Downregulation of microRNA-605 indicates poor prognosis and promotes the progression of osteosarcoma

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Received June 3, 2020; Accepted September 16, 2020

DOI: 10.3892/ol.2020.12233

Abstract. Osteosarcoma (OS) is a type of primary bone tumor, which is one of the leading causes of cancer-related death. MicroRNA (miR)-605 has been demonstrated to act as a prognostic biomarker and therapeutic target in various cancers, such as breast cancer and non-small cell lung cancer, but its function in OS remains unclear. The aim of the present study was to investigate the prognostic value of miR-605 in patients with OS by evaluating its expression levels and to explore the biological function of miR-605 in OS progression. For this purpose, tumor tissues and adjacent normal tissues were collected from OS patients, and the expression of miR-605 in the collected tissues and OS MG63, U2OS, HOS, and SAOS-2 cell lines was detected by quantitative real-time PCR. The prognostic value of miR-605 was evaluated by Kaplan-Meier survival curves and Cox regression analysis. The effects of miR-605 on OS cell proliferation, migration and invasion were analyzed by the CCK-8 and transwell assays, respectively. The results of the present study revealed that miR-605 was significantly downregulated in OS tissues compared with adjacent normal tissues, which was associated with the clinical stage and distant metastasis of patients. Additionally, the downregulation of miR-605 predicted the poor prognosis of patients with OS and served as an independent prognostic indicator. The downregulation of miR-605 enhanced cell proliferation, migration, and invasion of OS cells, which suggested that miR-605 may be involved in the progression of OS. The findings of the present study provide a new therapeutic target for the treatment of patients with OS.

Introduction

Osteosarcoma (OS) is an aggressive tumor of the bone that has a high level of chromosomal instability and very complex karyotypes (1,2). Although extensive studies have been conducted on OS, the prognosis of OS remains unsatisfactory and OS remains a leading cause of tumor-related death (3,4). Statistical data between 1994 and 2013 show that the word-wide 5-year survial rate of OS patients wihout metastasis is ~70%, but this rate in the patients with metastasis is <20% (5). In recent decades, the 5-year overall survival rate of OS has improved significantly and a number of patients have died from local relapse or distant metastasis (6). Therefore, identifying a sensitive and effective biomarker that is involved in the progression of OS can help explore novel insights into the treatment of OS. Currently, the role of microRNAs (miRNAs), especially dysregulated miRNAs in the prognosis or diagnosis of tumors or cancers has attracted growing attention (7).

miRNAs are small endogenous non-coding RNAs with lengths of ~18-21 nucleotides (8). Previously, miRNAs have been demonstrated to regulate the expression of genes and mRNAs and the differentiation and apoptosis of cells by binding to the 3' untranslated regions of their targets (9,10). An increasing amount of evidence has revealed that miRNAs have functions in various cancers and tumors, such as serving as tumor suppressors or promoters. For example, miR-1285-5p acts as a tumor suppressor in breast cancer as it can inhibit the cell proliferation of breast cancer cells (11). In gastric cancer, miR-19a serves as a tumor promoter that facilitates cell growth and tumorigenesis (12). Previous studies also have investigated a variety of miRNAs that were associated with the progression of OS (13-15). miR-21 can inhibit the progression of OS by targeting reversion inducing cysteine rich protein with Kazal motifs, which regulates the Wnt/β-catenin signaling pathway (16). miR-206 is upregulated in patients with OS compared to normal controls and induces the proliferation and migration of OS cells (17).

miR-605 has been reported to serve roles in non-small cell lung cancer, as cell proliferation and metastasis are inhibited by the downregulation of miR-605 (18). In prostate cancer, miR-605 is downregulated, which promotes the proliferation and invasion of prostate cancer by targeting engrailed-2 (EN2) (19). As miR-605 is downregulated in OS, it may be involved in the progression of OS (20). In the present study, the expression of miR-605 was investigated in OS tissues and cells using reverse transcription-quantitative (RT-q) PCR. The present study aimed to investigate the function of miR-605 in the progression and prognosis of OS to determine the potential therapeutic and prognostic value of miR-605 in OS.

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Key words: osteosarcoma, microRNA-605, proliferation, migration, invasion, prognosis

Materials and methods

Patients and tissue collection. A total of a 110 patients with OS that underwent resection surgery in Weifang People's Hospital (Weifang, China) from April 2009 to August 2014 were recruited in the present study. OS tissues and adjacent normal tissues (5 cm from the edge of the tumor) were collected during the surgery and immediately frozen in liquid nitrogen at -80˚C for the following experiments. The inclusion criteria of patients were as follows: i) None of the patients had received any antitumor therapy, such as radiotherapy or chemotherapy; ii) patients had complete clinicopathological data. The exclusion criteria were as follows: i) Patients with severe organ dysfunction and injury; ii) patients who were not willing to partipate in this study. The clinicopathological features of the patients were summarized in Table I. There were 62 males and 48 females in the OS patients, and the average age of patients was 19.84±12.69 years. The clinical stage of the tumor tissues was determined using the criteria by the Musculoskeletal Tumor Society system (21), and 72 patients had I-IIA stage tumors, whereas 38 patients had IIB-III stage tumors. A 5-year follow-up survey was performed by telephone to obtain the survival information of patients. The present study was approved by the Ethics Committee of Weifang People's Hospital (Weifang, China) with the approval no. WFRM01-082009, and all the patients signed informed consent for tissue use and analysis.

Cell lines and cell transfection. OS cell lines (MG63, U2OS, HOS, and SAOS-2) and the normal cell line hFOB 1.19 were purchased from the American Type Culture Collection. hFOB 1.19 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM)/F-12 (1:1; Invitrogen; Thermo Fisher Scientific, Inc.) and osteosarcoma cells in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.). All cells were maintained at 37˚C in a humidified incubator with 5% CO_2 in media supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 1% of 100 U/ml penicillin and streptomycin (both Invitrogen; Thermo Fisher Scientific, Inc.) and 2 mM L-glutamine (Gibco, Thermo Fisher Scientific, Inc.).

Cells were transfected with miR-605 mimic (50 nM), miR-605 inhibitor (100 nM), mimic negative control (mimic NC; 50 nM) and inhibitor negative control (inhibitor NC; 100 nM), in order to regulate the expression of miR-605. The sequences for cell transfection were as follows: miR-605 mimic 5'-UAAAUCCCAUGGUGCCUUCUCCU-3'; miR-605 inhibitor 5'-AGGAGAAGGCACCAUGGGAUUUA-3'; mimic NC 5'-UUCUCCGAACGUGUCACGU-3'; and inhibitor NC 5'-CAGUACUUUUGUGUAGUACAA-3'. All the sequences were synthesized by Shanghai GenePharma Co, Ltd. Cell transfection was performed using Lipofectamine 2000® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 6 h according to the manufacturer's instructions. After that, the culture medium was replaced, and the subsequent experiments were carried out at 48 h post-transfection.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the collected tisues and OS cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and miRNeasy Mini kit (Qiagen, Inc.) according to the manufacturer's instructions. Then cDNA was synthesized using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the following temperature protocol: 42˚C for 30 min and 85˚C for 5 sec. RT-qPCR was performed using the SYBR-Green I Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) with U6 as the internal control. The thermocycling conditions were as follows: 95˚C for 10 min, 95˚C for 20 sec, 60˚C for 15 sec, 72˚C for 20 sec, a total of 40 cycles. Following were the sequences of primers: miR-605 forward 5'-GCCGAGTAA ATCCCATGGTG-3', and reverse 5'-CTCAACTGGTGTCGT GGA-3'; U6 forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'. The $2^{-\Delta\Delta Cq}$ method (22) was used to calculate the relative quantitation of miRNA expression.

Cell counting kit-8 (CCK-8) assay. Cell proliferation of OS was assessed by the CCK-8 assay. Briefly, MG63 and U2OS cells with a density of $5x10^3$ cells/well were seeded into 96-well plates and incubated for 0, 24, 48 and 72 h at 37˚C. After the incubation, 10 μl of CCK-8 reagent (Sigma-Aldrich; Merck KGaA) was added to each well for 4 h at 37˚C with 5% CO_2. The absorbance at 450 nm was measured to evaluate the proliferation of OS cells.

Transwell assay. Transwell insert chambers (Corning, Inc.) with 8-μm pores were used to evaluate the migration and invasion of MG63 and U2OS cells. A concentration of $1x10^4$ cell/well cells were seeded into the upper chambers with serum-free DMEM and DMEM with 10% FBS was contained in the lower chamber as chemoattractant. Following 24 h of incubation at 37˚C, cells migrated to the lower chamber were fixed in 70% ethanol for 30 min and stained with 0.2% crystal violet at room temperature for 10 min. For invasion assay, the upper chambers were pre-coated with Matrigel (BD Biosciences) for 6 h at 37˚C. Stained cells were counted under a light microscope using a x200 magnification.

Statistical analysis. All data was collected from at least 3 independent replicates and were represented as mean value ± standard deviation (SD). Data analysis was performed using SPSS 20.0 software (IBM Corp.) and GraphPad Prism 5.0 software (GraphPad Software, Inc.). Differences between two groups were analyzed using the paired Student's t-test and multiple groups by one-way ANOVA followed by the post hoc Tukey's test. The association of miR-605 with the clinicopathological features of the patients was assessed using χ^2 test. Kaplan-Meier analysis with the log-rank test and multivariate Cox regression analysis were used to assess the prognostic value of miR-605. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-605 expression is downregulated in OS tissues and cell lines compared with normal tissues and cells. miR-605 expression in tissues from patients with OS and cell lines was detected by RT-qPCR. miR-605 was significantly downregulated in OS tissues compared with adjacent normal tissues ($P<0.001$; Fig. 1A). Similarly, in OS cell lines, the

Table I. Association between miR-605 expression and clinical features in patients with OS.

Parameters	No. of cases (n=110)	miR-605 low (n=60)	miR-605 high (n=50)	P-value
Sex				0.483
Male	62	32	30	
Female	48	28	20	
Age, years				0.423
<20	57	29	28	
≥20	53	31	22	
Anatomical location				0.175
Tibia/femur	67	40	27	
Elsewhere	43	20	23	
Tumor size, cm				0.117
<8	57	27	30	
≥8	53	33	20	
Clinical stage				0.012
I-IIA	72	33	39	
IIB-III	38	27	11	
Distant metatasis				0.006
Negative	78	36	42	
Positive	32	24	8	

OS, osteosarcoma.

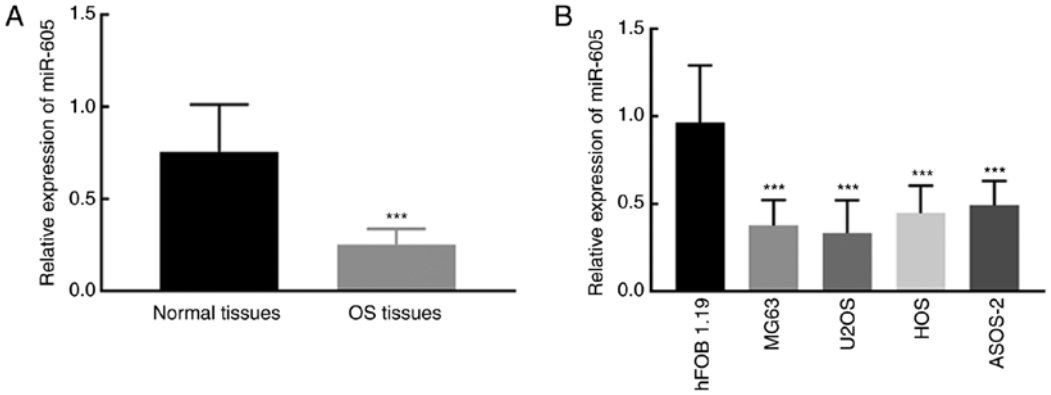

Figure 1. miR-605 expression measured by reverse transcription-quantitative PCR in OS tissues and cell lines. (A) miR-605 was significantly downregulated in OS tissues (n=110) compared with adjacent normal tissues. (B) miR-605 was significantly downregulated in OS cells (MG63, U2OS, HOS and ASOS-2) compared with a normal cell line (hFOB 1.19). ***P<0.001. OS, osteosarcoma; miR, microRNA.

expression of miR-605 was significantly lower compared with that in the normal cell line hFOB 1.19 (P<0.001; Fig. 1B). Additionally, MG63 and U2OS were used for subsequent cell experimentation due to their relatively lower miR-605 expression among the four OS cell lines.

Relationship between miR-605 expression clinical features of patients with OS. Mean value of miR-605 mRNA expression (0.253) in tissues from patients with OS was used to divided 110 patients into the high miR-605 expression group (n=50) and low miR-605 expression group (n=60). The expression of miR-605 demonstrated significant association with the clinical stage (P=0.012) and distant metastasis (P=0.006) of patients with OS, while the age, sex, anatomical location and tumor size showed no significant relationship with miR-605 expression (P>0.05; Table I).

Prognostic value of miR-605. Kaplan-Meier curves demonstrated that patients with low miR-605 expression had a lower overall survival rate compared with patients with higher miR-605 expression (log rank P=0.016; Fig. 2). In addition, Cox regression analysis was used to evaluate the prognostic value of miR-605 and the clinical features of patients with OS. miR-605 expression was significantly associated with the overall survival rate of patients with OS (Fig. 2). In addition, the multivariate Cox regression analysis results indicated that

Table II. Multivariate Cox regression analysis of clinical features and miR-605 in patients with OS.

Indicators	HR	95% CI	P-value
miR-605	2.533	1.150-6.107	0.029
Age	1.187	0.582-2.420	0.637
Sex	1.247	0.601-2.587	0.553
Anatomical location	1.340	0.603-2.979	0.473
Tumor size	1.787	0.812-3.932	0.149
Clinical stage	1.896	1.055-3.885	0.040
Distant metastasis	2.015	1.094-4.123	0.036

miR, microRNA; HR, hazard ratio; CI, confidence interval; OS, osteosarcoma.

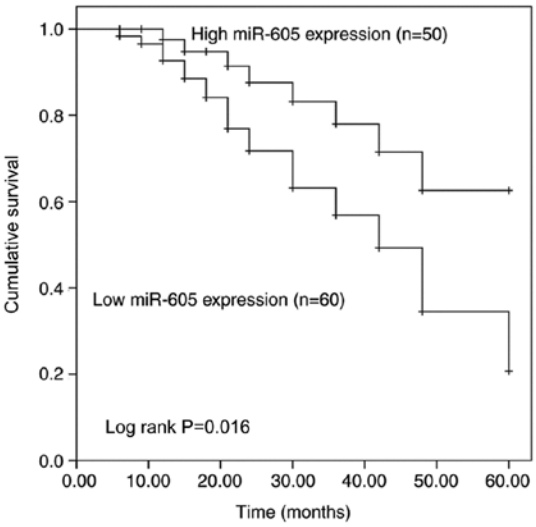

Figure 2. Kaplan-Meier overall survival curve of patients with high or low miR-605 expression. miR, microRNA.

miR-605 served as an independent factor in the prognosis of OS with the hazard ratio (HR)=2.533; 95% confidence interval (CI)=1.150-6.107; P= 0.029; Table II).

Downregulation of miR-605 promotes cell proliferation in OS cells. MG63 and U2OS cells were transfected with miR-605 mimic or miR-605 inhibitor to overexpress or downregulate the expression of miR-605. RT-qPCR results demonstrated that miR-605 was significantly upregulated after transfection with miR-605 mimic, while the transfection with miR-605 inhibitor significantly downregulated the expression of miR-605 in both MG63 and U2OS cells (P<0.001; Fig. 3A). The proliferation of transfected cells was assessed using a CCK-8 assay. It was found that the downregulation of miR-605 significantly promoted the proliferation of MG63 and U2OS cells, while the upregulation of miR-605 significantly inhibited the proliferation of MG63 and U2OS cells (P<0.05; Fig. 3B).

Effect of miR-605 on OS cell migration and invasion. Transwell assays were used to evaluate the migration and invasion abilities of MG63 and U2OS cells. The migration ability of MG63 and U2OS cells was significantly inhibited by the overexpression of miR-605 but promoted by the downregulation of miR-605 (P<0.001; Fig. 4A). For cell invasion, the knockdown of miR-605 significantly enhanced the invasion of MG63 and U2OS cells, while the overexpression of miR-605 inhibited OS cell invasion (P<0.001; Fig. 4B).

Discussion

With the development of molecular biology, an increasing number of biomarkers, such as miRNAs, that play roles in the progression of cancers and tumors have been identified, and the therapy of different cancers, including OS, has improved rapidly (23-25). miRNAs have been reported to play vital biomarker roles in various cancers, such as lung cancer, ovarian cancer, breast cancer, and gastric cancer (11,26-29). For example, miR-593 can inhibit the migration and invasion of non-small cell lung cancer cells by targeting SLUG-associated signaling pathways (30). miR-486 can promote the cell proliferation and mobility of renal cell carcinoma and inhibit its apoptosis (31). Several miRNAs have also been identified as effective biomarkers for the prognosis and progression of OS (32,33). miR-1225-5p serves as a tumor suppressor in OS and inhibits the proliferation, migration and invasion of OS cells by targeting Sox9 (32). miR-384 is downregulated in OS tissues and cells compared to normal controls and upregulates stem loop binding protein and promotes the growth and metastasis of OS (33). A previous study reported the downregulation of miR-605 in OS, which indicates that miR-605 may play a role in the progression of OS (20).

The present study demonstrated that miR-605 was significantly downregulated in OS tissues and cell lines compared with adjacent normal tissues and cells. Additionally, miR-605 expression was found to be associated with the clinical stage and distant metastasis of patients with OS in the present study. In terms of the prognosis of OS, patients with high miR-605 expression demonstrated an improved prognosis compared with patients with low miR-605 expression. In the present study, miR-605 was found to be an independent prognostic indicator of OS based on the results of Cox regression analysis. These findings of the present study revealed the potential prognostic biomarker role of miR-605 in OS, which is consistent with a previous study (12).

miR-605 is reported to play roles in the progression of numerous other cancers and tumors, where miR-605 is dysregulated and affects the proliferation, migration, invasion and apoptosis of tumor cells (34-36). For example, miR-605 inhibits the progression of intrahepatic cholangiocarcinoma by suppressing gankyrin (34). miR-605 also functions as a tumor suppressor in melanoma by regulating the expression of inositol polyphosphate-4-phosphatase type II B (35). In addition, miR-605 was also demonstrated to affect the proliferation, migration and invasion of non-small cell lung cancer, prostate cancer, and breast cancer (18,19,36). In the present study, miR-605 was found to be a vital factor in the progression of OS, as the downregulation of miR-605 significantly promoted cell proliferation, migration, and invasion of OS, which indicates that miR-605 may be involved in the progression and development of OS.

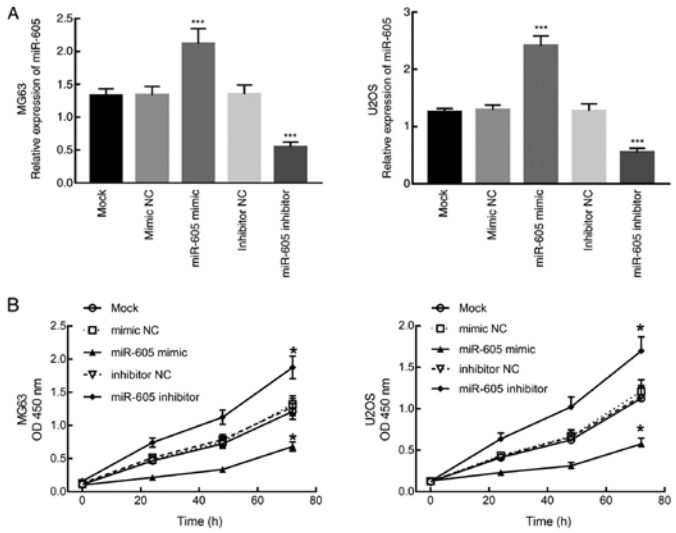

Figure 3. Effect of miR-605 expression on the proliferation of MG63 and U2OS cells. (A) miR-605 was significantly upregulated in MG63 and U2OS transfected with miR-605 mimic and downregulated in MG63 and U2OS cells transfected with miR-605 inhibitor. ***P< 0.001. (B) The proliferation of MG63 and U2OS cells was significantly promoted by the downregulation of miR-605 and inhibited by the upregulation of miR-605. *P<0.05. NC, negative control; miR, microRNA; OD, optical density; Mock, cells that only received transfection reagent.

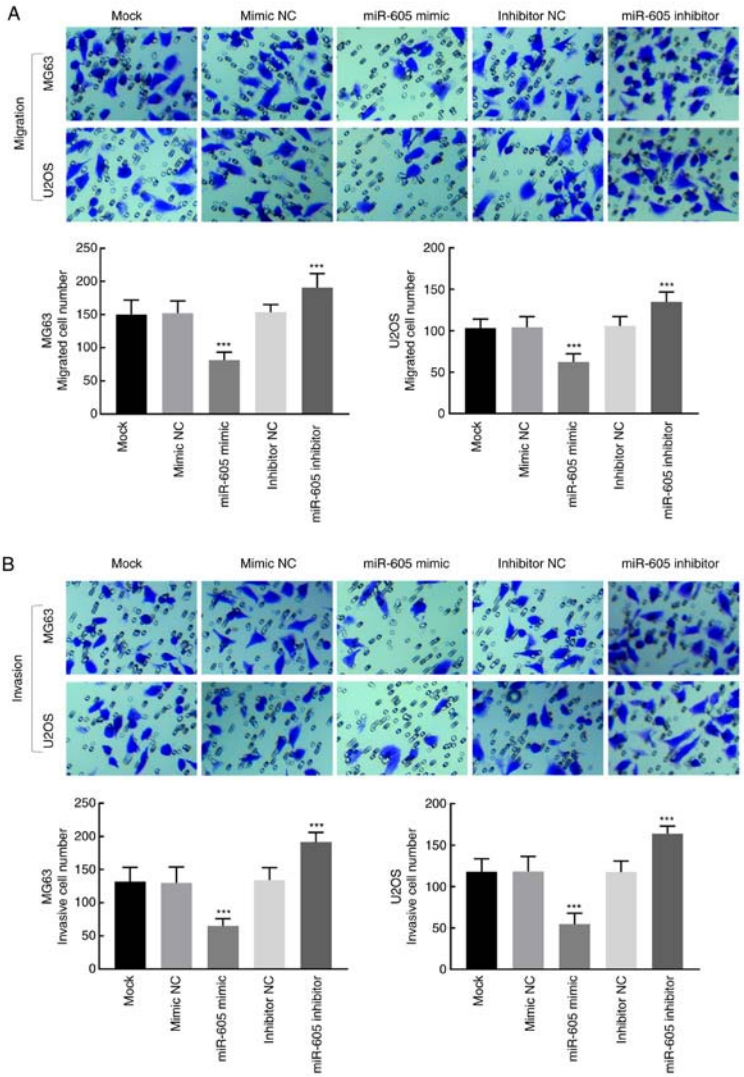

Figure 4. Effects of miR-605 expression on the migration and invasion of MG63 and U2OS cells. (A) Migration of MG63 and U2OS cells was significantly promoted by the downregulation of miR-605 and inhibited by the upregulation of miR-605. (B) Invasion of MG63 and U2OS was significantly promoted by the downregulation of miR-605 and inhibited by the upregulation of miR-605. ***P<0.001. NC, negative control; miR, microRNA; Mock, cells that only received transfection reagent.

In the present study, the expression and function of miR-605 were investigated in OS tissues and cells in vitro, however, the role of miR-605 in the progression and prognosis of OS should be verified in in vivo experiments. On the other hand, previous studies have provided some potential targets of miR-605, such as tumor necrosis factor α-induced protein 3, EN2, enhancer of zeste 2 (EZH2) and forkhead Box P1, which mediate the functional effects of miR-605 in human malignancies (18,19,37,38). EZH2 is a widely investigated oncogene in several human malignancies, such as non-small cell lung cancer and bladder cancer (39,40). In OS tissues, EZH2 has been found to be upregulated and associated with aggressive tumor behavior and poor prognosis and the knockdown of EZH2 could significantly inhibit OS cell proliferation, migration and invasion (31). Thus, the functional role of miR-605 in OS progression may also be investigated by targeting EZH2. To corroborate this deduction, further mechanical studies and *in vivo* experiments are needed.

In conclusion, miR-605 is downregulated in OS and this dysregulation is associated with clinical stage and distant metastasis in patients with OS. The downregulation of miR-605 indicates a poor prognosis in terms of OS, which shows that miR-605 serves as an independent prognostic indicator for OS. In addition, the downregulation of miR-605 promotes OS cell proliferation, migration, and invasion, which suggests that miR-605 may be involved in the progression of OS. The present study provides a novel insight into the prognosis and progression of OS and provides new therapeutic targets for the treatment of patients with OS.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and material

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

Authors' contributions

XY conducted the clinical study, analyzed clinical data and wrote the manuscript. CL designed this research and conducted the cell experiments. Both authors have read and approved this manuscript.

Ethics approval and consent to participate

The research was approved by the Ethics Committee of Weifang People's Hospital (Weifang, China) with the approval no. WFRM01-082009, and the participants provided signed informed consent for tissue collection and analysis.

Consent for publication

Consent for publication was obtained from the patients.

Competing interests

The authors declare that they have no competing interests.

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