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Three LHPP gene-targeting co-expressed microRNAs (microRNA-765, microRNA-21, and microRNA-144) promote proliferation, epithelial-mesenchymal transition, invasion, and are independent prognostic biomarkers in renal cell carcinomas patients

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Abstract

Background: Renal cell carcinoma (RCC) is one of the highly malignant tumors in the world. Global Cancer Statistics 2020 estimated that there were 179,368 deaths from kidney tumors. Therefore, exploring the prognostic biomarkers of RCC is of great significance for RCC patients. This study aims to explore the potential mechanism and prognostic value of phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) gene-targeting co-expression microRNAs in RCC patients.

Methods: A total of 60 RCC patients were included. Quantitative real-time PCR (qRT-PCR), western blotting, and immunohistochemistry were used for LHPP, microRNA-765, microRNA-21, and microRNA-144 levels evaluation. Cell Counting Kit-8 assay, dual-luciferase reporter gene assay, invasion assay, and RNA fluorescence in situ hybridization were used for functional analyses.

Results: Compared with adjacent tissues, LHPP levels in cancer tissues were significantly increased ($p < .001$). Herein, we confirmed that microRNA-765, microRNA-21, and microRNA-144 were direct biological targets of LHPP. MicroRNA-765 ($r = -0.570$, $p < 0.001$), microRNA-21 ($r = -0.495$, $p < .001$), and microRNA-144 ($r = -0.463$, $p < .001$) expression levels were negatively correlated with LHPP expression levels. The high expression levels of microRNA-765, microRNA-21, and microRNA-144 in RCC tissues were associated with poor differentiation, recurrence, and poor prognosis ($p < .05$). *In vitro*, microRNA-765, microRNA-21, and microRNA-144 act as oncogenes to promote proliferation, invasion, and epithelial-mesenchymal transition (EMT) through targeting LHPP.

Conclusions: MicroRNA-765, microRNA-21, and microRNA-144 are independent risk biomarkers for RCC patients. Inhibiting the expression levels of microRNA-765, microRNA-21, and microRNA-144 can reduce the proliferation, EMT, and invasion of

RCC cells. Therefore, the above three microRNAs are expected to become molecular biomarkers for RCC therapy.

1 | INTRODUCTION

Renal cell carcinoma (RCC) is one of the highly malignant tumors in the world, accounting for 80% to 90% of renal malignant tumors.[1] Global Cancer Statistics 2020 estimated that there were 431,288 new cases of kidney tumors and 179,368 deaths from kidney tumors.[2] Hence, exploring prognostic biomarkers is of great significance for RCC patients.

A recent study has discovered a new type of tumor suppressor protein, namely pholosysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP).[3] Researchers have found that low expression of LHPP can lead to the upregulation of histidine phosphorylation and promote tumor growth.[4-6] Endogenous microRNAs are small, evolutionarily conserved non-coding ribonucleotide acids that are crucial players in many biological processes, as well as the occurrence and development of RCC.[7]

In this study, first, we detected the protein and mRNA levels of LHPP in the RCC cancer tissues. Next, we applied bioinformatics software, luciferase reporter gene technology to predict and verify the expression profile of microRNAs involved in the regulation of LHPP. Then, functional experiments were employed to check the biological behaviors of LHPP and microRNAs in RCC cells. Finally, we analyzed the relationship between the levels of LHPP and microRNAs and the long-term prognosis of patients. Our data indicated that microRNA-765, microRNA-21, and microRNA-144 can promote the proliferation, epithelial-mesenchymal transition (EMT), and invasion of RCC by downregulating LHPP, which can serve as therapeutic targets for RCC.

2 | MATERIAL AND METHODS

2.1 | Clinical tissues

From February 2017 to April 2018, a total of 60 RCC patients from the Second Affiliated Hospital, Harbin Medical University were included. Among them, 39 were males and 21 were females, aged from 42 to 75 years old, with an average of 57.3 years old. The histological classification of 60 patients was confirmed by pathology, including 42 cases of clear cell carcinoma and 18 cases of papillary carcinoma. According to the standards of the World Health Organization, 19 cases were poorly differentiated, 14 cases were moderately differentiated, and 27 cases were highly differentiated. In addition, normal kidney tissue adjacent to cancer (2–5 cm from the side of the cancerous tissue) was chosen as a control. None of the included 60 patients received chemotherapy or radiotherapy before surgery. All tissues were acquired with written informed consent. Our research plans were approved by the Human Ethics Committee of The Second Affiliated Hospital, Harbin Medical University (NO.: KL2017013-V02), according to the Declaration of Helsinki. All patients were followed up for 36 months.

2.2 | Cell culture

Normal human renal fibroblast cell line (HEK-293T) and human RCC cell lines (KTCTL-140, RCC23, A498, and OSRC2) were obtained from American Type Culture Collection (ATCC). Human RCC cell lines (KTCTL-140, RCC23, A498, and OSRC2) and normal human renal fibroblast cell line HEK-293T were cultured in RPMI-1640 and DMEM/F12 medium containing 10% fetal bovine serum, respectively. Cells were maintained at 37°C in an incubator with 5% CO_2. The medium was changed every 3 days.

2.3 | Transfections and transductions

MicroRNA-765, microRNA-21, and microRNA-144 mimics or inhibitors were obtained from Shanghai GenePharma. The RCC cell lines in the logarithmic growth phase were seeded on a 6-well culture plate with approximately 2×10^5 cells per well. When the growth confluence of the cells reached 50%, they were divided into microRNA mimic group, microRNA inhibitor group, and microRNA negative control group. The transfection was conducted using Lipofectamine 3000® Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

pLenti-CMV vector for LHPP (pLV-LHPP) was constructed by Public Protein/Plasmid Library (Jiangsu, China) to permanently overexpress LHPP. The empty vector was used as the negative control (pLV-NC). All these vectors were packaged with lentivirus. Viral supernatants were used to transduce cells.

2.4 | Cell invasion assay

A 24-well Transwell chamber was used to evaluate the invasion ability of RCC cells. After 24 h of transfection, a total of 3×10^4 cells were plated in the upper chamber. The lower chamber contains 600 μL RPMI-1640 medium, 10% fetal bovine serum, and 1%

glutamine. At 48 h, the cells were fixed with 0.1% paraformalde-hyde and stained with 4% crystal violet at room temperature for 25 min.[9] The cells were counted in three randomly selected fields of view.

2.5 | Cell counting kit-8 (CCK-8) assay

Approximately 1×10^3 cells in the logarithmic growth phase were seeded in a 96-well plate. Then, add 10 μL of CCK-8 solution to each well and incubate for 30 min in a 37°C, 5% CO_2 incubator. After the cells were incubated for 0, 24, 48, and 72 h, the absorbance at 450 nm was measured with a microplate reader.[8] The experiment was performed three times.

2.6 | Subcellular localization of LHPP

A nuclear/cytoplasmic fractionation kit (Life Technologies) was used to purify the nucleus and cytoplasmic components of each group according to the kit's instructions. Quantitative real-time PCR (qRT-PCR) was used to detect the expression level of LHPP in each group. Subcellular localization of LHPP was identified with fluorescence in situ hybridization (FISH) method using a Ribo™ FISH Probe Mix (Green) (RiboBio) according to the manufacture's protocol.[10] The FISH probe mix for LHPP was synthesized by Sangon Biotech. The nuclei of the RCC cell were stained with 2.5 μM DAPI (Abcam) for 30 min. The fluorescence was scanned using a fluorescence micro-scope (Eclipse 80i; Nikon Corporation).

2.7 | RNA extraction and qRT-PCR

Total RNA was isolated from RCC cell lines and 60 paired tissues, then, the first-strand cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara). qRT-PCR was performed using the SYBR Green qPCR Kit (Thermo Fisher Scientific) with a StepOne™ Real-Time PCR System (Applied Biosystems). The cycling condition includes hold (95°C for 30 s), PCR (95°C for 5 s, followed by 60°C for 25 s, 45 cycles), and dissociation (95°C for 15 s, then 60°C for 30 s, followed by 95°C for 15 s). The $2^{-\triangle Ct}$ (U6 or GAPDH as control) formula was applied to determine RNA relative expression level. Their primer sequences are listed in Table S1. The amplifica-tion efficiencies of LHPP, microRNA-765, microRNA-21, microRNA-144, GAPDH, and U6 were 96.5%, 97.6%, 100.6%, 98.2%, 102.6%, and 97.7%, respectively. The test was repeated three times for each sample.

2.8 | Luciferase reporter assay

A total of 2×10^3 RCC cells were plated onto 96-wells plates. The partial sequences of the LHPP 3′-untranslated region (UTR), which

contain the putative microRNA-765 or microRNA-21 or microRNA-144-binding site, were constructed into the pmirGLO Luciferase vector (Promega) to generate wild-type LHPP reporter (LHPP-WT). The GeneArt™ Site-Directed Mutagenesis System (Thermo Fisher Scientific) was used to produce microRNA-765 or microRNA-21 or microRNA-144 target site-mutation LHPP (LHPP-MUT) reporter. The luciferase reporters and microRNA-765 or microRNA-21 or mi-croRNA-144 mimic or control mimic were co-transfected into RCC cells using Lipofectamine 3000® Transfection Reagent (Invitrogen). 48 h after transfection, cells were collected, and the relative firefly luciferase activities were measured using a dual-luciferase reporter assay system (Promega).

2.9 | Western Blot

The total protein from RCC cells was isolated using RIPA lysis buffer (Beyotime), and the protein concentration was measured by the BCA Kit (Pierce). A total of 30 μg protein was separated by SDS-PAGE electrophoresis. Then, the membrane was incubated with LHPP (ab116175, Abcam), N-cadherin (ab76011, Abcam), E-cadherin (ab40772, Abcam), β-catenin (ab32572, Abcam), Vimentin (ab92547, Abcam), Caspase-3 (ab32351, Abcam), Bcl-2 (ab32124, Abcam), Bax (ab32503, Abcam), and β-actin (ab8227, Abcam) primary antibodies and goat derived IgG H&L secondary antibodies, respectively. The immunoreactive bands were visualized by an enhanced chemilumi-nescence ECL kit and then scanned by a LAS-4000 imaging system (Fujifilm).

2.10 | Immunohistochemistry

Immunohistochemistry was performed in paraffin-embedded sections using an immunohistochemistry kit (SV0002, BOSTER). RCC samples were treated with 3% hydrogen peroxide, then covered with 3% BSA. The tissue samples were incubated with primary antibodies against LHPP (ab116175, Abcam) overnight at 4°C, followed by incubation with the secondary antibody pro-vided in the kits for 30 min. The section was then stained with 3,3′-diaminobenzidine and was photographed by an Olympus in-verted microscope.

2.11 | Statistics

The miRDB (http://www.mirdb.org/, version 6.0), miRanda (http://www.miranda.org/, version 3.3), PicTar (https://pictar.mdc-berlin.de/, version 5.0), and Targetscan (http://www.targetscan.org/, Version 7.1) online prediction systems were used to predict the tar-geted microRNAs of LHPP. The R version 3.6.1 and GraphPad Prism version 7.0 were used in this study. The t test and one-way ANOVA were used for the two groups or multiple groups comparison. $p < .05$ was considered to be statistically significant.

3 | RESULTS

3.1 | LHPP expression levels in RCC cells and cancer tissues were decreased and were related to the malignant biological behavior

Firstly, we used the Human Protein Atlas project online prediction system to evaluate the prognostic value of LHPP for RCC (Figure 1A). Compared with RCC tissues with low LHPP levels, patients with high LHPP levels had a higher long-term survival rate ($p < .001$). Compared with adjacent tissues, LHPP protein (Figure 1B-D) and mRNA (Figure 1E) levels in RCC tissues were lower ($p < .05$). Besides, we found that the expression level of LHPP mRNA in RCC tissue was collected with poor differentiation, recurrence, and poor prognosis (Table 1) and was also associated with the disease-free survival and overall survival ($p < .05$, Figure 1F).

In vitro, qRT-PCR and the FISH results demonstrated that LHPP was preferentially localized in the cytoplasm (Figure S1A,B). Then, the relatively lower LHPP mRNA expression was detected in the RCC23 cell line, which was thus selected as the subject of subsequent experiments (Figure 2A). To ascertain whether LHPP exerts a tumor suppressor function, pLV-LHPP was transfected into RCC23 cells to overexpress the endogenous LHPP expression. LHPP overexpression was verified by qRT-PCR (Figure 2B). pLV-LHPP#2
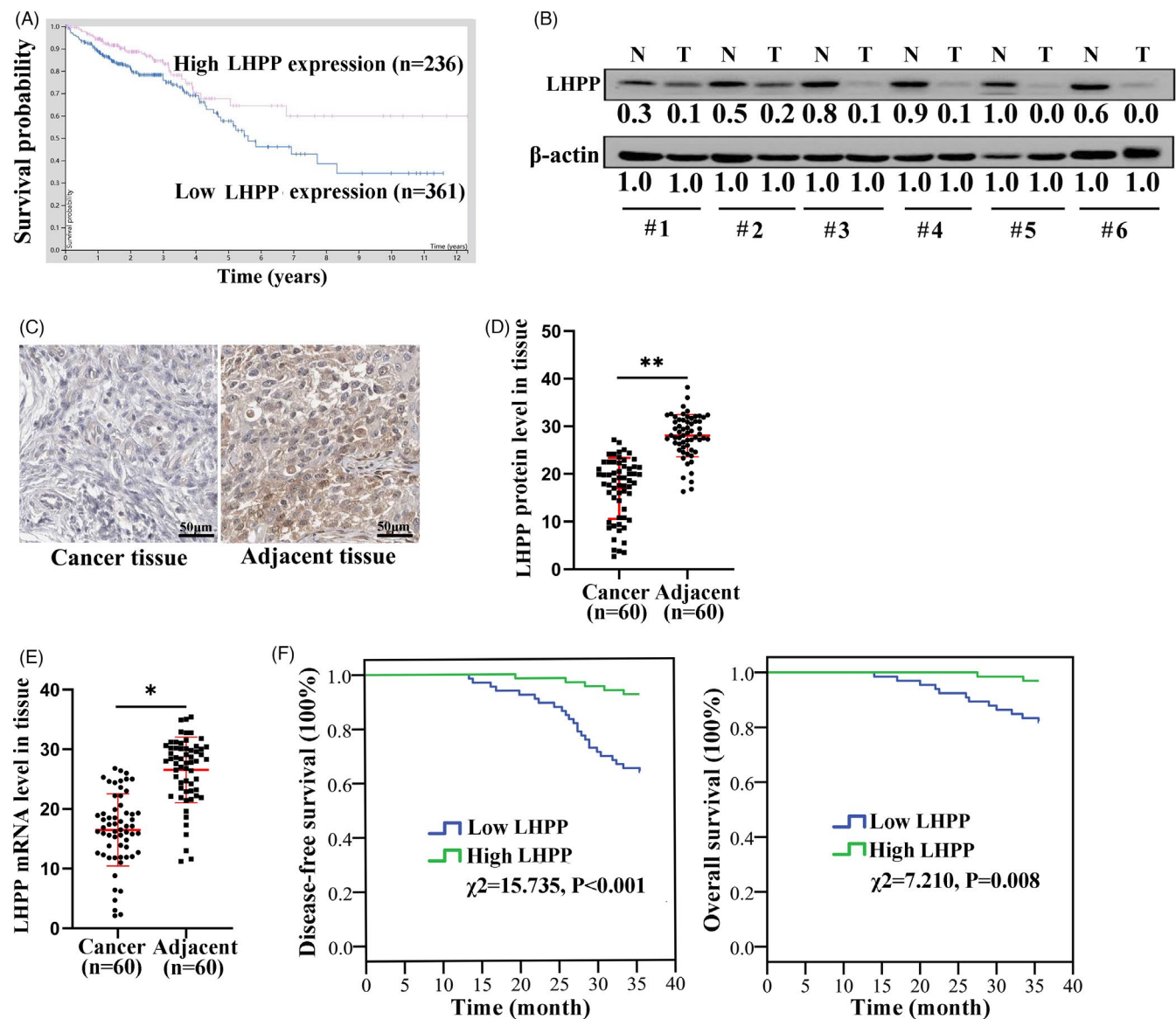

FIGURE 1 LHPP expression in RCC tissue was significantly decreased. A, The Human Protein Atlas project online prediction system was used to evaluate the prognostic value of tissue LHPP in RCC. B,C, Compared with adjacent tissues, the level of LHPP protein in cancer tissues was significantly decreased. T: cancer tissue; N: adjacent tissue. The experiment was performed three times. D, LHPP protein levels in cancer and adjacent tissues of sixty RCC patients. Each point represents an independent sample. E, LHPP mRNA levels in cancer and adjacent tissues of sixty RCC patients. F, Compared with the low LHPP group, the high LHPP group has significantly higher disease-free survival and overall survival. *$p < .05$, compared with adjacent tissue; **$p < .001$, compared with adjacent tissue

TABLE 1 Relationships between LHPP mRNA, microRNA-765, microRNA-21, and microRNA-144 levels in RCC tissues and patients' clinical characteristics

Characteristics	Case (n = 60)	LHPP mRNA levels	P value	microRNA-765 levels	P value	microRNA-21 levels	P value	microRNA-144 levels	P value
Gender			.896		.302		.794		.885
Male	39	16.30 ± 3.12		16.87 ± 2.45		8.43 ± 1.21		1.63 ± 0.22	
Female	21	16.41 ± 3.05		16.67 ± 2.43		8.32 ± 1.04		1.58 ± 0.18	
Age			.873		.895		.931		.734
<50	19	16.27 ± 2.96		16.84 ± 2.37		8.37 ± 1.11		1.70 ± 0.19	
≥50	41	16.37 ± 3.08		16.78 ± 2.59		8.40 ± 1.14		1.58 ± 0.20	
Smoking			.719		.806		.270		.800
No	24	16.42 ± 2.88		16.85 ± 2.09		8.50 ± 0.98		1.57 ± 0.24	
Yes	36	16.29 ± 3.31		16.77 ± 2.50		8.32 ± 1.04		1.65 ± 0.13	
Alcohol using			.698		.487		.866		.979
No	17	16.24 ± 3.18		16.91 ± 2.44		8.34 ± 1.06		1.60 ± 0.21	
Yes	43	16.38 ± 2.85		16.76 ± 2.26		8.41 ± 1.13		1.62 ± 0.18	
Pathological typing			.909		.920		.273		.067
Clear cell carcinoma	42	16.31 ± 3.07		16.82 ± 2.11		8.29 ± 1.01		1.58 ± 0.19	
Papillary carcinoma	18	16.41 ± 3.14		16.76 ± 2.09		8.62 ± 1.17		1.69 ± 0.25	
Histological differentiation			<.001		<.001		<.001		<.001
Well/moderate	41	18.42 ± 3.29		13.96 ± 2.47		7.57 ± 0.94		1.42 ± 0.17	
Poor	19	11.85 ± 2.78		22.93 ± 2.65		10.15 ± 1.32		2.04 ± 0.23	
T stage			<.001		<.001		<.001		<.001
Tis-2	22	19.35 ± 3.22		12.78 ± 2.35		3.93 ± 0.88		0.76 ± 0.13	
T3-4	38	14.60 ± 3.03		19.13 ± 2.58		10.97 ± 1.34		2.11 ± 0.19	
N stage			<.001		<.001		<.001		<.001
N0	25	20.23 ± 3.31		14.04 ± 2.41		4.57 ± 0.69		1.12 ± 0.18	
N1	35	13.56 ± 2.93		18.77 ± 2.59		11.12 ± 1.29		1.97 ± 0.25	

Note: Data are expressed as mean ± standard deviation. Unpaired student's *t* test (two-tailed) was used for comparison between two groups.

presented higher efficiency; thus, it was used in the following experiments. Transfection with pLV-LHPP caused an obvious decrease in proliferation rate (P_{48h}=0.008, P_{72h}<0.001, Figure 2C). Furthermore, the invasive (Figure 2D) property of the RCC23 cell was strikingly hindered by pLV-LHPP treatment in contrast to pLV-NC treatment. Western blot results showed that, compared with the pLV-NC group, the levels of EMT-related protein markers in the pLV-LHPP group were inhibited, while the levels of apoptosis-related protein markers were increased (Figure 2E). Overall, the observations mentioned above supported the hypothesis that LHPP acts as a tumor suppressor gene in RCC.

3.2 | MicroRNA-765, microRNA-21, and microRNA-144 are involved in the regulation of LHPP

We respectively applied miRDB, miRanda, PicTar, and Targetscan online prediction systems and find out the LHPP gene-targeting co-expressed microRNAs (Figure S2). Then, three microRNAs were predicted, namely microRNA-765, microRNA-21, and microRNA-144.

Next, we applied the dual-luciferase reporter gene method (Figure S3A-C) to verify the targeting relationships between micro-RNA-765, microRNA-21, and microRNA-144 and LHPP.

3.3 | MicroRNA-765, microRNA-21, and microRNA-144 expression levels in RCC cells and cancer tissues were significantly increased and were related to the malignant biological behavior

Firstly, we detected microRNA-765, microRNA-21, and microRNA-144 expression levels in 60 paired tissues and analyzed their correlation with LHPP mRNA levels (Figure 3). Compared with adjacent tissues, the levels of microRNA-765 (Figure 3A), microRNA-21 (Figure 3B), and microRNA-144 (Figure 3C) in cancer tissues were significantly increased (p < .001). Moreover, the levels of microRNA-765 (r = −0.570, p < .001, Figure 3D), microRNA-21 (r = −0.495, p < .001, Figure 3E), and microRNA-144 (r = −0.463, p < .001, Figure 3F) were all significantly negatively correlated with LHPP (p < .001).

In vitro, transfection with microRNA-765 (P_{48h} = .011, P_{72h} < .001), microRNA-21 (P_{48h} = .017, P_{72h} < .001), or microR-NA-144 (P_{48h} < 0.001, P_{72h} < .001) inhibitor caused an obvious decrease in proliferation rate (Figure 4A). Furthermore, the invasive (Figure 4B) ability of RCC23 cells was strikingly hindered in contrast to the control group. Western blot results showed that compared
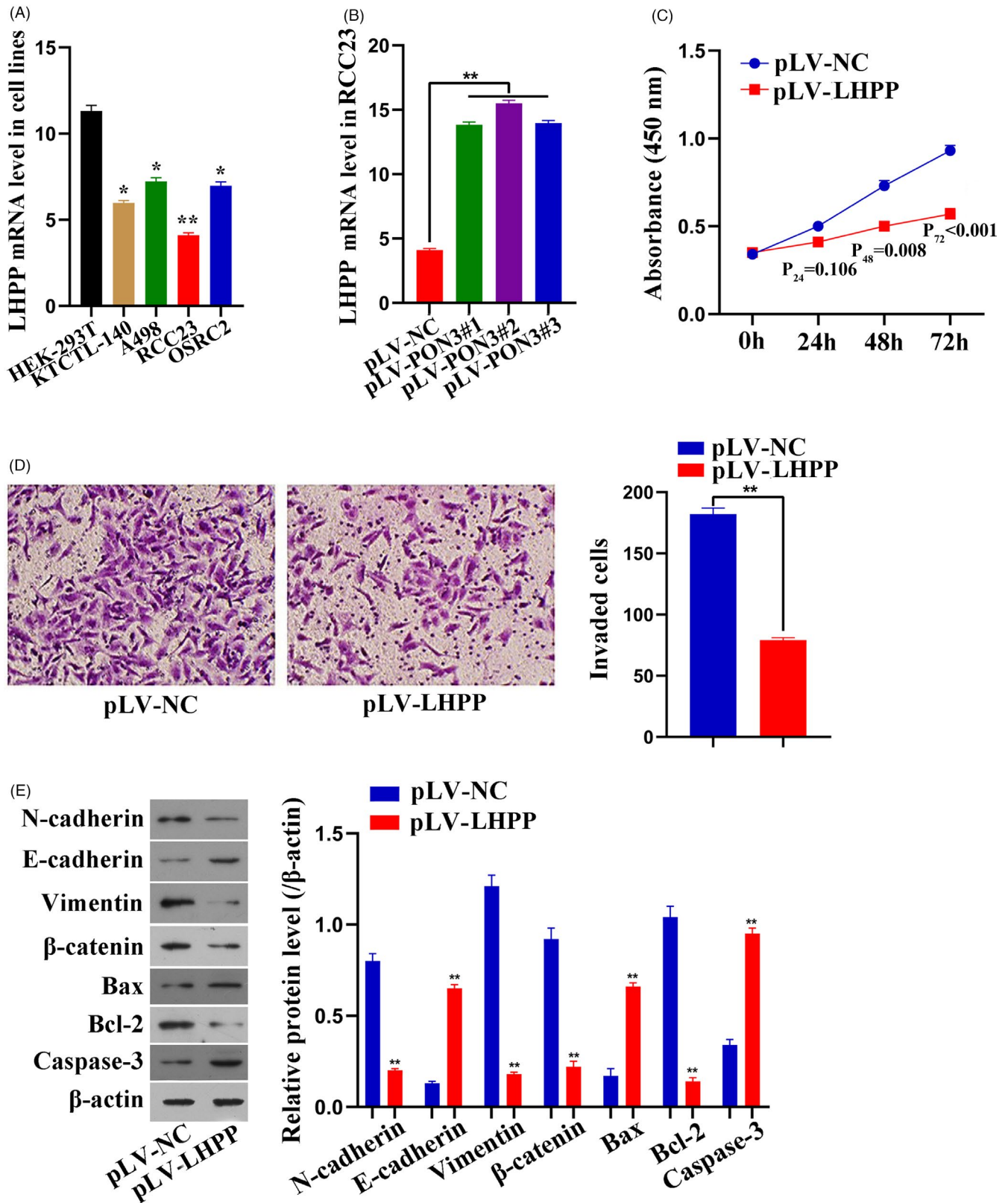
FIGURE 2　LHPP is related to the malignant biological behavior in RCC. A, A relatively higher LHPP mRNA expression was detected in the RCC23 cell line. The test was repeated three times for each sample. B, RCC23 cells were transfected with pLV-LHPP#1, pLV-LHPP#2, or pLV-LHPP#3. The test was repeated three times for each sample. C, CCK-8 assay detected the change of proliferative capacity in the RCC23 cell line after the introduction of pLV-LHPP. The experiment was performed three times. D, An invasion assay was performed to assess the effect of LHPP on the invasion of RCC23 cells. The experiment was performed three times. E, Western blot results showed that compared with the pLV-LHPP group, the expression levels of N-cadherin, β-catenin, Vimentin, and Bcl-2 were decreased, while the expression levels of E-cadherin, Bax, and Caspase-3 were increased. The experiment was performed three times. *$p < .05$; **$p < .001$, compared with HEK-293T or pLV-NC
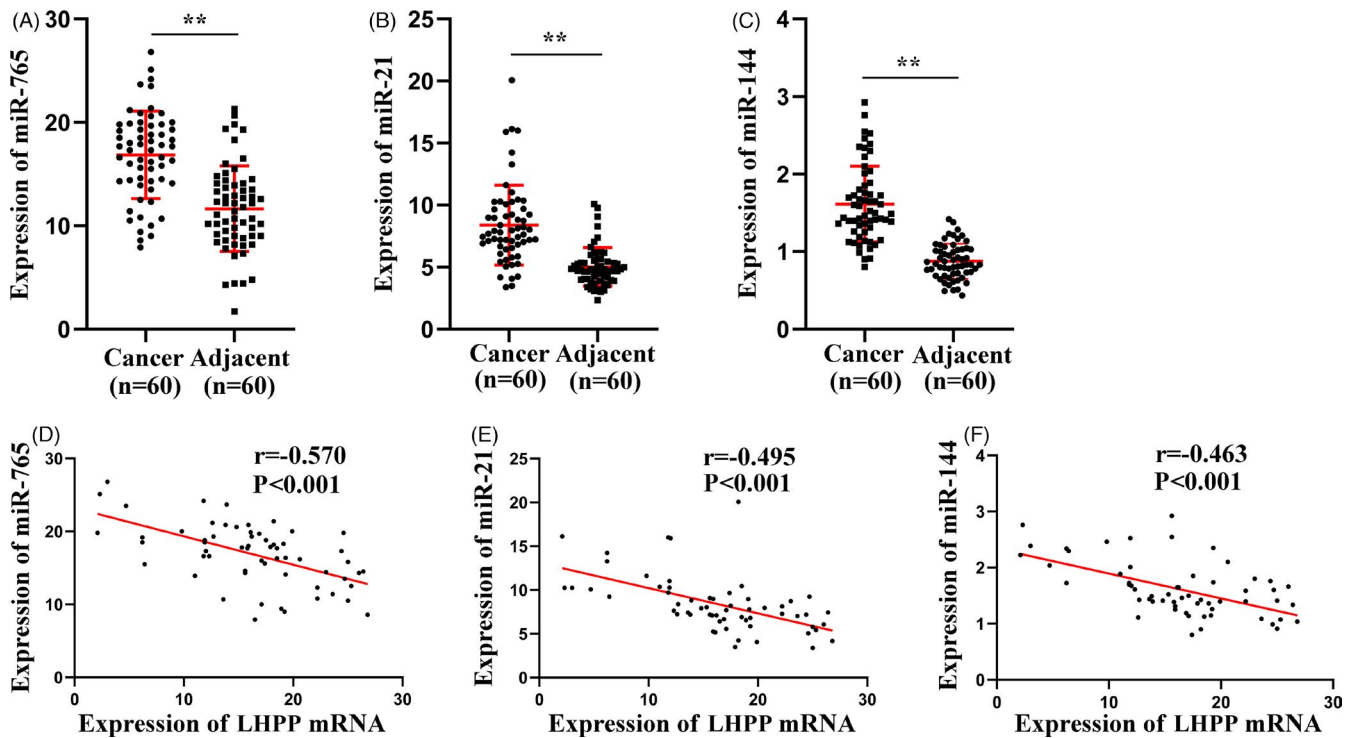

FIGURE 3　MicroRNA-765, microRNA-21, and microRNA-144 expression levels in RCC tissues were significantly increased. A, microRNA-765 level in RCC and adjacent tissues. B, microRNA-21 level in RCC and adjacent tissues. C, microRNA-144 level in RCC and adjacent tissues. D, The correlation between microRNA-765 and LHPP in the RCC tissue. E, The correlation between microRNA-21 and LHPP in the RCC tissue. F, The correlation between microRNA-144 and LHPP in the RCC tissue. **$p < .001$, compared with adjacent tissues

with the control group, the expression levels of N-cadherin, β-catenin, Vimentin, and Bcl-2 were decreased, while the expression levels of E-cadherin, Bax, and Caspase-3 were increased (Figure 4C).

3.4 | Survival analysis of microRNA-765, microRNA-21, and microRNA-144 on the recurrence and prognosis of patients with RCC

Furthermore, we found that the expression levels of microRNA-765, microRNA-21, and microRNA-144 in RCC tissue were correlated with poor differentiation (Table 1) and were also negatively correlated with the 3-year prognosis. Survival analysis results are shown in Figure 5. Compared with patients in the high microRNA-765 group, the high microRNA-21, and the high microRNA-144 groups, the disease-free survival and overall survival of patients in the low microRNA-765 (Figure 5A,D), the low microRNA-21 (Figure 5B,E),

and the low microRNA-144 (Figure 5C,F) groups were increased ($p < .05$).

4 | DISCUSSION

The combination treatment of surgery and chemoradiotherapy has not made any further breakthrough to improve patients' quality of life and survival rate.[11-13] Therefore, searching for the relationship between LHPP gene-targeting co-expressed microRNAs and RCC metastasis will lay the foundation for the development of new treatments.

At present, there is still little research on LHPP in RCC, and its molecular mechanisms remain unclear. In this study, we found that the expression levels of LHPP in cancer tissues were significantly lower than that in adjacent cancer tissues, and the low expression level of LHPP in cancer tissue was associated with poor
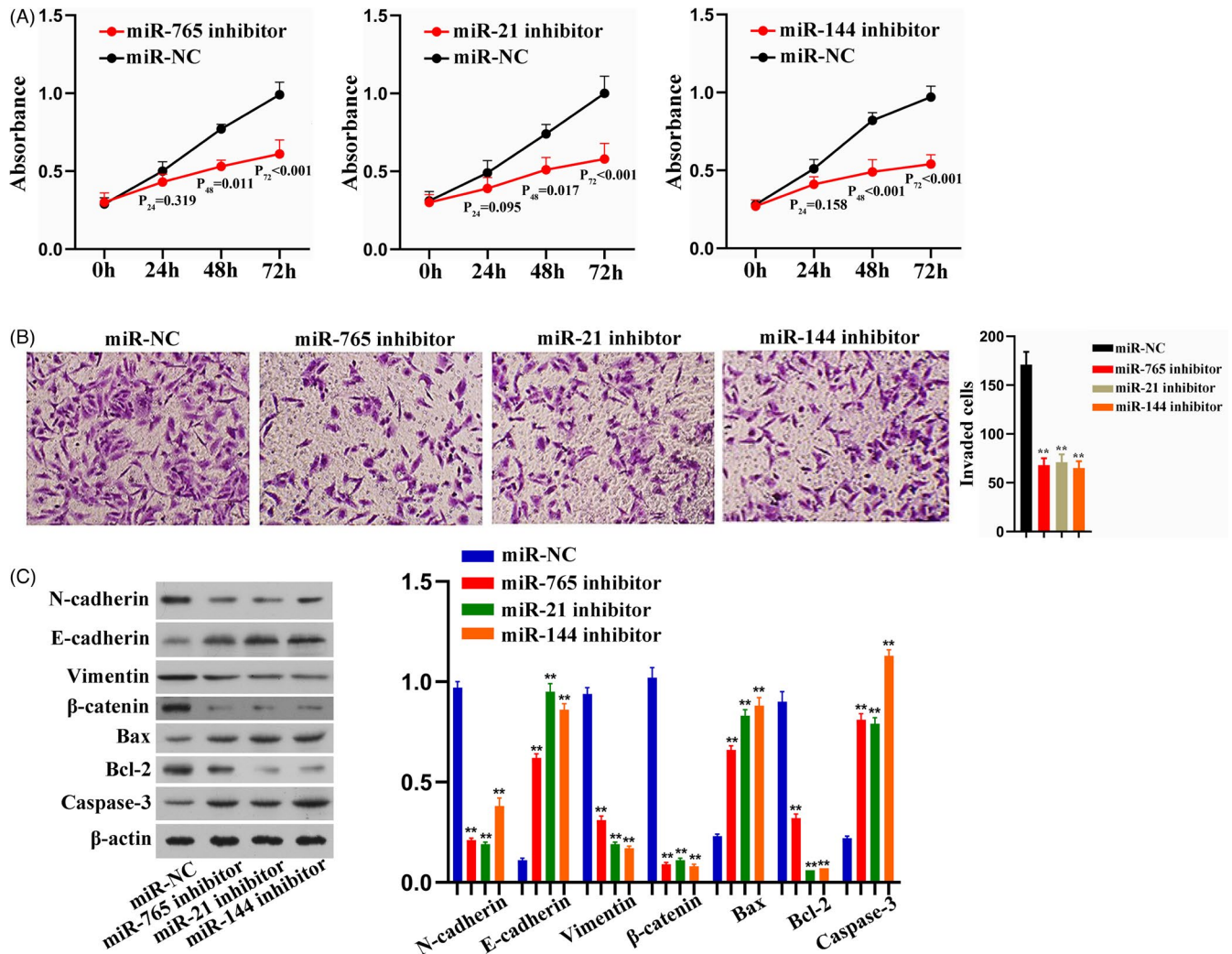
FIGURE 4 Effects of microRNA-765, microRNA-21, and microRNA-144 on RCC23 cell proliferation, apoptosis, invasion, and metastasis. A, Proliferative capacity in the RCC23 cell line after the introduction of microRNA-765, microRNA-21, or microRNA-144 inhibitor. The experiment was performed three times. B, Invasion assay was performed to assess the effect of microRNA-765, microRNA-21, or microRNA-144 inhibitor interference on the invasion of RCC23 cells. The experiment was performed three times. C, Western blot results showed that the expression levels of N-cadherin, β-catenin, Vimentin, and Bcl-2 were decreased, while the expression levels of E-cadherin, Bax, and Caspase-3 were increased in microRNA-765, microRNA-21, or microRNA-144 inhibitor group. The experiment was performed three times. **$p < .05$, compared with microRNA normal control group

differentiation, recurrence, and poor prognosis. *In vitro* study, we further found that LHPP expression was significantly decreased in the highly metastatic RCC cell line. Furthermore, we explored the effect of upregulated LHPP expression on RCC cells. We found that increasing the expression of LHPP can significantly inhibit the proliferation, EMT, and invasion of RCC cells. For the first time, our study confirmed the tumor suppressor appearance of LHPP in RCC tissues and cell lines.

Previous studies indicated that microRNA participates in tumor formation and progression by modulating their downstream target genes.[7,9] However, which microRNAs are involved in the regulation of LHPP in RCC? To solve this problem, we explored the relationship between LHPP and potential upstream target microRNAs in the present study. Herein, we found that microRNA-765,

microRNA-21, and microRNA-144 were direct targets of LHPP in RCC cells. Consistently, we confirmed that the high expression levels of microRNA-765, microRNA-21, and microRNA-144 in RCC tissues were significantly related to the patient's poor differentiation, T stage, N stage, recurrence, and poor prognosis. *In vitro* cell experiments also further verified that microRNA-765, microRNA-21, and microRNA-144 promote the malignant progression of RCC.

A recent study pointed out that microRNA-144 can promote the progression of nasopharyngeal carcinoma through the phosphatase and tensin homolog deleted on the chromosome ten pathway.[14] However, there is still no report about microRNA-144 and RCC. In the current study, we confirmed for the first time that microRNA-144 plays an oncogene in RCC patients from the clinical and cellular levels. MicroRNA-21 and microRNA-765 have been confirmed to be
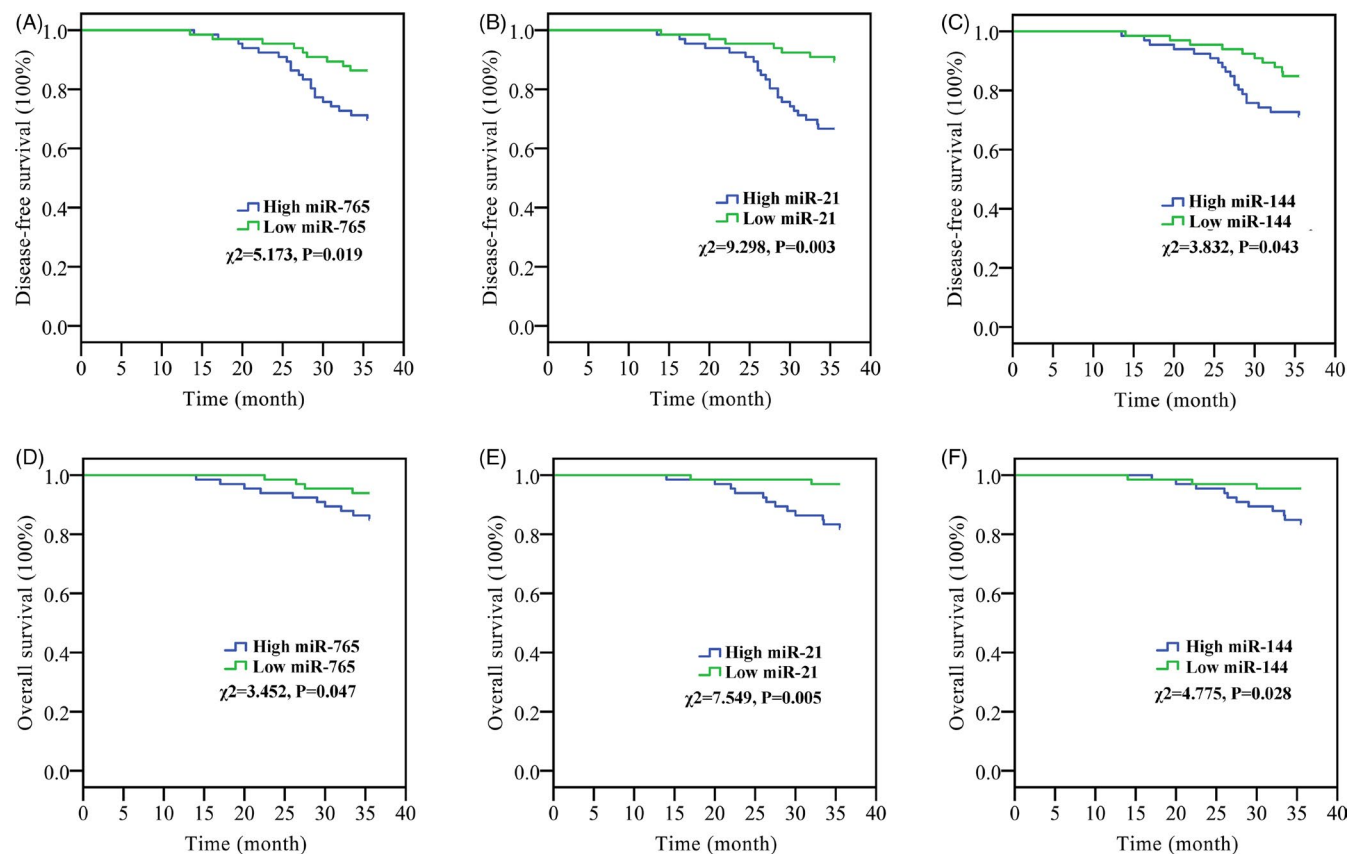
FIGURE 5 MicroRNA-765, microRNA-21, and microRNA-144 expression levels in RCC tissues were related to the prognosis of patients. A, Analysis of microRNA-765 level in RCC tissue and patient's disease-free survival. B, Analysis of microRNA-21 level in RCC tissue and patient's disease-free survival. C, Analysis of microRNA-144 level in RCC tissue and patient's disease-free survival. D, Analysis of microRNA-765 level in RCC tissue and patient's overall survival. E, Analysis of microRNA-21 level in RCC tissue and patient's overall survival. F, Analysis of microRNA-144 level in RCC tissue and patient's overall survival

oncogenes,[15,16] but there is no report about the prognostic values of the two microRNAs in evaluating RCC patients. In this study, our results suggested that microRNA-765, microRNA-21, microRNA-144, and LHPP had effective values in the evaluation of RCC patients' recurrence and prognosis.

However, due to the lack of sufficient evidence, we will also conduct animal experiments to further support the reliability of our conclusion. In summary, our research demonstrated that microRNA-765, microRNA-21, and microRNA-144 can promote the proliferation, EMT, and invasion of RCC by downregulating LHPP.

CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

XC researched literature and conceived the study. KM and ZL were involved in gaining ethical approval, patient recruitment, and data analysis. KM and XC wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

ETHICS APPROVAL

Our research plans were approved by the Human Ethics Committee of The Second Affiliated Hospital, Harbin Medical University (NO.: KL2017013-V02), according to the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

The data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.