

MicroRNA-337-5p participates in the development and progression of osteosarcoma via ERBB, MAPK and VEGF pathways

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Abstract. – OBJECTIVE: To investigate the role of microRNA-337-5p in osteosarcoma (OS) and its underlying mechanism.

PATIENTS AND METHODS: The microRNA (microRNA-337-5p) that may be related to OS development was screened out by GEO (Gene Expression Omnibus) database. Survival analysis and ROC curve were performed according to microRNA-337-5p expressions in OA patients. Besides, the correlation between microRNA-337-5p expression and clinical parameters was evaluated by Chi-square analysis. Cox regression analysis was performed to detect the relationship between the overall survival and clinical parameters of OA patients. Subsequently, enriched functions and pathways of microRNA-337-5p were predicted by GESA (gene enrichment sets analysis). MicroRNA-337-5p expression was detected in 65 OS tissue samples and 30 normal tissue samples by qRT-PCR (quantitative Real-Time Polymerase Chain Reaction). For *in vitro* experiments, after microRNA-337-5p mimics or microRNA-337-5p inhibitor was transfected into OS cells, proliferative and invasive abilities were detected by CCK-8 (Cell Counting Kit-8) and transwell assay, respectively. Finally, Western blot was used to explore the underlying mechanism of microRNA-337-5p in regulating OS.

RESULTS: MicroRNA-337-5p was overexpressed in serum and tissue samples of OS patients, which was valuable in diagnosing OS. Besides, microRNA-337-5p expression was correlated with the overall survival and necrosis range of OA patients, whereas not correlated with age and sex. GESA indicated that microRNA-337-5p was enriched in ERBB, MAPK, and VEGF pathways. *In vitro* experiments indicated elevated proliferative and invasive abilities in MG63 and U2OS cells after microRNA-337-5p overexpression. Furthermore, increased expressions of ERBB2, Erk1/2, and VEGF121 were observed in OS cells after microRNA-337-5p overexpression.

CONCLUSIONS: MicroRNA-337-5p is upregulated in OS tissues, which is an independent prognostic factor in OS. Overexpressed microRNA-337-5p can promote proliferative and invasive abilities of OS cells via activating ERBB, MAPK, and VEGF pathways.

Key Words:

Osteosarcoma, MicroRNA-337-5p, ERBB, MAPK, VEGF.

Introduction

Osteosarcoma (OS) is a primary bone tumor with a high malignancy. In recent years, the 5-year survival rate of OS has greatly increased from 20%-30% to 60%-70% because of the progressed combination treatment of surgery and chemotherapy^{1,2}. However, 10% of OS patients experience local recurrence after comprehensive treatment and their prognosis is often poor³⁻⁵. Therefore, exploration of the molecular mechanism is beneficial to search for new prognostic biomarkers and targeted therapeutic drugs of OS.

MicroRNAs are newly discovered non-encoding small RNAs with 22-28 nucleotides in length, which are widely found in eukaryotes. The precursors of microRNAs are pre-miRNAs, and they are cleaved and hydrolyzed *via* Dicer enzymes and hydrolases to form a single-stranded microRNA^{6,7}. Functionally, microRNAs can completely or incompletely bind to 3'-UTR of target mRNAs, leading to its degradation or translational inhibition, thereby affecting the *in vitro* biological processes⁸⁻¹¹. Accumulating studies have shown that microRNAs are involved in the development of tumors as oncogenes or tumor suppressor genes.

Each microRNA has various target genes, which forms a complex regulation system¹². Therefore, microRNAs provide a new alternative strategy for gene therapy of malignant tumors. Furthermore, microRNA expression profile in peripheral blood is parallel to that of lesion tissues in many diseases, including OS^{13,14}.

MicroRNA-337 has been reported in many diseases. For example, microRNA-337 can regulate gene expressions and transcription during liver cell differentiation¹⁵. MicroRNA-337 promotes progression of gastric cancer by inhibiting MZF1 (myeloid zinc finger 1) expression¹⁶. MicroRNA-337 inhibits progression of neuroblastoma by inhibiting MMP-14 (matrix metalloproteinase-14) expression¹⁷. In addition, the role of microRNA-337 in osteoarthritis¹⁸, melanoma¹⁹, and pancreatic cancer²⁰ has already been reported. The role of microRNA-337 in OS, however, has not been studied. Therefore, it is of great significance to investigate the effect of microRNA-337-5p on OS, so as to provide a theoretical basis for the diagnosis and treatment of OS.

Patients and Methods

Microarray Data Collection and Analysis

The Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) is a public database that stores microarray and sequencing data. We screened out microarrays of OS in the GEO database and GSE65071 that contains serum samples of 15 normal individuals and 20 OS patients were selected. Expression levels of 750 microRNAs in GSE65071 were detected using Exiqon human V3 microRNA PCR panel I+II platform. Generally, GSE39040 included clinical information about the prognosis, age, and sex of 65 OS patients. Moreover, expression levels of 858 microRNAs were detected using the Illumina Human v2 MicroRNA Expression BeadChip. Differentially expressed microRNAs were analyzed by the Limma package. The prognostic analysis was performed by the survival function. $p < 0.05$ and $|\log FC| > 1$ were considered as cut-off values.

Correlation Analysis

The normal bone and OS tissue samples expressing microRNA-337-5p were downloaded, as well as the corresponding clinical parameters and prognostic data of these OS patients. OS cases with unknown or incomplete clinical parameters and those without complete prognostic follow-up data were

excluded. OS tissue samples were further assigned to high-expression and low-expression group based on their microRNA-337-5p expressions.

Gene Enrichment Sets Analysis

Gene enrichment sets analysis (GESA) 2.2.3 software was used for data analysis. A dataset of c2.cp.kegg.v6.0.symbols.gmt was obtained from the Msig-DB database of GESA website. GESA is then performed according to the default weighted enrichment statistics method, and the random combination number was set to 1000 times.

Sample Collection

Paraffin slices, peripheral blood samples of OS and corresponding control samples were collected at the Orthopedic Department, The Affiliated Jiangyin Hospital of Southeast University Medical College from July 2012 to June 2017. OS patients were surgically treated and pathologically diagnosed. All patients did not have other systemic tumors except for OS. Trauma patients during the same period were selected as the control group. General characteristics of enrolled subjects, including sex, age, and percentage of bone necrosis were shown in Table I. This study was approved by the Affiliated Jiangyin Hospital of Southeast University Medical College Ethics Committee. All the subjects signed the informed consent.

RNA Extraction and qRT-PCR (quantitative Real-Time Polymerase Chain Reaction)

The total RNA was extracted from tissue samples by TRIzol method and then transcribed into cDNA according to the instructions of PrimeScript RT Master Mix (Invitrogen, Carlsbad, CA, USA). QRT-PCR was then performed following the instructions of SYBR[®] Green Master Mix (TaKaRa, Otsu, Shiga, Japan). Primer sequences used in this study were as follows: MicroRNA-337-5p, F: 5'-ACACTCCAGCTGGGTTCAGCTCCTATATGAT-3', R: 5'-CTCAACTGGTGTCTGTTGGA-GTCGGCAATTCAGTTGAGAAAGGCAT-3'.

Cell Culture and Transfection

Cell lines used in this experiment (hBOB 1.19, MG63, U2OS, Saos2) were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin, and 100 µg/mL streptomycin (Hyclone, South Lo-

Table 1. Correlation between miR-337-5p expression and clinicopathological characteristics of osteosarcoma.

Clinical parameters	Numbers	has-miR-337-5p		<i>p</i> -value
		Low level	High level	
Age				
≤12	35	15	20	0.2669
>12	30	17	13	
Gender				
Female	30	14	16	0.7018
Male	35	18	17	
Necrosis percent				
≤60	23	10	13	0.4924
>60	42	22	20	

gan, UT, USA), and incubated in a 5% CO₂ incubator at 37°C.

Cells in good growth condition were selected and seeded in the 6-well plates. Cell transfection was performed when the cell confluence was up to 60%-80% according to the instructions of Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmids (microRNA-NC, microRNA-337-5p mimics, microRNA-337-5p inhibitor) were constructed by GenePharma (Shanghai, China).

CCK-8 (Cell Counting Kit-8) Assay

Transfected cells were seeded into 96-well plates at a density of 2×10^3 per well, with 5 replicates in each group. 10 µL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added in each well after cell culture for 6, 24, 48, 72, and 96 h, respectively. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell Assay

The upper chamber of transwell chamber was previously coated with 100 µL of Matrigel (BD Biosciences, San Jose, CA, USA) and maintained in an incubator for 2 h. After cell density was adjusted to 1×10^6 /mL, 100 µL of cell supernatant and 600 µL of DMEM containing 10% FBS were then added in the upper and lower chamber, respectively. Transwell chamber was removed after incubation for 24 h, and the non-migrated cells in the chamber were gently wiped off with a cotton swab. The chamber was fixed with methanol for 15 min, washed with phosphate-buffered saline (PBS) twice and stained in 1% crystal violet for 30 min. Finally, 5 randomly selected fields were captured for cell count.

Western Blot

Total protein was extracted from treated cells by RIPA (radioimmunoprecipitation assay) solution (Beyotime, Shanghai, China). The protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene difluoride) membrane (Roche, Basel, Switzerland). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBS-T (Tris-buffered saline with Tween 20) and followed by the incubation of secondary antibody. The protein blot on the membrane was exposed by enhanced chemiluminescence (ECL).

Statistical Analysis

SPSS (Statistical Product and Service Solutions) 22.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis and GraphPad Prism (La Jolla, CA, USA) was introduced for image editing. Measurement data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$) and compared using the *t*-test. Chi-square test was performed to test the classification data. COX regression analysis was used for evaluating the correlation between gene expression and clinical parameters. *p* < 0.05 considered the difference was statistically significant.

Results

Upregulated MicroRNA-337-5p in OS Was Negatively Correlated to Its Prognosis

We screened out OS microarrays in the GEO database and GSE65071 that contains serum

samples of 15 normal individuals and 20 OS patients were selected. A total of 40 differentially expressed microRNAs were found in the microarray, including 29 upregulated microRNAs and 11 downregulated microRNAs (Figure 1A). Among them, microRNA-337-5p was remarkably upregulated in serum samples of OS patients (Figure 1B). We then collected clinical information of 65 OS patients in GSE39040, including prognosis, age, sex, and their microRNA-337-5p expression. Kaplan-Meier and log-rank were introduced to evaluate the correlation between microRNA-337-5p expression and prognosis of OS patients. Based on the median expression of microRNA-337-5p, OS patients were further assigned to high-expression group and low-expression group. Our data suggested that the overall survival of OS patients in the high-expression group was shorter than those in the low-expression group ($p=0.01949$, Figure 1D).

GESA of MicroRNA-337-5p Functions

We next explored the effect of microRNA-337-5p on OS patients. Chi-square analysis indicated no significant differences in sex, age, and necrosis range between high-expression group and low-expression group (Table I). Cox regression demonstrated that microRNA-337-5p expression and necrosis range were the independent prognostic factors in OS (Table II). Moreover, *t*-test indicated there were no significant differences between microRNA-337-5p expression and age, sex, and necrosis range of OA patients (Figure 2A-2C). GESA elucidated that microRNA-337-5p functions were mainly enriched in regulations of adherens junction (Figure 2D), focal adhesion (Figure 2E), and actin cytoskeleton (Figure 2F). It also suggested that microRNA-337-5p was enriched in ERBB (Figure 2G), MAPK (Figure 2H), and VEGF (Figure 2I) pathways.

MicroRNA-337-5p Was Overexpressed in OA Tissues

Expression levels of microRNA-337-5p were detected in 65 OS tissues and 30 normal tissues by qRT-PCR. Higher expression of microRNA-337-5p was observed in OS tissues than that of normal tissues (Figure 3A). After OS patients were assigned to the high-expression group and low-expression group as previously described, we found a shorter postoperative overall survival in OS patients of high-expression group than those of low-expression group ($p=0.0127$, Figure 3B). Higher expression of microRNA-337-5p was also found in OS patients with pulmonary metastasis than those without metastasis (Figure 3C). Furthermore, ROC curve elucidated that the area under ROC was 0.7761, indicating a higher sensitivity and specificity of microRNA-337-5p in predicting OS (Figure 3D). The above data suggested that microRNA-337-5p may participate in the occurrence and development of OS.

Overexpressed miR-337-5p Promoted Proliferation of OS Cells

To investigate the effect of microRNA-337-5p on OS cells, we first detected microRNA-337-5p expressions in normal osteoblast cell line (hFOB 1.19) and OS cell lines (MG63, U2OS, HOS, Saos2). The data showed a higher expression of microRNA-337-5p in OS cells than that of osteoblasts (Figure 4A). MG63 and U2OS were selected for further *in vitro* experiments since these two cells lines presented a higher microRNA-337-5p expression. MicroRNA-337-5p expression was altered in OS cells by transfection of microRNA-337-5p mimics or inhibitor, respectively (Figure 4B and 4C). Subsequently, the CCK-8 assay was carried out for detecting cell viability. Elevated cell viability was found after transfection with microRNA-337-5p mimics in OS cells (Figure 4D and 4E). Transwell assay obtained the similar re-

Table II. Univariate and multivariate Cox regression analyses miR-337-5p for OS of patients in study cohort.

Variables	Univariate analysis			Multivariate analysis		
	<i>p</i> -value	HR	95% CI	<i>p</i> -value	HR	95% CI
Age (≤ 12 vs. >12 years)	0.959	1.001	0.954, 1.050	0.362	0.959	0.877, 1.049
Gender (Female vs. Male)	0.616	0.764	0.268, 2.183	0.606	0.74	0.236, 2.321
Percent necrosis ($\leq 60\%$ vs. $>60\%$)	0.006	0.621	0.442, 0.872	0.02	0.662	0.467, 0.938
has-miR-337-5p (High level vs. Low level)	0.005	4.7	1.594, 13.857	0.02	2.96	1.189, 7.371

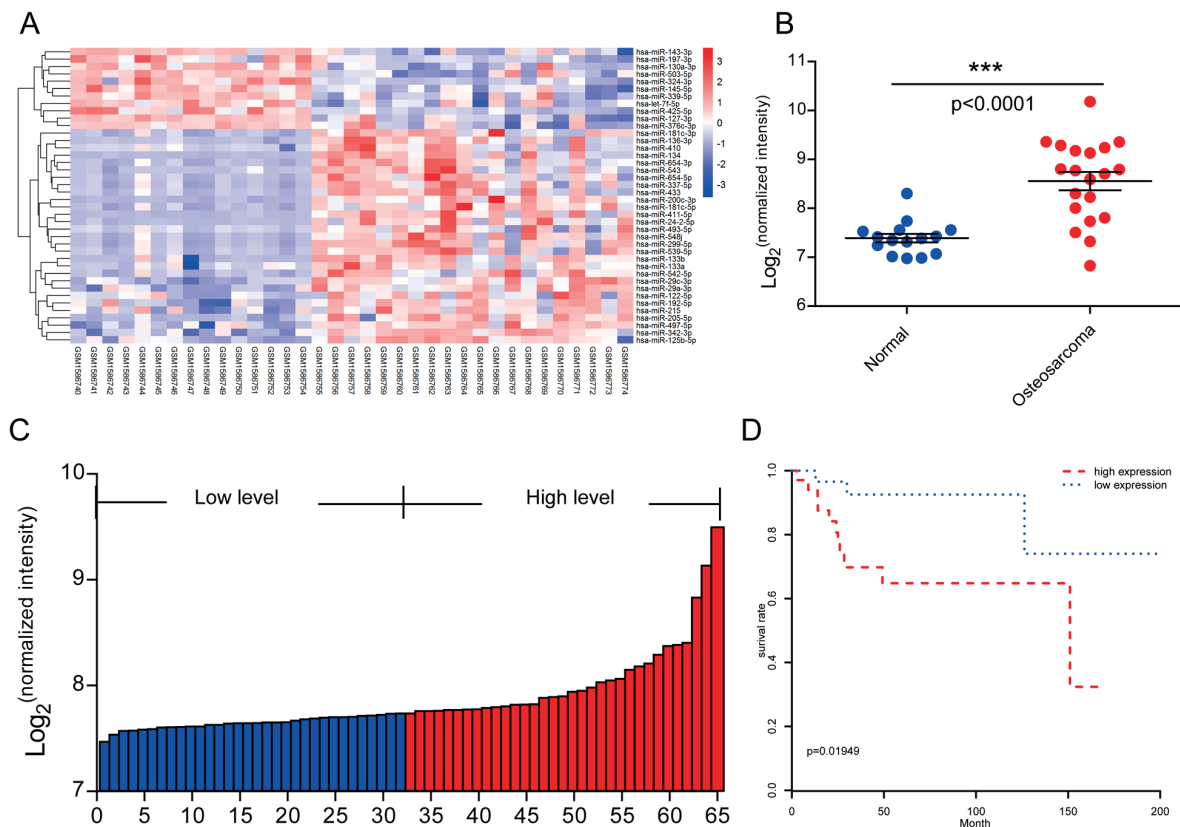


Figure 1. MicroRNA-337-5p was upregulated in OS. **A, B**, MicroRNA-337-5p was upregulated in GSE65071. **C, D**, The overall survival in high-expression group was lower than that of low-expression group.

sults (Figure 4F and 4G), indicating that microRNA-337-5p promotes proliferation and invasion of OS cells.

MicroRNA-337-5p Participated in the Development and Progression of OS Via ERBB, MAPK and VEGF Pathways

GESA demonstrated that microRNA-337-5p is enriched in ERBB, MAPK, and VEGF pathways. Hence, we detected expressions of ERBB2, Erk1/2, and VEGF121 in MG63 and U2OS cells after microRNA-337-5p overexpression. Western blot results indicated that overexpressed microRNA-337-5p resulted in elevated expressions of ERBB2, Erk1/2, and VEGF121 in OS cells (Figure 5A and 5C). The opposite results were obtained after microRNA-337-5p inhibition in OS cells (Figure 5B and 5D), suggesting that microRNA-337-5p participates in OS development *via* ERBB, MAPK, and VEGF pathways.

Discussion

Osteosarcoma is a common malignant bone tumor in children and adolescents, which is characterized as poor prognosis, low survival and difficult treatment. In recent years, progressed development of biological technology has provided new ideas for cancer treatment, especially gene therapy. Current researches^{21,22} have shown that microRNAs have a wide range of regulatory functions and can regulate all aspects of gene activities. MicroRNAs are capable of regulating various biological processes in tumor cells, such as proliferation, apoptosis, and cell cycle.

MicroRNA, as a crucial molecule in eukaryotic cells, is highly conserved among various species²³. Researches have shown that microRNAs are involved in almost all cellular activities, including cell proliferation and apoptosis, organogenesis, xenogenesis, hematopoiesis, and lipometabolism by regulating expressions of their target genes²⁴.

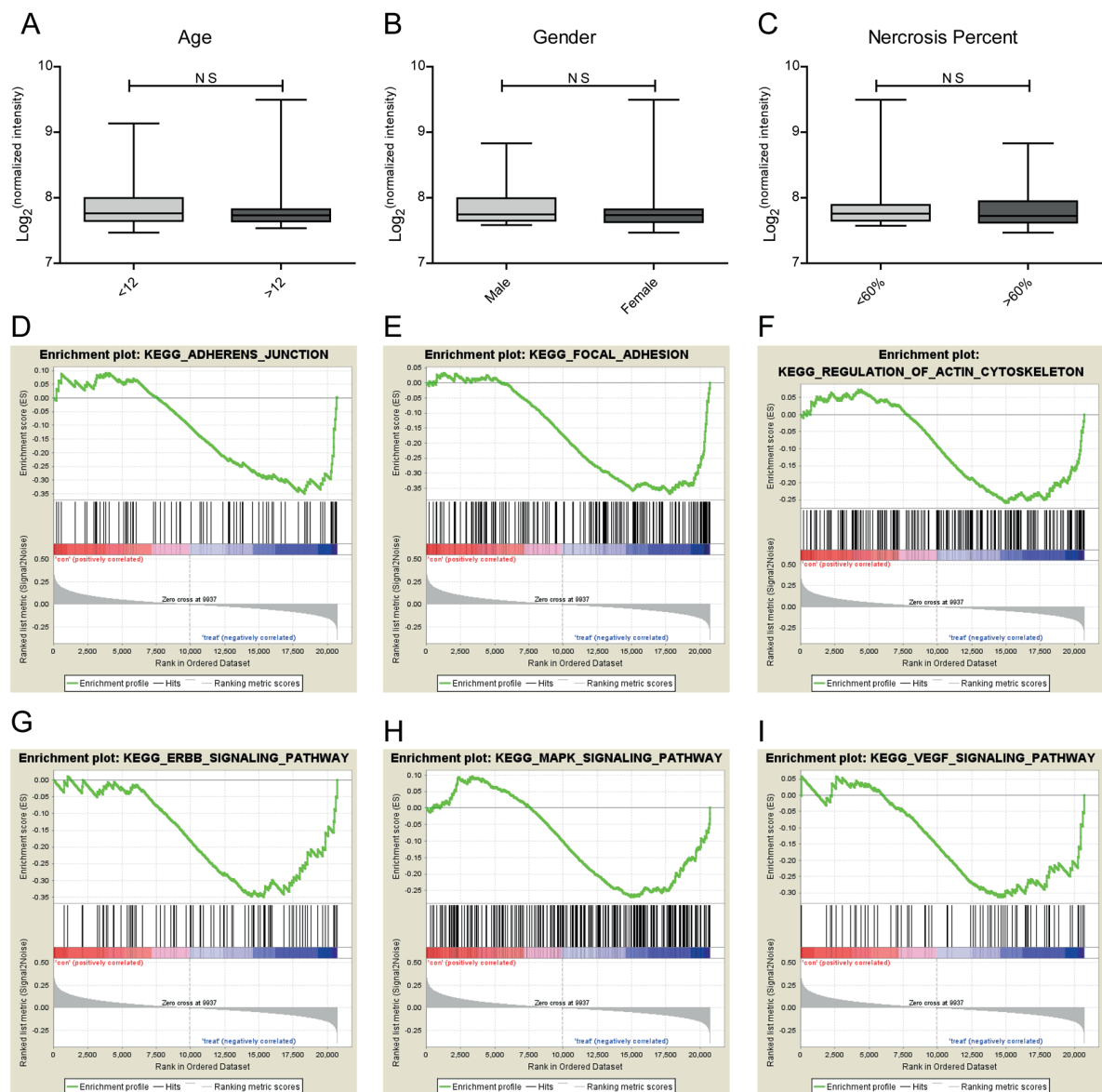


Figure 2. GESA of microRNA-337-5p functions. **A-C**, No significant differences were found between microRNA-337-5p and age, sex and necrosis range. **D-F**, GESA suggested that microRNA-337-5p functions were mainly enriched in regulations of adherens junction (**D**), focal adhesion (**E**), and actin cytoskeleton (**F**). **G-I**, GESA suggested that microRNA-337-5p was enriched in ERBB (**G**), MAPK (**H**), and VEGF (**I**) pathways.

Therefore, to study the role of microRNA in OS help to better understand its pathogenesis. At present, some differentially expressed microRNAs have been found in OS. Among them, microRNA-34 can promote OS through a p53-dependent manner²⁵. Overexpressed microRNA-24 is involved in the pathogenesis of OS by upregulating LPAAT β expression²⁶. Besides, microRNA-20a is overexpressed in OS and induces pulmonary metastasis *via* inhibiting Fas expression²⁷. In this study, microRNA-337-5p was found to be up-

regulated in OS by database analysis. Prognostic analysis showed that microRNA-337-5p expression was negatively correlated with the prognosis of OS patients. Furthermore, overexpressed microRNA-337-5p was correlated with poor outcomes and pulmonary metastases in OS patients.

Tumor cell invasion refers to the adhesion and translocation of tumor cells to the extracellular matrix, which involves three important steps, including adhesion to the basement membrane, gap formation of lysate membrane proteins, and

cell translocation to the gap²⁸. Many factors in the body are associated with cell invasion, such as matrix metalloproteinases (MMPs)²⁹. Briefly, primary tumor cells are transformed from epithelial cells to mesenchymal cells. Tumor cells are subsequently dislocated from the primary site and invaded the surrounding tissues. These invaded tumor cells are still functionally proliferated in the surrounding tissues and further damage the healthy tissues³⁰.

Epithelial-mesenchymal transition (EMT) is a hallmark of tumor cell invasion, which is manifested as cell polarity alteration, cell morphology transformation, and enhanced motility. Both reduced adhesion ability and elevated cell invasiveness are beneficial to cell exfoliation from the

primary tumor tissues. Subsequently, destroyed or degraded integrity of the extracellular matrix leads to invasion of detached tumor cells into the surrounding healthy tissues³¹. Moreover, adherents junction³², focal adhesion³³, and regulation of cytoskeletal proteins³⁴ are all involved in the biological processes of invasion and migration of tumors. Therefore, inhibition of tumor invasion can remarkably improve prognosis of tumor patients. In this study, our results showed that microRNA-337-5p mainly regulates pathways that are related to tumor invasion and metastasis.

The proto-oncogene ERBB2 is widely expressed during embryonic development and is down-regulated in adult normal tissues³⁵. ERBB2 receptor is a member of the tyrosine kinase

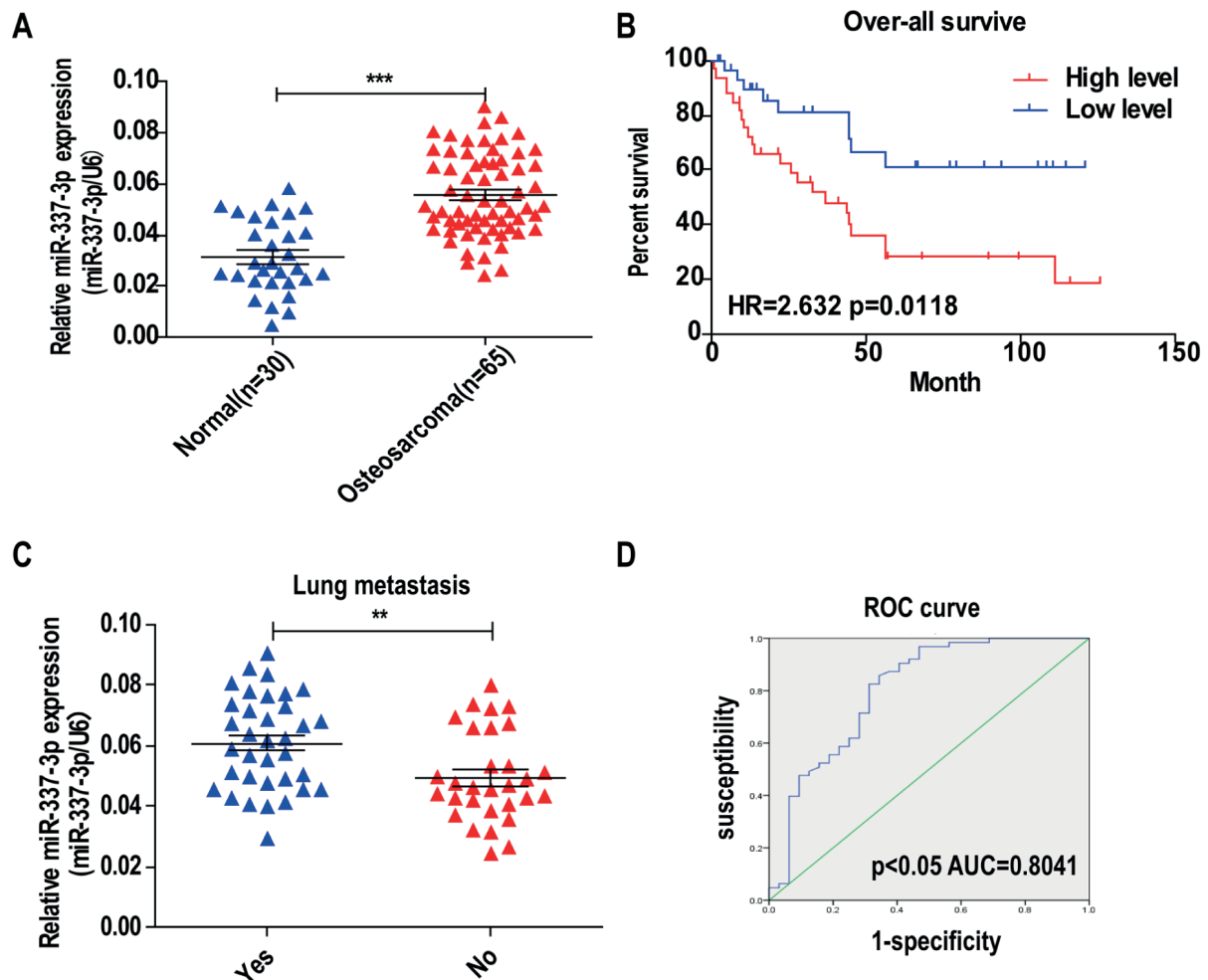


Figure 3. MicroRNA-337-5p was overexpressed in OS tissues. **A**, Higher microRNA-337-5p expression was found in 65 OS patients than that of 30 normal controls. **B**, Lower overall survival was found in OS patients with higher microRNA-337-5p expression than those with lower expression. **C**, Higher microRNA-337-5p expression was found in OS patients with pulmonary metastasis than those without metastasis. **D**, ROC curve of microRNA-337-5p expression and diagnostic value.

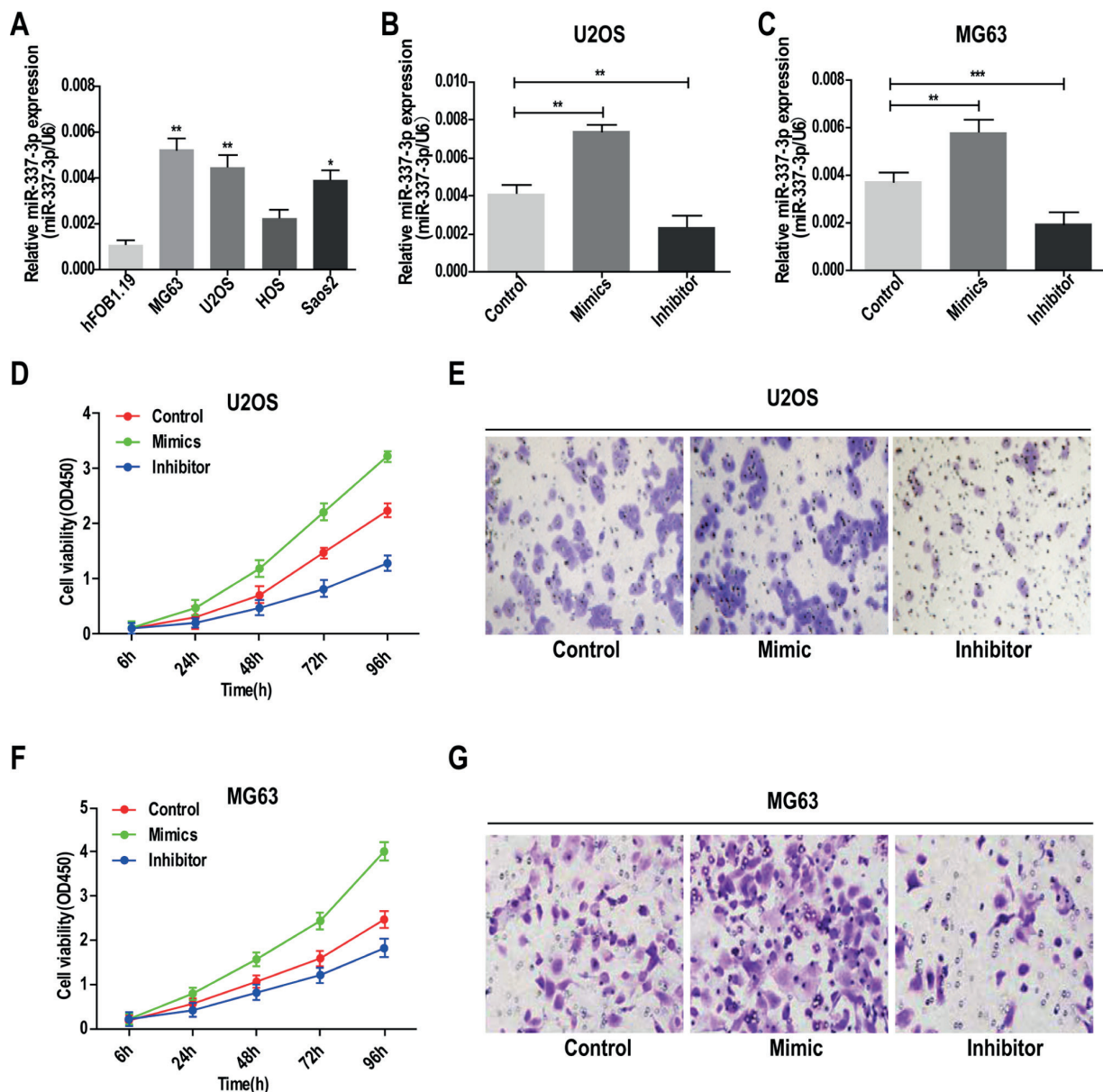


Figure 4. Overexpressed microRNA-337-5p promoted proliferation of OS cells. **A**, Expression levels of microRNA-337-5p in hFOB 1.19, MG63, U2OS, HOS, and Saos2. **B-C**, Transfection efficacy in U2OS (**B**) and MG63 (**C**) cells. **D**, Cell viability in U2OS cells after overexpression or knockdown of microRNA-337-5p. **E**, Cell invasion in U2OS cells after overexpression or knockdown of microRNA-337-5p. **F-G**, Cell viability in MG63 cells after overexpression or knockdown of microRNA-337-5p.

receptor superfamily, which exerts a crucial regulatory role in the development of most tumors. Overexpressed key factors in ERBB2 pathway are significant mediators of tumorigenesis. ERBB2 stimulates cell proliferation and even malignant transformation *via* formation of heterodimers³⁶. Slamon et al³⁷ first discovered ERBB2 amplification in breast cancer. ERBB2 participates in the development of various ma-

lignant tumors, such as OS and gastric cancer. Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase (MAPK) family. Functionally, ERK controls many biological responses such as cell proliferation and differentiation, cell morphology maintenance, apoptosis, and cell malignancy³⁸. ERK pathway has different effects on different types of cells. On the one hand, ERK pathway

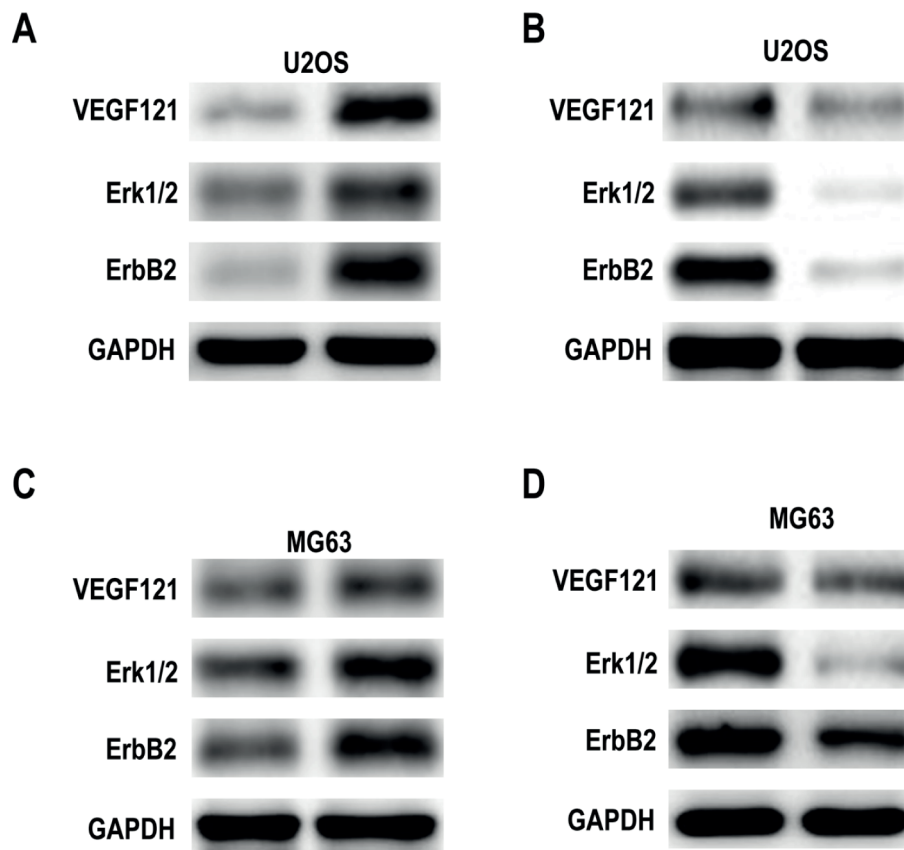


Figure 5. MicroRNA-337-5p participated in the development and progression of OS *via* ERBB, MAPK, and VEGF pathways. **A, C,** Protein expressions of VEGF121, Erk1/2, and ERBB2 in OS cells after microRNA-337-5p overexpression. **B, D,** Protein expressions of VEGF121, Erk1/2, and ERBB2 in OS cells after microRNA-337-5p knockdown.

can be strongly and persistently activated by cell growth factors such as EGF and RDGF. On the other hand, ERK activation can remarkably inhibit cell growth, such as smooth muscle cells³⁹.

VEGF (vascular endothelial growth factor) is the most significant proangiogenic factor discovered so far, and neovascularization is a necessary condition for tumor development and infiltration. A great number of studies⁴⁰⁻⁴² have suggested that VEGF overexpression is greatly involved in the development of multiple tumors⁴⁰. VEGF receptor consists of an intracellular domain containing a receptor tyrosine kinase, a transmembrane domain, and an extracellular domain containing seven immunoglobulin-like domains. VEGF not only promotes the formation of new blood vessels, but also increases vascular permeability, which is conducive to the hematogenous metastasis^{41,42}. In

this study, overexpressed microRNA-337-5p in OS cells resulted in the elevated proliferative and invasive abilities. Besides, ERBB, MAPK and VEGF pathways were all activated after microRNA-337-5p overexpression.

Conclusions

We showed that microRNA-337-5p is upregulated in OS tissues, which is an independent prognostic factor in OS. Overexpressed microRNA-337-5p can promote proliferative and invasive abilities of OS cells *via* activating ERBB, MAPK, and VEGF pathways.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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