ± 0.18
Control group	45	0.89 ± 0.13	1.02 ± 0.14	1.05 ± 0.18	0.55 ± 0.13	0.43 ± 0.11
t	–	19.846	10.570	8.173	11.077	18.762
P	–	$P < 0.001$				

LIFR, leukemia inhibitory factor receptor.

Expression of Hippo–YAP signaling pathway-related proteins

The study group exhibited higher LIFR, P-YAP, and P-TAZ protein expression and lower YAP and TAZ protein expression than the control group, as presented in Table 1 and Figure 2.

The invasion and migration of LIFR-overexpressing and control SiHa cells

We transfected a lentiviral vector overexpressing LIFR or an empty vector into SiHa cells. Subsequently, we compared LIFR expression in the groups by qPCR. We then compared the invasiveness and

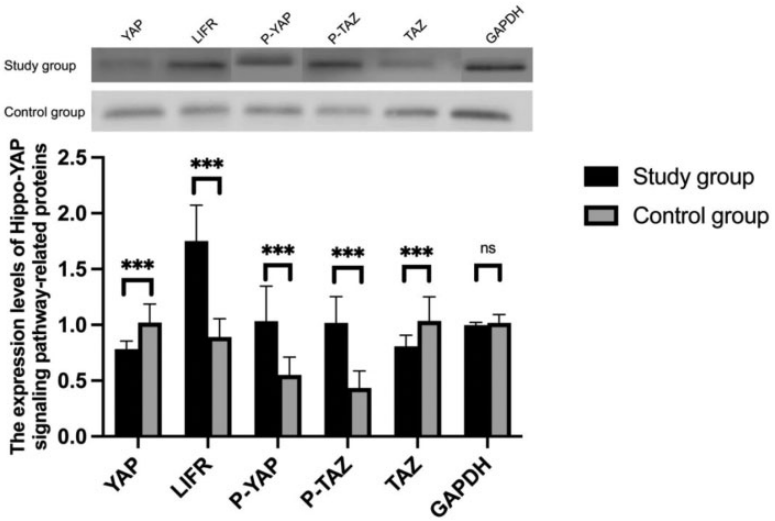

Figure 2. Comparison of the expression of Hippo–YAP signaling pathway-related proteins. LIFR, P-YAP, and P-TAZ protein expression were higher in the study group than in the control group, whereas YAP and TAZ protein expression was higher in the control group than in the study group. ***$P < 0.001$.

migration of the two groups of cells (Figure 3). LIFR-overexpressing cells migrated a longer distance than control cells ($47.03 \pm 5.35\,\mu m$ vs. $28.67 \pm 4.19\,\mu m$, $P < 0.05$).

Number of lung cell metastases

First, qPCR was performed to determine the expression of LIFR in the tumor tissues of mice in the two groups, as presented in Figure 4. LIFR levels were higher in the study group than in the control group (1.97 ± 0.45 vs. 0.93 ± 0.32, $P < 0.05$).

The mean number of lung metastases in nude mice was 9.4 ± 1.2 in the study group, versus 23.3 ± 5.4 in the control group ($P < 0.05$; Figure 5). The area of lung metastases was $5.87 \pm 1.33\,mm^2$ in the study group, compared with $8.33 \pm 2.43\,mm^2$ in the control group ($P < 0.05$).

Discussion

LIFR expression is low in many malignant tumor tissues, indicating its correlation with the recurrence and metastasis of malignant tumors.[16] Prior research[17,18] found that P-YAP is aberrantly expressed in many tumor tissues. P-YAP expression in esophageal cancer tissue is closely related to the clinical parameters of patients.[19]

Patients with cervical cancer were recruited in this study, and the mRNA expression of LIFR was compared in different tissues. The results demonstrated that LIFR mRNA expression was lowest in lung metastases, followed by cervical cancer tissues and adjacent tissues. Moreover, LIFR expression was absent in metastatic tissues, which was consistent with previous findings.[20,21] To further explore the mechanism of action of LIFR, the relationship between Hippo–YAP signaling pathway-related proteins and LIFR expression was examined. Compared with the findings in normal tissues, LIFR mRNA expression and P-YAP and P-TAZ protein expression were elevated in tumors, whereas YAP and TAZ expression was reduced. The results also illustrated that the increase in LIFR mRNA expression promoted the phosphorylation of

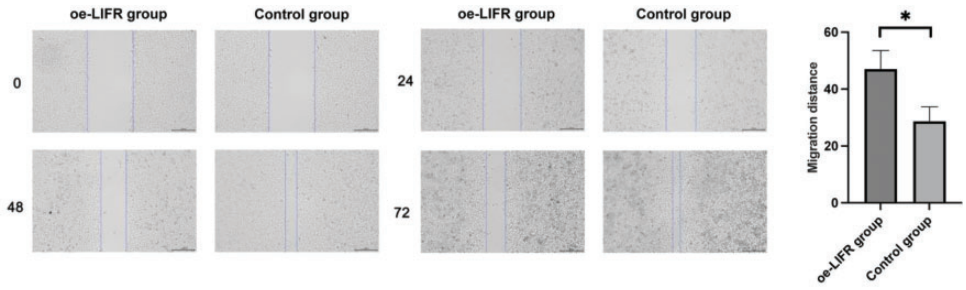

Figure 3. The invasiveness and migration of SiHa cells. The cell migration distance was 47.03 ± 5.35 μm in the LIFR overexpression group, versus 28.67 ± 4.19 μm in the control group ($P < 0.05$). LIFR, leukemia inhibitory factor receptor.

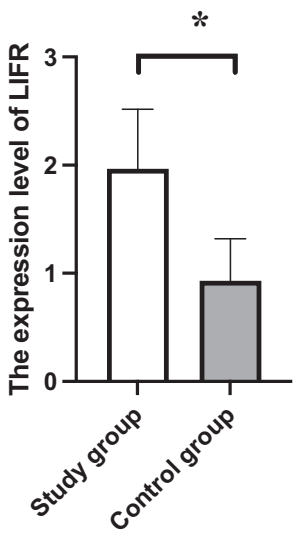

Figure 4. LIFR mRNA expression. LIFR mRNA expression was higher in the study group than in the control group. *$P < 0.05$, study group vs. control group.
LIFR, leukemia inhibitory factor receptor.

YAP and TAZ, leading to increased P-YAP and P-TAZ expression and decreased YAP and TAZ expression. Current research has confirmed that YAP is an oncogene that enhances the activity of transcription factors and promotes the expression of oncogenes in cancer cells.[22] Therefore, YAP loses its activity upon phosphorylation, leading to a decline in the activity of YAP-mediated transcription factors, thereby inhibiting the expression of oncogenes.[23] TAZ is a transcriptional co-activator that is homologous to YAP, and its function is similar to that of YAP. TAZ is similarly inactivated by phosphorylation.[24] Therefore, LIFR overexpression in SiHa cells led to increased YAP and TAZ phosphorylation, thereby inhibiting the downstream related signaling pathways, destabilizing the Hippo–YAP signaling pathways, and disturbing the balance of tumor cell proliferation and apoptosis.[25] YAP and TAZ inactivation can also suppress tumor cell invasion and metastasis. Other studies[26,27] demonstrated that YAP could accelerate the migration of prostate epithelial cells and boost the development and metastasis of gastric cancer. At present, there are few reports about LIFR in cervical cancer. LIF and LIFR might have different biological effects on different tumor types. LIF can be used as a key paracrine factor to activate pancreatic stellate cells and promote the invasion and migration of tumor cells. Blockage of LIFR can significantly delay tumor progression and prolong overall survival in pancreatic cancer mouse models.[28] However, Ma *et al.*[29] found that LIFR suppressed the expression β-catenin and vimentin and elevated the expression of E-cadherin, thereby negatively regulating the migration of pancreatic cancer cells. Lei *et al.*[30] also reported that LIFR could inhibit tumor cell metastasis by

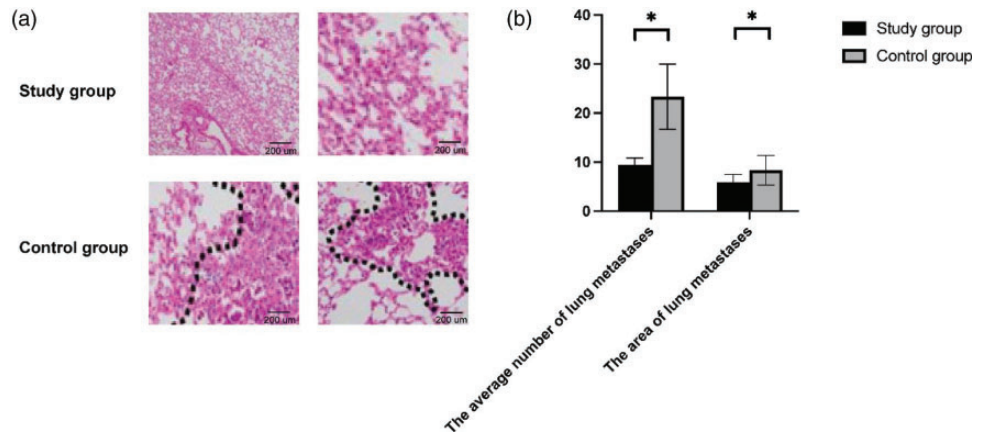

Figure 5. Histological image of lung metastasis in the study and control groups (hematoxylin–eosin staining). The number (9.4 ± 1.2 vs. 23.3 ± 5.4, $P < 0.05$) and area of lung metastases ($5.87 \pm 1.33\,mm^2$ vs. $8.33 \pm 2.43\,mm^2$, $P < 0.05$) were lower in the study group than in the control group.

inhibiting YAP expression in renal clear cell carcinoma. Therefore, the role of LIFR in tumor metastasis is worth further exploration. The present study demonstrated that LIFR could accelerate the metastasis of cervical cancer cells by enhancing the level of phosphorylation in Hippo-related signaling pathway-related proteins.

In conclusion, the decreased expression of LIFR and the decreased phosphorylation of Hippo–YAP signaling pathway-related proteins might represent the underlying mechanisms promoting lung metastasis in cervical cancer.

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Author contributions

Hengfen Hu wrote the manuscript. Yanqing Xiao performed experiments. Na Jiang prepared and edited the figures. Yufei Sang edited the manuscript. All authors read and approved the final manuscript.

Aailability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interests

The authors declare that they have no competing interests.

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