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# MicroRNA-145 inhibits tumour growth and metastasis in osteosarcoma by targeting cyclin-dependent kinase, CDK6

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**Abstract. – OBJECTIVE: Osteosarcoma (OS) is reported to be one of the most familiar forms of the primary malignant tumor to adolescents and adults, which is usually found in their long bones. Evidence and data have shown that abnormality in micro-RNA expression is closely related to tumorigenesis. The aim of this study is to investigate the expression, function and underlying mechanism of miR-145 in OS.**

**MATERIALS AND METHODS: MiR-145 expression in tumor tissues of patients and cell lines were detected by SYBR green qPCR. Kaplan-Meier method and log-rank test were applied to analyze the overall survival of patients with different miR-145 expression. MG-63 and U2OS cells were transfected with miR-145 or miR-NC, after which we recorded and analyzed the proliferation, migration and invasion respectively. Potential binding sites of miR-145 in CDK6 was identified using TargetScan 6.2. Meanwhile, OS cells were transfected with miR-NC, miR-145, or transfected with miR-145 and CDK6 vector together to find out whether the effect of miR-145 inhibit growth and mobility of OS cells were via targeting CDK6. Moreover, the expression of miR-145 was mediated by a constructed lentivirus vector to inoculate nude mice to observe the inhibiting effect of miR-145 in OS *in vivo*.**

**RESULTS: MiR-145 downregulation was remarkably related with the occurrence of a phenotype of OS with more aggressiveness. The univariate analysis was applied which showed that with a lower miR-145 level in OS patients led to a more unfavorable prognosis, or, a lower overall survival rate. In OS cells, cell proliferation, motility and invasion were inhibited by ectopic overexpression of miR-145 *in vitro*. Further studies into the mechanism indicated that its expression probably has a close interrelationship with the 3'- untranslated region (3'-UTR) of CDK6 mRNA, whose mRNA and expression levels were inhibited. And it is found that CDK6 expression was also reversely related to miR-145 expression in clinical specimens. In *in vivo* experiment, the miR-145 inhibits the growth of OS tumor.**

**CONCLUSIONS: miR-145 plays a crucial role in inhibiting invasive and metastatic of OS, whose underlying mechanism probably is directly targeting CDK6. Therefore, MiR-145 is believed to have an important role in growth and metastasis of OS.**

*Key Words:*
MicroRNA-145, CDK6, Osteosarcoma, Proliferation, Invasion, Migration.

## Introduction

Osteosarcoma (OS) is reported to be one of the most familiar forms of the primary malignant tumor to adolescents and adults, which is usually found in their long bone[1,2]. OS is closely related to the abnormality in differentiation induced by genetic and epigenetic changes. Current treatments include chemotherapy, surgery, and sometimes radiotherapy all together, but the prognosis remains very poor, the 5-year survival rate of patients with recurrent or metastatic osteosarcomas is only 50%-60%[3]. Despite researchers having identified many carcinogens and tumor suppressor genes, which are believed to account for the growth of OS, the possible mechanism of migration and invasion of OS remains mysterious in many aspects.

MicroRNAs (miRNAs) are a kind of RNA with the length of 22-25 nucleotide that down-regulate the expression of the gene in plants and animals[4,5]. MicroRNAs are believed to play vital roles in oncogenesis, cellular differentiation programs and developmental biology[6]. Decades of studies have found that the proliferation, apoptosis, metastasis, and invasion are tightly associated with abnormal expression of miRNAs in human cancer cells, including osteosarcoma. Several miRNAs were reported to be associated with OS growth and metastasis, which includes miR-32[7], miR-142-3p[8], miR-194[9], miR-202[10], miR-217[11], and let-7a[12]. A decrease in the level of miR-145 has been detected in OS. Decreased le-

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vel of miR-145 is closely related to metastasis potential of OS and ensuing poor prognosis[13]. Despite an increasing number of studies on the biogenesis and mechanisms of miR-145 in the pathogenesis of OS, the underlying mechanisms of miR-145 expression abnormality remain vague.

To reveal the possible mechanism, we found that level of miR-145 were significantly lower in osteosarcoma tissues than those in corresponding noncancerous bone tissues and more miR-145 down-regulation was detected in osteosarcoma specimens with positive metastasis. Additionally, the univariate analysis was applied which showed that OS patients who had lower miR-145 expression ended up with a poorer prognosis. In OS cell lines, proliferation, motility and invasion were inhibited by ectopic over-expression of miR-145. Moreover, it is shown that miR-145 binds to CDK6-3'-UTR, directly down-regulating the expression of CDK6. In OS tissues, the expression of CDK6 was negatively related to miR-145 level. Moreover, miR-145 has an inhibitory effect on tumor growth and mobility of osteosarcoma *in vivo*.

## Materials and Methods

### Ethics Statement

Tissue specimens involved was permitted to be used in the study exclusively by the Ethics Review Board at Shaanxi Provincial People's Hospital in advance. All animals involved in the experiments were also permitted by the Animal Ethical and Experimental Committee of Shaanxi Provincial People's Hospital. All of the surgeries were conducted with sodium pentobarbital anesthesia being applied all through the surgery, and sufficient efforts have been made to minimize the pain of animals.

### OS Cell Lines, Tissue, and Transfection

Fifty OS tissue specimens and corresponding non-tumor tissue specimens were collected in the laboratory from all subjects. Cell lines of NHOst, HOS, MG-63, SOSP-9607 and U2OS were purchased from the American Type Culture Collection (Hill, NJ, USA). All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, South Logan, UT, USA) with 10% fetal bovine serum supplement (FBS; Life Technologies, Carlsbad, CA, USA). According to the manufacturer's protocol, transfection was conducted using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

### Real-time qPCR

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied to extract total RNA from cells and tissues. qRT-PCR assays for CDK6 and miR-145 were conducted using SYBR Green Reagents (TaKaRa, Dalian, China). Primers used were as following: (1) CDK6: forward, 5'-GGACTTTCTTCATTCACACCG-3'; reverse, 5'-GACCACTGAGGTTAGGCCA-3'. (2) GAPDH: forward, 5'-TCAACGACCACTTTGTCAAGCTCA-3'; reverse, 5'-GCTGGTGGTCCAGGGGTCTTACT-3'. The $2^{-\Delta\Delta Ct}$ method was applied to quantify the expression. For average data, all operations in experiments above were conducted for three times.

### Cell Proliferation Assay

MG-63 and U2OS cells were cultured in 96-well plates at the density of $10^3$ per well, respectively. Then, cells were transfected with miR-NC, miR-145, while negative control was transfected with miR-145 and CDK6, respectively. A microplate reader (Bio-Rad, Hercules, CA, USA) was applied to assess cell viability. All experiments were run in triplicate, and averages were calculated.

In invasion and migration assays, 1.0 X $10^5$ cells/ml of MG-63 and U2OS cells were prepared after transfection with miR-NC, or miR-145 for 24 h, respectively, and 1.0 X $10^5$ cells/ml of MG-63 and U2OS cells were prepared after transfection with miR-NC or miR-145, or co-transfection with the miR-145 and CDK6 vector. Transwell insert chambers were used to determine assays of cell migration and invasion. Cells which migrated or invaded through the membrane were fixed with 4% polyoxymethylene, stained with 0.2% crystal violet, imaged, and counted under an inverted microscope (Olympus, Tokyo, Japan).

### Vector Construct and Luciferase Assay

The DNA oligonucleotides containing wild-type or mutant 3'-UTR of CDK6 were synthesized with flanking NotI and XbaI restriction enzyme digestion sites, respectively. Luciferase report vectors were built using DNAs and pMIR-REPORT™ Luciferase vectors. And the normal control was the mutant 3'-UTR of CDK6. For CDK6 vector, Homo sapiens full open reading frame cDNA clone was transcribed, and the product was amplified, followed by the DNAs were inserted into the pcDNA3.1 vector. After that, the wide-type or mutant pMIR-miR-145-3'UTR and Renilla luciferase control vector (pRL-TK, Promega, Madison, WI, USA) were used to transfect

the HEK293T cells through the Lipofectamine 2000, and then the miR-NC or miR-145 was used to transfect respectively. After transfection, the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) was used to exam the luciferase activity. Renilla activity was normalized to firefly activity.

### Lentivirus Packaging and Transduction

Lentiviral vector pLVX-shRNA1 (Clontech Laboratories, Hercules, CA, USA) was cloned using amplified miR-145 and miR-control precursor sequences. In HEK293T cells, virus packaging was conducted. pLV-miR-145 or pLV-miR-control and Lenti-X HTX Packaging Kit were co-transfected using the Xfect transfection reagent. Then, the MG-63 cells were transduced with pLV-miR-145 or pLV-miR-control. 48 hours after transfection, cells infected with the lentivirus were selected using 2 μg/ml of puromycin. The cell lines that expressed miRNA-145 and the cell line transfected with control vector were labeled as LV-miR145-MG-63 and LV-miRcontrol-MG-63 respectively.

### Animal Studies

Female nude mice (5 weeks, BALB/c,nu/nu; Shaanxi Provincial People's Hospital) used in this animal study were raised following a strict protocol of Laboratory Animal Care and Use. To test the cytoactivity of the proliferation of LV-miR145- MG-63 and LV-miRcontrol-MG-63 in living tissue, cells (1 X $10^6$) were injected into the proximal tibia (n=10). The volumes of individual orthotopic tumor were calculated according to the formula:

½ X length X width$^2$. After inoculation for 42 d (length and width were gauged with calipers, per 7 d) and the volume data was depicted into the curve of orthotopic tumor growth. Mouse orthotopic tumors were harvested and weighed.

### Western Blot Assay

Proteins from cells were lysed in a lysis buffer containing 10 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% deoxycholic acid. Then, 30 μg of protein samples were subjected to sodium salt-Polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). After blocked with 5% skim milk, antibodies against CDK6 or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied to probe the membranes. After all those procedure, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies.

### Statistical Analysis

Data are expressed as mean±SD or it was noted otherwise. The SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and the two-tail Student's t-test were applied to test the results. Kaplan-Meier method and log-rank test were applied to determine patient survival and their differences. Whether miR-145 and CDK6 mRNA was correlated was determined by Pearson's correlation. When $p<0.05$, they were considered as significantly correlated.

## Results

MiR-145 down-regulation in tumor tissues and cells was significantly correlated with tumor growth and metastasis in tumor tissues of OS patients and down-regulation of miR-145 confers unfavorable overall survival of patients with osteosarcomas.

MiR-145 expression in tumor tissues of patients and cell lines were detected by SYBR green qPCR. Expression levels of miR-145 were detected in five cell lines: decrease of the expression level of miR-145 was observed in four OS cell lines (HOS, MG-63, SOSP-9607 and U2OS), compared with the human osteoblast cells (NHOst) (Figure 1A). Additionally, the miR-145 expression in the 50 tumorous tissues and their paired non-tumor tissues specimen were also detected and recorded. The outcome manifested that expression level of miR-145 was significantly lowered in OS tissues in comparison with the matched non-tumorous tissues (Figure 1B). With the 50 tumors being classified on the basis of clinical stage, miR-145 expression was found to be diminished in primary tumours that subsequently metastasised compared with those that did not metastasise (Figure 1C). Moreover, the Kaplan-Meier method and the log-rank test was applied, which showed the lower expression of MiR-145 led to a significantly shorter overall survival of patients in comparison with those who with higher miR-145 expression ($p=0.0124$; Figure 1D).

### Inhibitory Ability of MiR-145 on the Proliferation, Invasion and Migration of OS Cells IN VITRO

In order to identify miR-145 as an inhibitor of the growth and progress potential of OS cel-

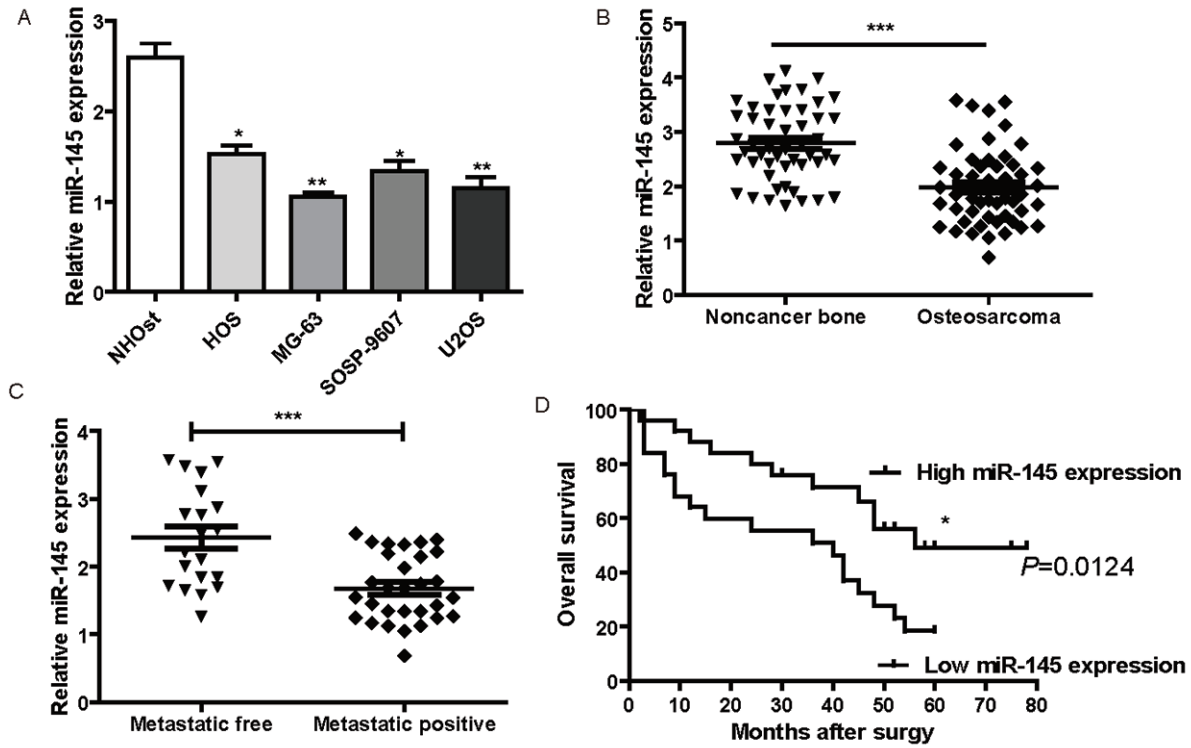


**Figure 1.** MiR-145 was decreased in OS tissues; cell lines and downregulation of miR-145 confers unfavorable prognosis in patients with osteosarcomas. ***A,*** qRT-PCR data detected of the level of miR-145 in normal osteoblast cells (NHOst) and four OS cell lines (HOS, MG-63, SOSP-9607 and U2OS). ***B,*** The level of miR-145 in 50 OS tissues and paired non-tumorous tissues. ***C,*** The level of miR-145 in primary OS (with or without metastasis). ***D,*** Overall survival curves of these two groups with a low and high expression of miR-145. The level of miR-145 was normalized to U6. All assays were performed in duplicates. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ comparing to the normal control.

ls, MG-63 and U2OS cells were transfected with miR-145 or miR-NC, after which we recorded and analyzed the proliferation, migration, and invasion respectively. As expected, results demonstrated that qRT-PCR increased the expression level of miR-145 in MG-63 and U2OS cells transfected with miR-145 (Figure 2A-B) and miR-145 did have an inhibitory effect on the proliferation (Figure 2C-D), invasion (Figure 2E) and migration (Figure 2F) of MG-63 and U2OS cells. These results indicated that proliferation and motility of OS cells were suppressed by ectopic expression of miR-145.

### CDK6 was Directly Targeting by miR-145

Bioinformatics analysis by using TargetScan 6.2 showed that CDK6 contains potential binding sites of miR-145. To identify whether CDK6 is a target of miR-145, vectors containing the wild-type or mutant 3'-UTR of CDK6 mRNA were constructed (Figure 3A). For the luciferase test, the results have shown that the wild type or mutant vector was transfected into HEK293T cells with miR-NC and miR-145. As given in Figure 3B, the relative luciferase activity of the wild-type 3'-UTR of CDK6 was significantly reduced by miR-145 ($p < 0.05$), while the luciferase activity of the mutant 3'-UTR was not significantly changed. We also found that the mRNA and protein expression of CDK6 was decreased by miR-145 significantly (Figure 3C-D). Altogether, it is evident that miR-145 have a suppressive effect on expression of CDK6 by directly targeting the 3'-UTR of CDK6.

### MiR-145 Inhibited OS cell Growth and Metastasis Through Directly Targeting CDK6

We conducted experiments in which the OS cells were transfected with miR-NC, miR-145, or transfected with miR-145 and CDK6 vector together with the purpose to find out whether the effect of miR-145 inhibit growth and mobility of OS cells were via targeting CDK6.

Function test showed that miR-145 transfection remarkably inhibited the growth and mobility of OS cells, whereas the restore of CDK6 led to the proliferation (Figure 4A-B), invasion (Figure 4C) and migration (Figure 4D) enhancement. Together, miR-145 expression led to the decrease of CDK6, which inhibited the growth and mobility capacity of OS cells by directly binding to CDK6.

### *MiR-145 was Inversely Correlated with CDK6 Expression in OS Tissues*

To validate that CDK6 expression and miR-145 were correlated, CDK6 mRNA expression levels and miR-145 were detected in 50 OS and paired non-tumorous tissues. The outcome manifested that average expression of CDK6 mRNA was significantly increased in OS tissues in comparison with that in the paired non-tumo-

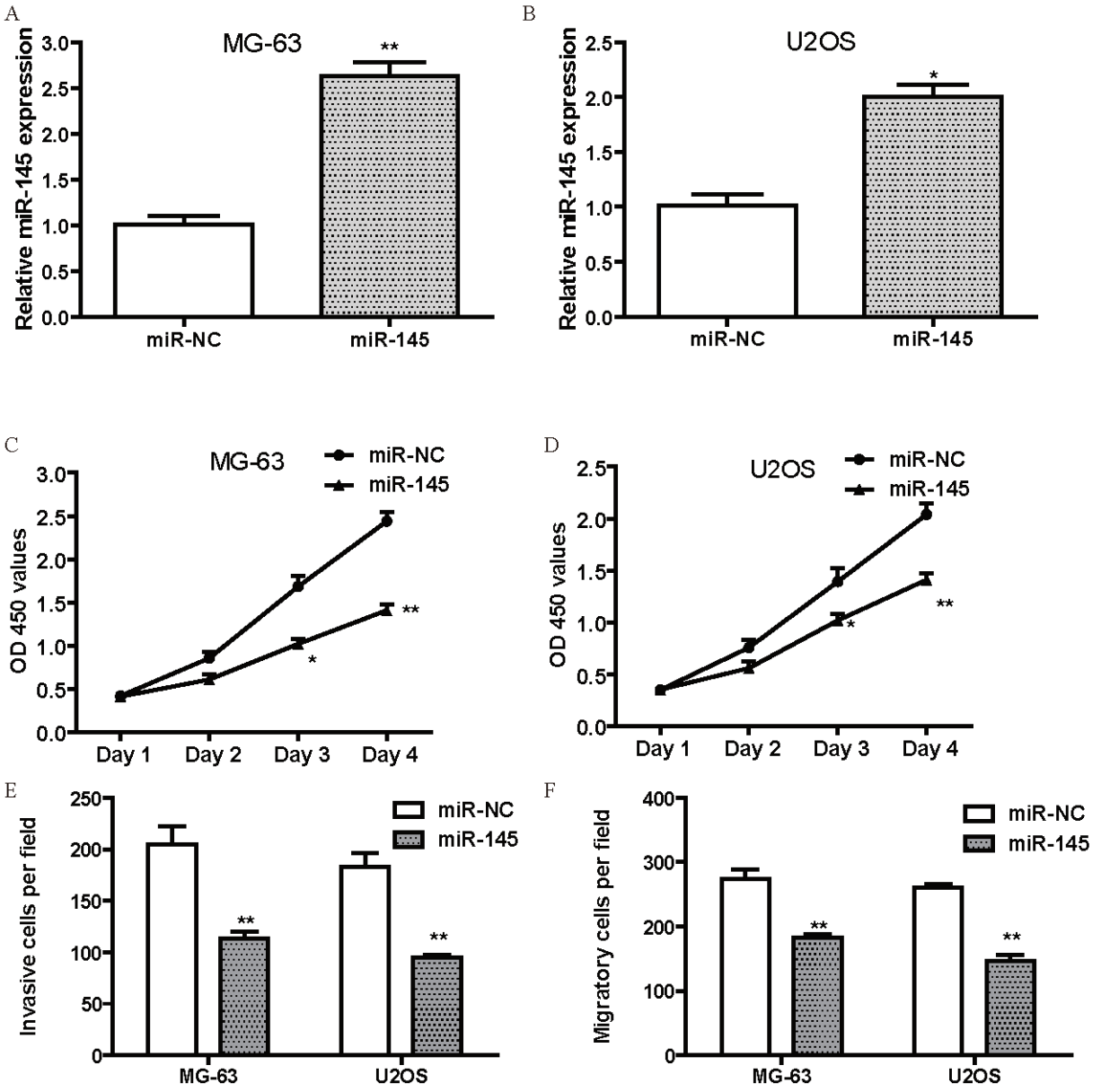


**Figure 2.** Proliferation, invasion and migration of OS cells were inhibited by miR-145. MG-63 and U2OS cells were transfected with miR-NC or miR-145, respectively. ***A-B,*** the level of miR-145 in MG-63 and U2OS cells transfected with miR-NC or miR-145. After transfection, the proliferation ***(C-D),*** invasion ***(E)*** and migration ***(F)*** assays were performed using MG-63 and U2OS cells. $^{*}p<0.05$, $^{**}p<0.01$ compared with the control. All assays were repeatedly performed in duplicates.

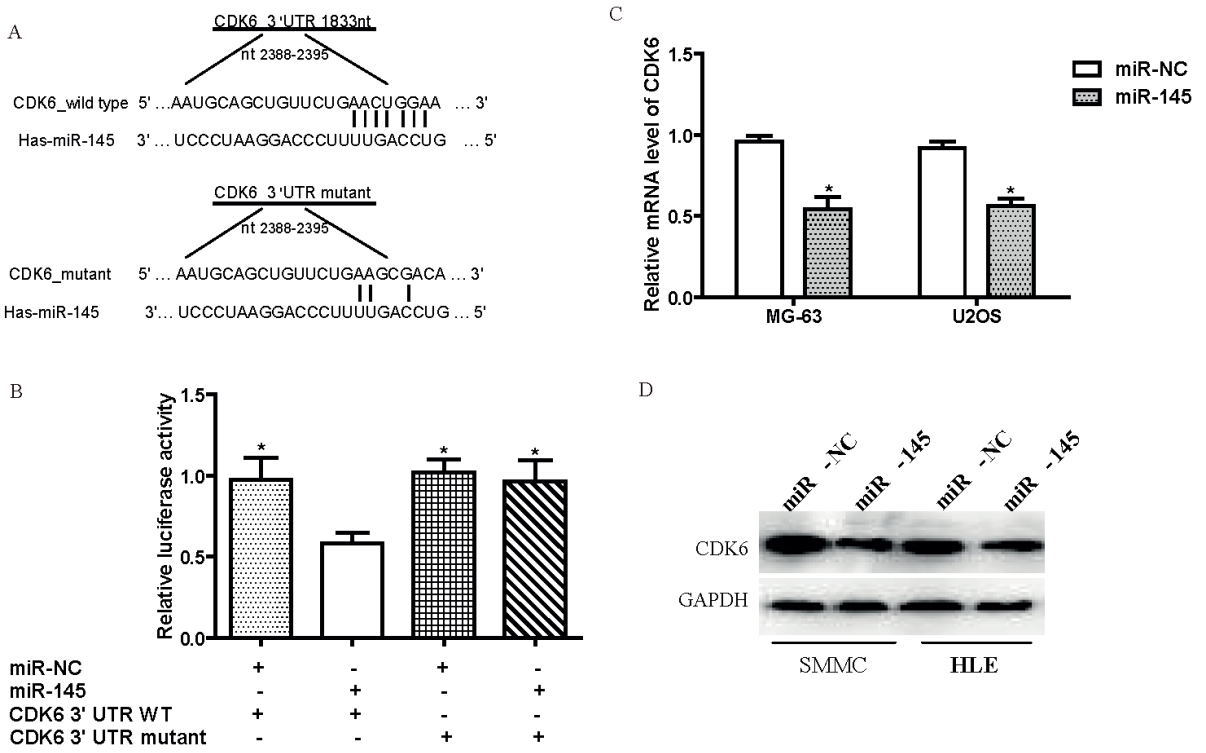


**Figure 3.** MiR-145 can directly target CDK6. ***A,*** The putative binding sequences of miR-145 in the 3'-UTR of CDK6. ***B,*** After transfection, Luciferase activity assays of luciferase vectors which contained wide-type or mutant CDK6-3'-UTR were conducted. Western blot (c) and qRT-PCR (c) assays were performed to detect the expressions of CDK6 mRNA and protein in MG-63 and U2OS cells, respectively. In Western blot assay, GAPDH was used as a control. And qRT-PCR assay was performed with GAPDH serving as an internal control for CDK6 and RNU6B for miR-145. $^{*}p<0.05$, $^{**}p<0.01$ comparing to the normal control.

rous tissues (Figure 5A). Additionally, CDK6 mRNA level and miR-145 level were inversely correlated (Figure 5B).

### *MiR-145 Inhibits Growth of Osteosarcoma IN VIVO*

To further investigate the contribution of miR-145 in vivo, the expression of miR-145 was mediated by a constructed lentivirus vector, and we established two cell lines: LV-miR145-MG63 and LV-miRcontrol-MG63, which were used to inoculate nude mice respectively, and data related to tumor growth was recorded. The length and width of the tumor were measured per 7 d post-inoculation in order to evaluate tumor growth. In all mice, progressive solid tumors were observed. The size of tumors from mice with LV-miRcontrol-MG63 inoculation was significantly bigger than those from mice with LV-miR145-MG63 inoculation (Figure 6A). The mice were sacrificed at 42 days after injection. The mean weight ± SD of tumors were 1.335±0.113 g (LV-miRcontrol-MG63 group) and 0.858±0.058 g (LV-miR145-MG63 group), corresponding to the tumor size; the tumor from LV-miRcontrol-MG63 group weighed significantly higher than those from LV-miR145- MG63 (Figure 6B). The data suggest that growth of MG-63-engrafted tumors is inhibited by miR-145.

## Discussion

In this study, we found that miR-145 was remarkably decreased in OS cell lines, HOS, MG-63, SOSP-9607, and U2OS, compared with the non-tumorous osteoblast cells NHOst. The miR-145 expression level was also significantly lowered in OS tissues in comparison with the corresponding non-tumorous tissues. Moreover, the miR-145 down-regulation occurred more frequently in osteosarcoma specimens that showed positive metastasis. Additionally, it is noted that low miR-145 expression contributes to the poorer overall survival of OS patients after being tested by univariate analysis. *In vitro*, cell proliferation, migration, and invasion in MG-63 and U2OS cells

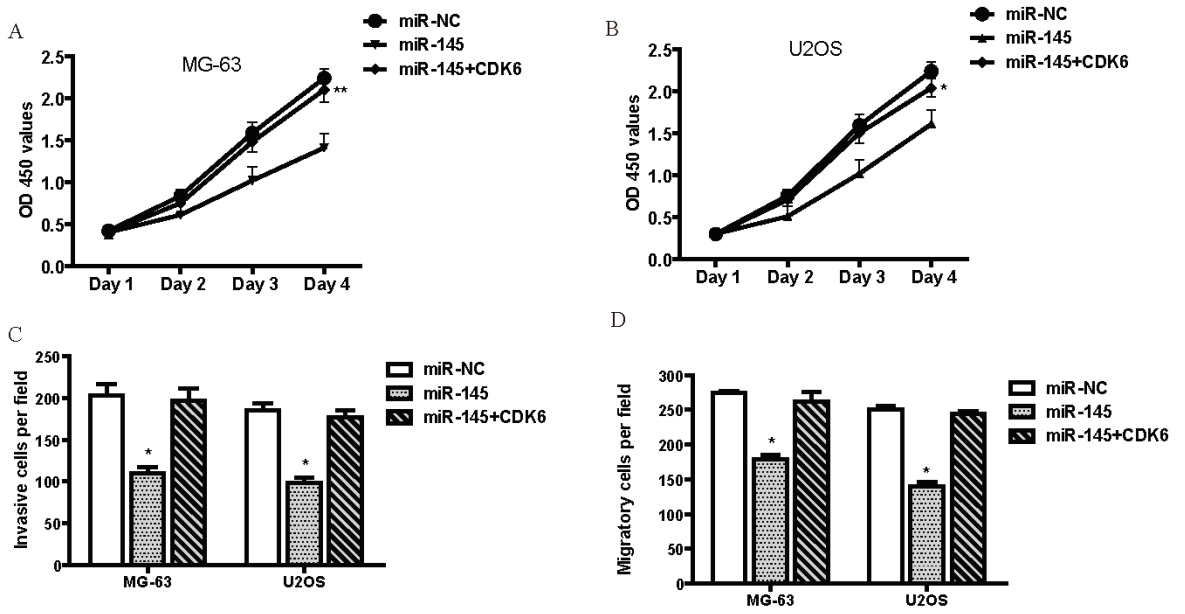


**Figure 4.** OS cells proliferation, invasion and migration were inhibited by miR-145 via targeting CDK6. ***(A-B)*** MG-63 and U2OS cells were transfected with both of the miR-145 and pcDNA3.1-CDK6 or the vector constructed. ***(C)*** *In vitro* invasion assay of MG-63 and U2OS cells with miR-145 and pcDNA3.1-CDK6 or the vector. ***(D)*** *In vitro* migration assay of MG-63 and U2OS cells co-transfected with miR-145 and pcDNA3.1-CDK6 or the vector. All assays were repeatedly performed in duplicates. $^{*}p<0.05$, $^{**}p<0.01$ comparing to miR-145 group.

were inhibited by ectopic expression of miR-145. In vivo tumor growth of osteosarcoma was inhibited by miR-145. And it is shown that miR-145 was in close relationship with silencing of CDK6 by using luciferase reporter assay. Moreover, an inverse correlation between CDK6 expression and miR-145 expression in OS tissues was observed, which offer solid evidence for an inhibitory ability of miR-145 on CDK6 in osteosarcoma. To conclude, results of this study indicated that novel strategies against OS metastasis could be achieved by using imiR-145.

Several evidences reveal that the miRNAs play a crucial role in regulating genes in cancer development, progression, and metastasis. MiR-145 has been reported to associate with several human cancers, including gastric cancer[14], colorectal cancer[15], endometrial cancer[16,17], ovarian cancer[18,19], glioblastoma[20,21], lung cancer[22], and OS[13]. Feng et al[15] reported that miR-145, by targeting Fascin-1, has an inhibitory effect on cell growth and mobility in colorectal cancer. In osteosarcoma, although Tang et al[13] offered evidence that the down-regulation of miR-145 is possibly related to tumor aggressiveness and metastasis of osteosarcoma, and that this miRNA may be a prognostic marker for osteosarcoma, the detailed mechanism of miR-145 in growth and mobility of OS remains unclear. In our data, we confirmed that miR-145 was decreased in tumor tissues of OS patients compared with matched non-tumor tissues. Moreover, we showed that proliferation, invasion and migration of OS cells were suppressed by ectopic expression of miR-145 *in vitro*, which indicated that miR-145 could in some degree affect the growth and mobility of OS.

CDK6 belongs to the family of serine-threonine kinases[23]. Cyclin D-cyclin-dependent kinase 6 (CDK6) or CDK4 activation facilitates cell cycle through the phosphorylation of substrates[24]. These kinase complexes also have shown the ability to target substrates with roles in centrosome duplication, mitochondrial function, cell growth, cell adhesion and motility, and cytoskeletal modeling. CDK6 has been reported frequently to amplify or overexpress in various cancers, including glioblastoma[25], lymphoid malignancies[26], and myxofibrosarcoma[27]. Consistent with this evidence, our data showed a level of CDK6 in OS tissues was increased compared with non-tumorous tissues. Additionally, CDK6 expression level was elevated in the primary OS tissues, which was inversely correlated with miR-145 levels. Western blot and the luciferase reporter assay were also applied

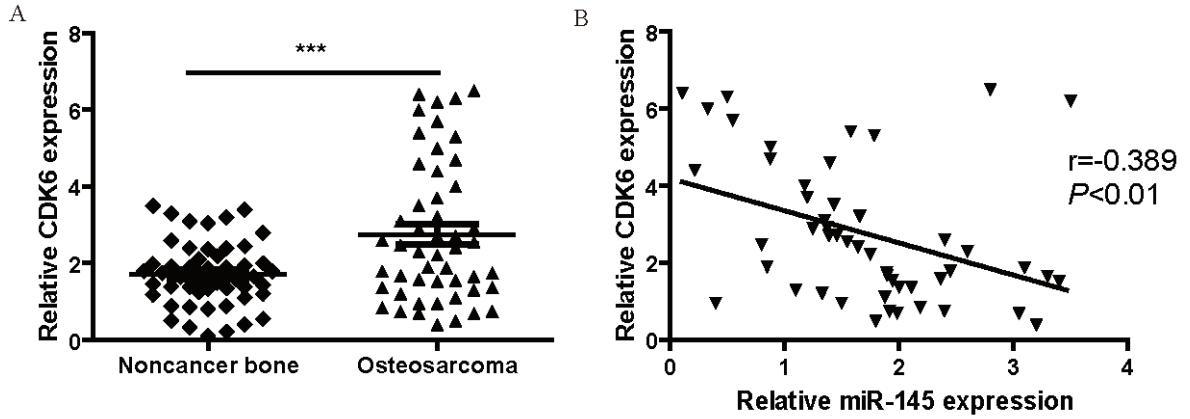


**Figure 5.** miR-145 and CDK6 expression in OS tissues were inversely correlated with each other. ***A,*** qRT-PCR data detected of expression of CDK6 in 50 OS tissues and that in the paired adjacent non-tumorous tissues. ***B,*** The result of correlation analysis between miR-145 and CDK6 mRNA level in OS tissues (Spearman's correlation analysis, r =- 0.389; $p<0.01$).

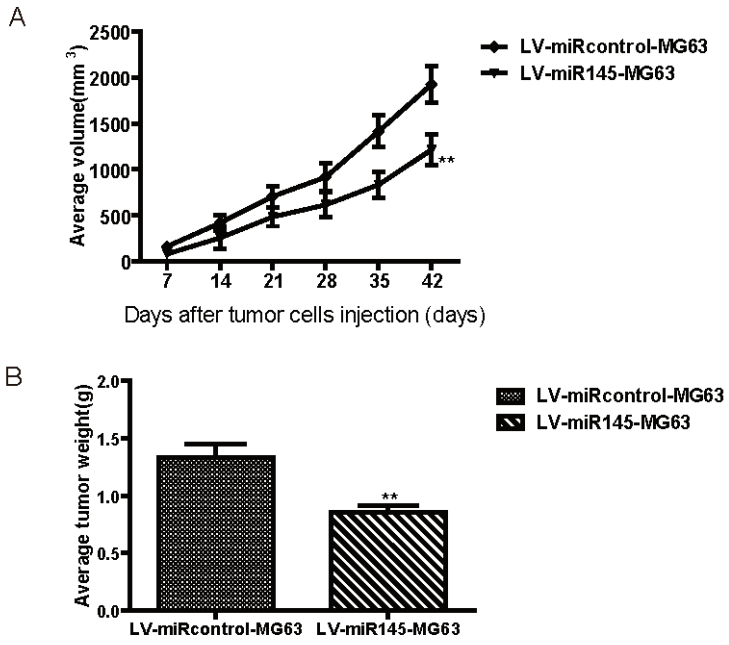


**Figure 6.** Tumor growth of osteosarcoma was inhibited by miR-145 *in vivo*. A, OS growth was inhibited by overexpression of miR-145 *in vivo*, (n=10; $^{**}p=0.0042$). B, Weights of orthotopic tumor 42 days post-inoculation (n=10; $^{**}p=0.0015$). Data are shown as means ± SD.

in the experiment of OS cell lines. Our findings also indicated that ectopic expression of CDK6 restores the effects of miR-145 on cell growth and mobility in OS cells. The results of this research clarified that CDK6 make a contribution to cell growth and mobility in OS, and is a direct target of miR-145.

## Conclusions

The miR-145 is confirmed as an effective tumor suppressor in the OS progression, whose suppressive effects are partially mediated via its downstream target gene, CDK6. Up to date, this is the first study to demonstrate that the growth

and mobility of OS cells were regulated by the miR-145/CDK6 axis. These findings provide a better understanding of the pathogenesis and development of OS and may be an important implication for future therapy of the OS.

### Conflict of interest

The authors declare no conflicts of interest.

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