MicroRNA-615 functions as a tumor suppressor in osteosarcoma through the suppression of HK2

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Abstract. At present, the regulatory mechanisms of various microRNAs (miRNAs/miRs) have been elucidated in human cancers including osteosarcoma (OS). This study mainly focused on the role of miR-615 in OS, which has not yet been reported. Ninety-two OS tissues and normal samples were used in this study. Human osteoblast hFOB1.19 cells and OS cell line HOS were utilized to detect the expression of miR-615. The expression of miR-615 and gene expression were assessed by RT-qPCR and western blot analysis. Transwell, MTT and luciferase reporter assays were used to investigate the regulatory mechanism of miR-615 in OS. The results revealed that miR-615 expression was reduced in OS tissues and cells, and was associated with poor clinical outcomes and prognosis in OS patients. In addition, overexpression of miR-615 restrained cell viability and metastasis in OS. Furthermore, hexokinase 2 (HK2) was confirmed as a direct target of miR-615. Upregulation of HK2 was detected in OS tissues. The upregulation of HK2 weakened the tumor-suppressive effect of miR-615 in OS. Moreover, miR-615 blocked epithelial-mesenchymal transition (EMT) and inactivated the PI3K/AKT pathway in OS. To conclude, miR-615 acts as a tumor suppressor in OS, thus miR-615 can be used as a target for OS treatment.

Introduction

Osteosarcoma (OS) is a malignant tumor that originates in bones, especially in children and adolescents. In addition, OS accounts for about 5% of all childhood tumors (1). Due to the high malignancy of OS and poor prognosis, patients may develop lung metastases within a few months or weeks. The 3- to 5-year survival rate of OS patients with amputation is usually between 5 and 20% (2). The key factors affecting the prognosis of OS are early diagnosis, complete tumor resection, chemotherapy and radiotherapy before and after surgery (3). In addition, it is also related to the tissue type, size and local lymph node status of the tumor cells (4). Although existing treatment methods have greatly improved the survival rate of OS patients, new and effective treatment methods are still needed.

MicroRNAs (miRNAs/miRs) are highly conserved endogenous RNAs, which play important regulatory roles in human cancers (5). It has been found that miRNAs are involved in biological activity through a complex regulatory network. This complex regulatory network can regulate multiple genes involved in cellular activities through an miRNA, and can also finely regulate a gene through the interaction of multiple miRNAs (6). In particular, it has been found that miRNAs can exhibit regulatory effect on bone cancers (7). For example, miR-214 was found to function as an oncogene in human OS by targeting TRAF3 (8). In contrast, miR-876-5p inhibited cell viability and metastasis in OS by targeting c-Met (9). Recently, the different roles of miR-615 in human cancers have attracted our attention. Upregulation of miR-615 has been found in gastric cancer. Moreover, overexpression of miR-615 was found to promote cell proliferation and migration and inhibit apoptosis in gastric cancer (10). However, downregulation of miR-615 was found in non-small cell lung cancer and renal cell carcinoma (11,12). In addition, miR-615 was found to play an inhibitory role in esophageal squamous cell carcinoma by targeting IGF2 (13). These findings indicate that miR-615 has a tissue-specific function in different types of cancer. At present, the specific role of miR-615 in OS is unclear and needs to be investigated.

As an important regulator, hexokinase 2 (HK2) has been found to be involved in various human cancers, such as laryngeal carcinoma and liver cancer (14,15). In detail, upregulation of HK2 was detected in hepatocellular carcinoma and breast cancer (16,17). Functionally, knockdown of HK2 was found to inhibit the growth of lung carcinoma A549 cells (18). In addition, it was found that upregulation of HK2 promoted the proliferation of ovarian cancer cells (19). Lu *et al* demonstrated that miR-603 inhibited the malignancy of ovarian cancer cells by targeting HK2 (20). However, the relationship between HK2 and miR-615 has not been reported in previous studies.

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In addition, the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/HK2 axis has been proposed to regulate the development of breast cancer (21). It has been suggested that the PI3K/AKT pathway is involved in the regulation of tumorigenesis, such as renal cancer and esophageal squamous cell carcinoma (22,23). Xu *et al* demonstrated that miR-149 inhibited cell growth by regulating the PI3K/AKT pathway in human OS (24). Therefore, the effect of miR-615 on HK2 expression and the PI3K/AKT pathway was also explored in OS. More importantly, the effect of miR-615 on OS cell viability and metastasis was investigated in this study. The present study facilitates the understanding of the pathogenesis of OS.

Materials and methods

Clinical tissues. A total of 92 paired OS tissues were collected from OS patients (60 males and 32 females, mean age 21 years, range from 14 to 33 years) who had undergone resection between January 2017 and January 2019 at the Shandong Provincial Third Hospital. Moreover, All OS patients underwent surgery, and none of them received preoperative radiotherapy or chemotherapy. All of the patients provided informed consents. Approval for this research was acquired from the Institutional Ethics Committee of Shandong Provincial Third Hospital (2016SPT-44).

Cell culture. Human osteoblast hFOB1.19 cells (CRL-11372) and OS cell line HOS (CRL-1543) were purchased from the American Type Culture Collection (ATCC). The cells were incubated in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin (all from HyClone; GE Healthcare). The cells were cultured at 37˚C in a humidified incubator containing 5% CO_2.

Cell transfection. miR-615 mimics (5'-UCCGAGCCUGGG UCUCCCUCUU-3'), mimics-NC (5'-UUCUCGAACGUG UCACGUUUU-3'), miR-615 inhibitor (5'-ACCGAGUCAGGG AUACCCACAA-3'), inhibitor-NC (5'-CAGUACUUUUGU GUAGUACAA-3') and the HK2 overexpression plasmid were obtained from GenePharma. These sequences and plasmid were transfected into HOS cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), respectively. For cell function assay, cells were collected 12 h after transfection. For RT-qPCR and western blot analysis, cells were collected 24 and 48 h after transfection. For the dual luciferase assay, cells were collected 48 h after transfection.

RNA isolation and RT-qPCR. Total RNA isolation was performed using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The miScript Reverse Transcription kit (Qiagen) was then used to obtain cDNA solution. RT-qPCR assay was performed using the miScript SYBR®-Green PCR kit (Qiagen) based on the manufacturer's instructions. In brief, 20 μl mixtures were heated at 95˚C for 3 min for enzyme activation, then the 20 μl reaction mixture was incubated as follows: 95˚C for 3 sec and 61˚C for 20 sec for 40 cycles. U6 or GAPDH was used as a control for miR-615 or HK2. The expression levels of miR-615 and HK2 were quantified using the $2^{-\Delta\Delta Cq}$ method (25). The forward primer for miR-615 was 5'-CTG CCTTTCACCTTGGAGAC-3', and the reverse primer was 5'-CGTTTCCTGGGGATGAGATA-3'. The internal control GAPDH was forward, 5'-CGGAGTCAACGGATTTGGTCG TAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAA GAC-3'. The primers for HK2 were 5'-CTTCTTCACGGA GCTCAACC-3 (forward) and 5'-AAGCCCTTTCTCCAT CTCCT-3' (reverse). The internal control was U6 (forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCT TCACGAATTTGCGT-3').

Transwell assay. For the invasion assay, Matrigel (BD Biosciences) was diluted 1:4 with serum-free DMEM and used to coat the Transwell inserts (pore size, 8-μm; EMD Millipore) to form a matrix barrier. For the migration assay, transfected cells were suspended in FBS-free DMEM containing 0.1% bovine serum albumin (BSA) (Bioworld Technology, Inc.). After 30 min, HOS cell suspension ($3x10^3$ cells/well) was added to the Transwell upper chamber, and RPMI-1640 medium (10% FBS) was added to a 24-well plate in the lower chamber. After 24 h, the cells that had migrated or invaded through the membrane were fixed with 95% ethyl alcohol for 15 min at room temperature and stained with 0.1% crystal violet for 10 min at room temperature. Observation and photographing were performed using a light microscope (magnification, x200).

MTT assay. First, the transfected HOS cells ($4x10^3$ cells/well) were prepared in a 96-well plate. Then, HOS cells were incubated for 24, 48, 72 or 96 h in fresh medium, respectively. After that, 10 μl of MTT solution was added to incubate the cells for 4 h. Next, the supernatant fractions were discarded, and 150 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve the crystals. The absorbance at 490 nm was examined using a spectrophotometric plate reader (Olympus Corp.).

Dual luciferase reporter assay. First, wild-type WT-HK2-3'UTR or mutant MUT-HK2-3'UTR was inserted into pmirGLO luciferase reporter vector (Promega Corp.). Next, a total of $4x10^5$ HOS cells were seeded per well into 6-well plates and co-transfected with either 50 nM miR-615-3p mimics or mimics-NC and 2 μg plasmid vector using Lipofectamine® 2000, according to the manufacturer's protocol. The cells were lysed and assayed for luciferase activity at 48 h post-transfection using a Dual-Luciferase Assay kit (cat. no. E1910, Promega Corp.). The firefly luciferase was used as a reference for normalization.

Western blot analysis. Protein samples were obtained using RIPA lysis buffer (Beyotime Institute of Biotechnology). Next, proteins were separated by 10% SDS-PAGE. A total of 30 μg protein samples were transferred to the PVDF membrane and were blocked with 5% skim milk. The protein samples were incubated with E-cadherin (rabbit monoclonal; dilution, 1:1,000; cat. no. ab1416; Abcam), N-cadherin (rabbit polyclonal; dilution, 1:1,000; cat. no. ab18203; Abcam), vimentin (rabbit monoclonal; dilution, 1:1,000; cat. no. ab217673; Abcam), PI3K (rabbit monoclonal; dilution, 1:1,000; cat. no. ab32089; Abcam), phosphorylated (p-)PI3K (rabbit monoclonal; dilution, 1:1,000; cat. no. ab154598; Abcam), AKT (rabbit polyclonal; dilution, 1:1,000; cat. no. ab8805; Abcam), p-AKT (rabbit monoclonal; dilution, 1:1,000; cat. no. ab81283; Abcam) and GAPDH (rabbit

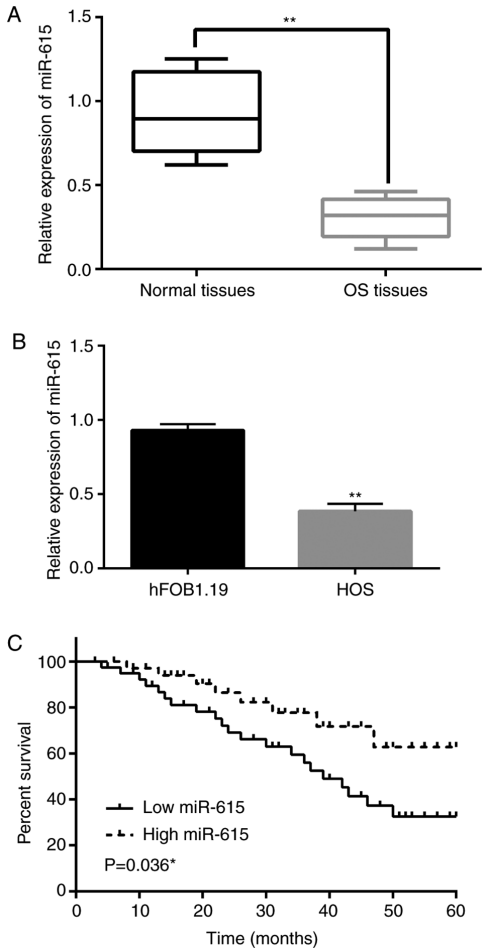

Figure 1. Downregulation of miR-615 is related to poor clinical outcome in OS. (A) miR-615 expression was examined in OS tissues and normal tissues by RT-qPCR. **P<0.01, OS tissues compared to normal tissues. (B) miR-615 expression was detected in human osteoblast hFOB1.19 cells and the OS cell line HOS. **P<0.01, compared with the hFOB1.19 cell line. (C) Low miR-615 expression was correlated with shorter overall survival in OS patients. OS, osteosarcoma.

monoclonal; dilution, 1:1,000; cat. no. ab181602; Abcam) primary antibodies overnight at 4˚C. After that, horseradish peroxidase-conjugated secondary antibodies (dilution, 1:5,000; cat. no. ab7090; Abcam) were added and the protein samples were incubated for 1 h. Protein bands were assessed using an ECL kit (Beyotime Institute of Biotechnology).

Statistical analysis. The data were analyzed by SPSS 17.0 (SPSS, Inc.) or Graphpad Prism 6 (GraphPad Software, Inc.). Data are shown as mean ± SD. Differences between groups were analyzed using Student's t-test or one-way analysis of variance with Tukey's post hoc test. The relationship between miR-615 expression and the clinic-pathological characteristics in OS patients was analyzed by Chi-square test. The univariate Kaplan-Meier method with log-rank test was used to calculate the overall survival rate and survival difference. P<0.05 was considered as indicative of statistical significance.

Results

Downregulation of miR-615 is related to poor clinical outcomes in OS patients. The expression of miR-615 was detected in OS tissues and cells by RT-qPCR. It was found that miR-615 expression in OS tissues was downregulated compared to that noted in the normal tissues (P<0.01; Fig. 1A). Similarly, the expression of miR-615 in HOS cells was lower than that in the hFOB1.19 cells (P<0.01; Fig. 1B). Next, we found that the low expression of miR-615 was associated with TNM stage and lymph node metastasis in OS patients (P<0.05; Table I). In addition, low miR-615 expression was associated with reduced overall survival of the OS patients (P=0.036; Fig 1C). These results indicate that miR-615 may be involved in the tumorigenesis of OS.

Table I. Association between miR-615 expression and the clinicopathological characteristics of the OS patients.

		miR-615		
Characteristics	Cases	High	Low	P-value
Age (years)				0.83
≥18	56	20	36	
<18	36	11	25	
Sex				0.27
Male	60	25	35	
Female	32	6	26	
Tumor size (cm)				0.08
<5	63	22	41	
≥5	29	9	20	
TNM stage				0.01[a]
I-II	71	26	45	
III-IV	21	5	16	
Lymph node metastasis				0.01[a]
No	75	26	49	
Yes	17	5	12	

Statistical analyses were performed by the χ^2 test. [a]P<0.05 is considered significant. OS, osteosarcoma.

miR-615 inhibits cell viability and metastasis in OS. Next, miR-615 mimics or inhibitor was transfected into HOS cells to explore the function of miR-615 in OS. RT-qPCR showed that the expression of miR-615 was increased by its mimics and reduced by its inhibitor in HOS cells (P<0.01; Fig. 2A). Functionally, overexpression of miR-615 inhibited cell proliferation in the HOS cells. In contrast, downregulation of miR-615 promoted the proliferation of HOS cells when compared to the NC group (P<0.05 or P<0.01; Fig. 2B). In addition, miR-615 mimics suppressed cell migration, while miR-615 inhibitor promoted cell migration in HOS cells when compared to the NC group (P<0.01; Fig. 2C). Consistently, cell invasion was also inhibited by miR-615 mimics and promoted by miR-615 inhibitor in HOS cells when compared to the NC group (P<0.01; Fig. 2D). These results indicate that miR-615 functions as a tumor suppressor in the pathogenesis of OS.

miR-615 directly targets HK2. In addition, TargetScan database (http://www.targetscan.org) indicated that miR-615 has a

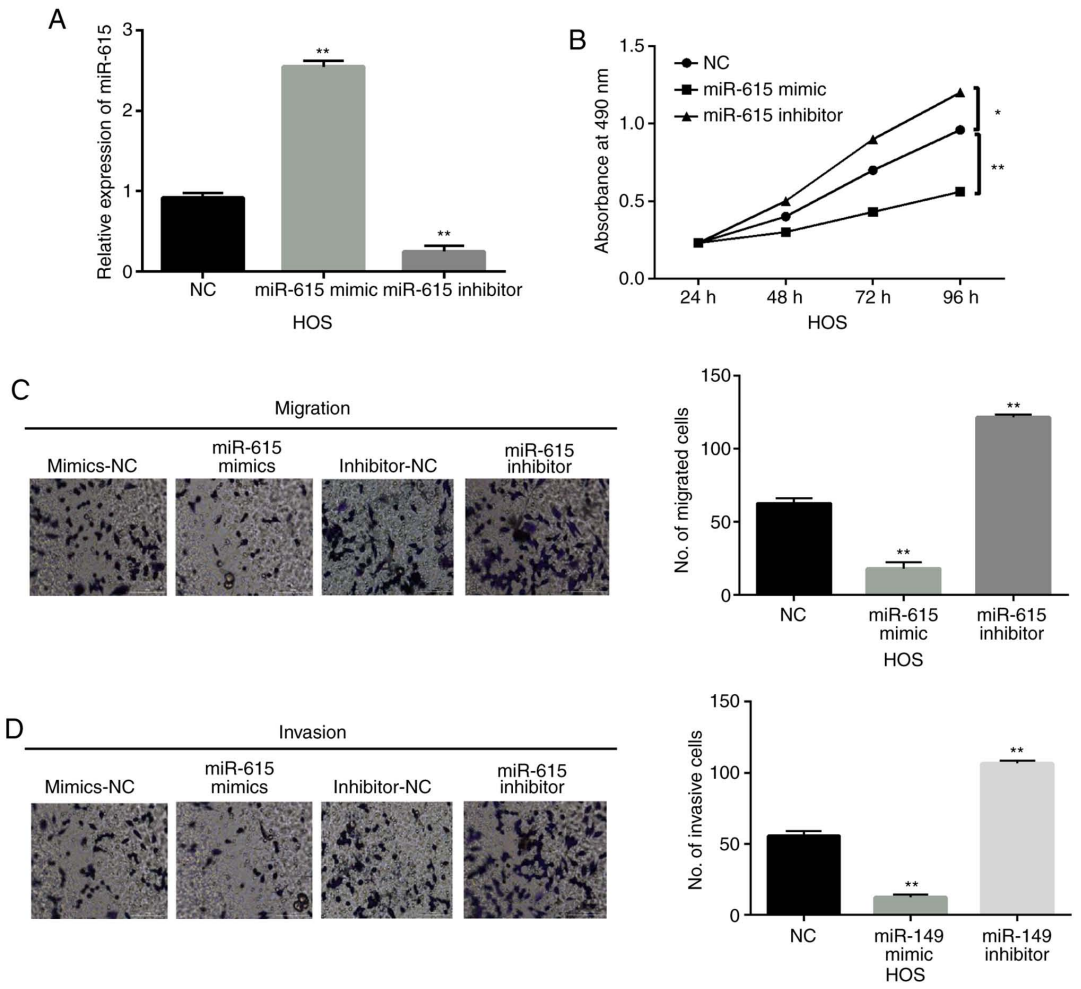

Figure 2. miR-615 inhibits cell viability and metastasis in OS. (A) miR-615 expression was detected by RT-qPCR in HOS cells transfected with the miR-615 mimic or inhibitor. **P<0.01, compared with the negative control (NC) group. (B) Cell proliferation, (C) migration and (D) invasion were assessed by MTT and Transwell assays in HOS cells following transfection with miR-615 mimics or inhibitor. *P<0.05, **P<0.01, compared with the NC group. OS, osteosarcoma.

binding site that binds to the 3'-UTR (3'-untranslated region) of HK2 (Fig. 3A). Next, this prediction was verified by luciferase reporter assay. We found that miR-615 mimics reduced the luciferase activity of WT-HK2 (P<0.01; Fig. 3B). However, the luciferase activity of Mut-HK2 was not affected by miR-615 mimics in the HOS cells. Then, HK2 expression levels were observed in HOS cells with miR-615 mimics or inhibitor. We found that HK2 expression was significantly reduced by miR-615 mimics and was significantly promoted by miR-615 inhibitor in HOS cells (P<0.01; Fig. 3C). Subsequently, the expression of HK2 was examined in OS tissues to explore the dysregulation of HK2 in the progression of OS. Compared to normal tissues, HK2 expression was significantly upregulated in the OS tissues (P<0.01; Fig. 3D). In addition, miR-615 was negatively correlated with HK2 expression in the OS tissues (P<0.0001, R^2=0.7319; Fig. 3E). Taken together, miR-615 directly targets HK2 and negatively regulates its expression in OS.

miR-615 is involved in OS progression through targeting HK2. To explore the interaction between miR-615 and HK2 in OS, HK2 overexpression vector was transfected into HOS cells with miR-615 mimics. First, the decreased expression of HK2 induced by miR-615 mimics was recovered by upregulation of HK2 in HOS cells (Fig. 4A). Similarly, overexpression of HK2 weakened the inhibitory effect of miR-615 on cell proliferation in HOS cells (P<0.01; Fig. 4B). Consistently, upregulation of HK2 also weakened the inhibitory effect of miR-615 on cell migration and invasion in HOS cells (P<0.01; Fig. 4C and D). These findings indicate that upregulation of HK2 impairs the tumor-suppressive effect of miR-615 in OS.

miR-615 blocks EMT and participates in the PI3K/AKT pathway in OS. Finally, the effect of miR-615 on EMT and the PI3K/AKT pathway in HOS cells was investigated to elucidate the molecular mechanisms of miR-615 in OS. The above results showed that miR-615 regulates cell migration and invasion in OS. Thus, we hypothesized that miR-615 may regulate cell metastasis by mediating EMT. N-cadherin, E-cadherin and vimentin are EMT-associated proteins. We investigated whether miR-615 regulates EMT by detecting the effect of miR-615 on N-cadherin, E-cadherin and vimentin expression. First, overexpression of miR-615 was found to suppress N-cadherin and vimentin expression in the HOS cells. In contrast, downregulation of miR-615 promoted N-cadherin and vimentin expression (Fig. 5). In addition, E-cadherin expression was promoted by miR-615 overexpression and reduced by miR-615 downregulation in HOS cells (Fig. 5).

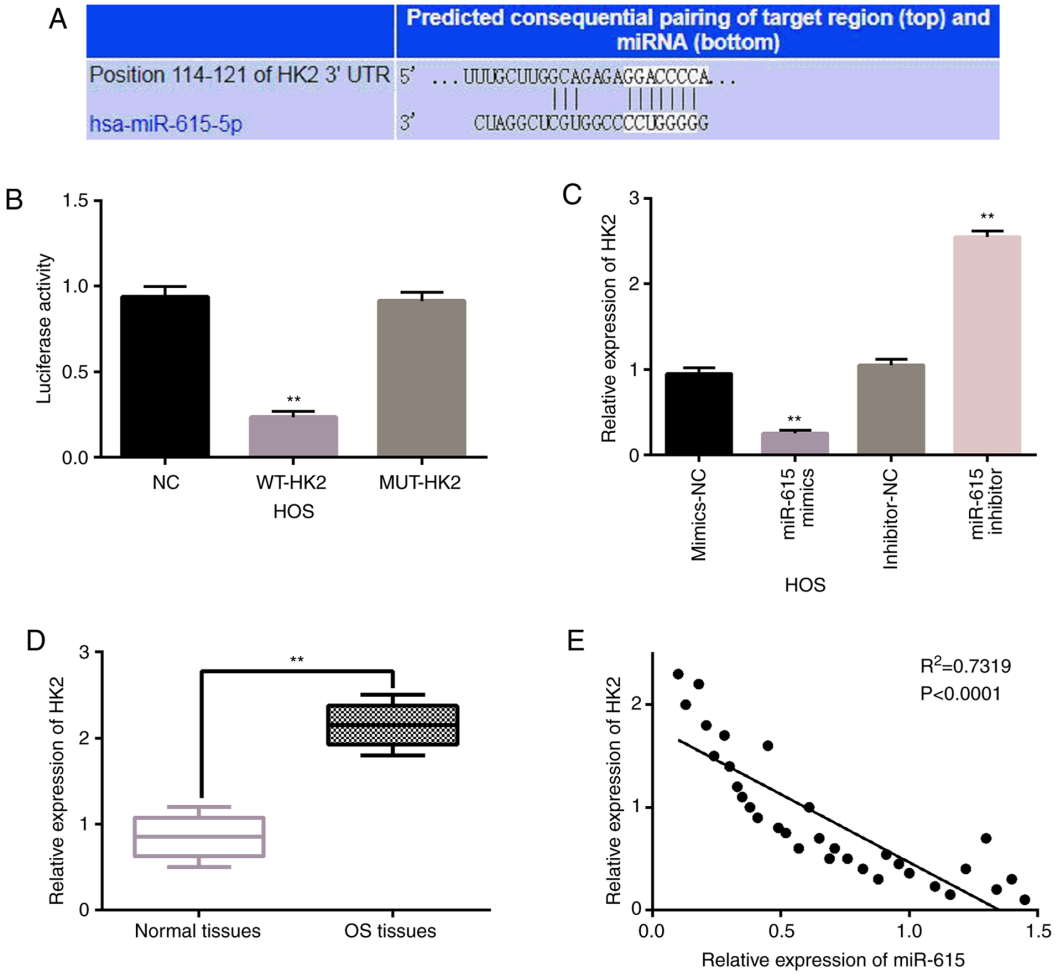

Figure 3. HK2 is a direct target gene of miR-615. (A) The binding site between miR-615 and HK2. (B) Luciferase reporter assay. **P<0.01, compared with the negative control (NC) group. (C) HK2 expression in HOS cells following transfection with the miR-615 mimics or inhibitor. **P<0.01 compared with the mimics-NC or inhibitor-NC group. (D) HK2 expression was detected in OS tissues and normal tissues. **P<0.01, OS tissues compared with normal tissues. (E) A negative correlation between miR-615 and HK2 was found in OS tissues. HK2, hexokinase 2; OS, osteosarcoma; WT, wild-type; MUT, mutant.

In addition, the expression levels of PI3K, p-PI3K, AKT and p-AKT involved in the PI3K/AKT pathway were detected in HOS cells with miR-615 mimics or inhibitor. The expression levels of phosphorylated (p-)PI3K and p-AKT were suppressed by miR-615 mimics and promoted by miR-615 inhibitor in HOS cells (Fig. 5). However, PI3K and AKT expression levels were not affected by miR-615 mimics or inhibitor in HOS cells (Fig. 5). Based on these results, miR-615 can block EMT and inactivate the PI3K/AKT pathway in OS.

Discussion

Many miRNAs have been found to participate in the pathogenesis of osteosarcoma (OS) by acting as tumor suppressors or promoters. For example, miR-493-5p was found to inhibit OS cell proliferation and metastasis by targeting Kruppel-like factor 5 (KLF5) (26). Similarly to the above study, miR-615 was found to act as a tumor suppressor in OS in the present study. Specifically, miR-615 expression was reduced in OS tissues and cells. In addition, the downregulation of miR-615 was related to the poor clinical outcomes in OS patients. Functionally, it was found that the overexpression of miR-615 inhibited OS cell viability and metastasis. In addition, miR-615 also blocked EMT in OS. EMT refers to the transformation of epithelial cells to mesenchymal cells, which provides cells with the ability to migrate and invade. N-cadherin, E-cadherin and vimentin are proteins associated with EMT. It is well recognized that EMT is activated by the abnormal expression of N-cadherin, E-cadherin and vimentin. In this study, we found that miR-615 inactivated EMT in OS cells by upregulating E-cadherin and downregulating N-cadherin and vimentin. In the present study, we also found that miR-615 blocked the PI3K/AKT pathway in OS by inhibiting the expression of p-PI3K and p-AKT. However, the more specific regulatory mechanisms of the miR-615/PI3K/AKT pathway are complex and require further study. Furthermore, miR-615 directly targets hexokinase 2 (HK2), and HK2 expression was upregulated in the OS tissues. More importantly, the upregulation of HK2 weakened the antitumor effect of miR-615 in OS. Taken together, the simple regulatory mechanism of miR-615 in OS is shown in a schematic diagram (Fig. 6).

Consistent with our results, downregulation of miR-615 was also observed in non-small cell lung (NSCLC) cancer and pancreatic ductal adenocarcinoma (27,28). In addition, it has been proposed that the low expression of miR-615 is negatively related to the clinicopathological parameter and

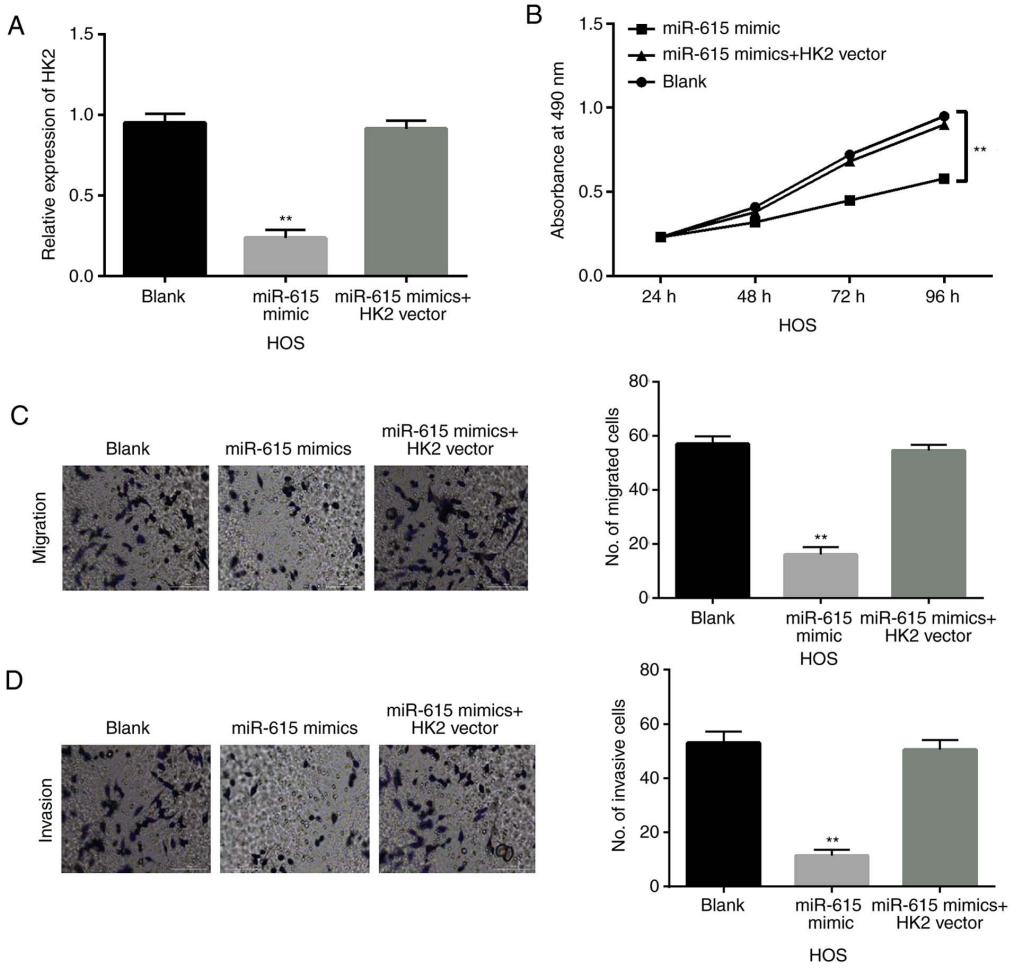

Figure 4. miR-615 is involved in OS progression through targeting HK2. (A) HK2 expression was determined in HOS cells following transfection with the miR-615 mimics and HK2 vector. **P<0.01, compared with the Blank group. (B) Cell proliferation, (C) migration and (D) invasion were assessed in HOS cells following transfection with the miR-615 mimics and HK2 vector **P<0.01, compared with the Blank group. HK2, hexokinase 2; OS, osteosarcoma.

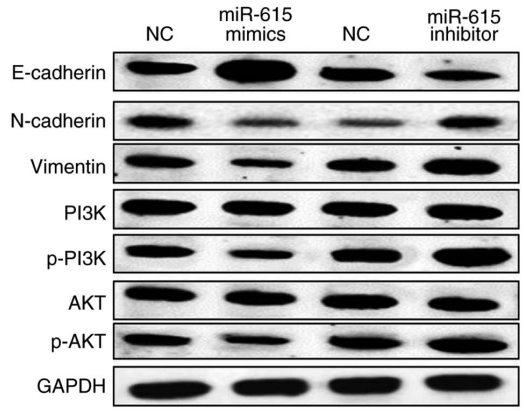

Figure 5. miR-615 blocks EMT and participates in the PI3K/AKT pathway in OS. The protein expression levels of E-cadherin, N-cadherin, vimentin, PI3K, p-PI3K, AKT and p-AKT were detected in HOS cells by western blot analysis following transfection with the miR-615 mimics or inhibitor. EMT, epithelial-mesenchymal transition; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; OS, osteosarcoma; p-, phosphorylated.

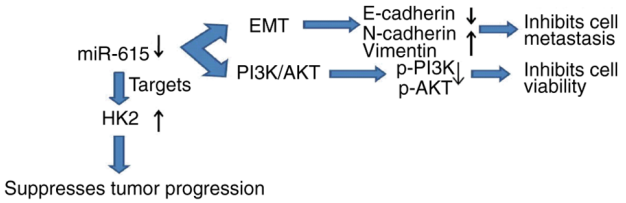

Figure 6. Simple regulatory mechanism of miR-615 in OS is shown in the schematic diagram. HK2, hexokinase 2; EMT, epithelial-mesenchymal transition; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; OS, osteosarcoma; p-, phosphorylated.

poor prognosis in patients with glioblastoma (29), which is similar to our results. Functionally, miR-615 has been reported to suppress cell proliferation and invasion in prostate cancer by directly targeting cyclin D2 (30). Moreover, miR-615-3p was found to suppress tumor growth and metastasis in NSCLC by inhibiting IGF2 (31). miR-615 was also found to restrain the proliferation of breast cancer cells by downregulation of AKT2 (32). Consistent with previous studies, we also found that miR-615 inhibited cell proliferation, invasion and migration in OS. However, there are still some limitations in the present study. Further in-depth study should be conducted at the cellular level, such as cell cycle distribution and apoptosis experiments. Moreover, miR-615 regulated the progression of OS by blocking EMT and inactivating the PI3K/AKT pathway, which has not been reported in previous studies. To make our results more convincing, we will design experiments to

explore whether the PI3K/AKT pathway can affect miR-615 function in OS.

In the present study, we found that miR-615 inhibited the development of OS by targeting HK2. As a tumor promoter, HK2 was also confirmed to be a direct target of miR-218 in glioma (33). Here, a negative correlation between miR-615 and HK2 expression was also identified in OS tissues. Similarly, it has been demonstrated that overexpression of miR-9, miR-98, and miR-199 is correlated with the downregulation of HK2 in colorectal cancer (34). More importantly, upregulation of HK2 impaired the tumor-suppressive effect of miR-615 in OS. The same interaction between HK2 and other miRNAs, such as miR-125b and miR-143, have also been identified in human cancers (35,36). In OS, upregulation of HK2 has been reported to attenuate the inhibition of cell growth and motility induced by miR-497 (37). In addition, it was found that miR-143 acted as a tumor suppressor in human prostate cancer by targeting HK2 (38). In the present study, miR-615 also inhibited the progression of OS by targeting HK2. All these findings prove the accuracy of our results concerning the relationship between miR-615 and HK2 in OS. Although we analyzed the role of miR-615 in the progression of OS, its detailed regulatory network in OS still needs further exploration.

In conclusion, our study reports the inhibitory effect of miR-615 on OS cell viability and metastasis. In addition, miR-615 acts as a tumor suppressor in OS by targeting HK2. Meanwhile, miR-615 blocks EMT and inactivates the PI3K/AKT pathway in OS. These findings suggest that miR-615 may be a new target for the treatment of OS.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

LS and JM conceived and designed the study. PW, ZZ, and KZ performed the experiments. LS wrote the paper. ZX and SL reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All of the patients in the present study provided informed consent. Approval for this research was acquired from the Institutional Ethics Committee of Shandong Provincial Third Hospital (2016SPT-44).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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