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MicroRNA-765 targets MTUS1 to promote the progression of osteosarcoma via mediating ERK/EMT pathway

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Abstract. – OBJECTIVE: Previous studies have shown that microRNA-765 (miR-765) is involved in certain biological behaviors of human cancers. However, abnormal expression and function of miR-765 have not been reported in osteosarcoma (OS).

PATIENTS AND METHODS: Changes in the expression of miR-765 and MTUS1 (Microtubule-associated tumor suppressor 1) were examined via Real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis. The function of miR-765 was investigated through Cell Counting Kit-8 (CCK-8) and transwell assays in OS. The target of miR-765 was identified using a Dual-Luciferase reporter assay.

RESULTS: MiR-765 was upregulated in OS tissues. And upregulation of miR-765 promoted cell proliferation, migration and invasion in OS. In addition, MTUS1 was confirmed as a direct target gene of miR-765. Moreover, miR-765 promoted the progression of OS through targeting MTUS1. Furthermore, miR-765 was involved in tumorigenesis of OS through activating extracellular-signal-regulated kinase/ epithelial-mesenchymal transition (ERK/EMT) pathway.

CONCLUSIONS: MiR-765 targets MTUS1 to promote the progression of OS via mediating the ERK/EMT pathway. Therefore, miR-765 may be used as a novel biomarker for the diagnosis of OS.

Key Words:
MiR-765, Osteosarcoma, MTUS1, ERK/EMT pathway.

Introduction

Osteosarcoma (OS) is a common malignant tumor that usually occurs in adolescents or children under the age of 20[1]. Moreover, OS accounts for about 5% of pediatric tumors. In addition, the malignant degree of OS accounts for about 34% of malignant bone tumors. Furthermore, the prognosis of OS patients is very poor[2]. Moreover, OS-induced lung metastasis can occur within a few months, with a survival rate of only 5-20% after amputation[3]. At present, OS is still a malignant tumor with high mortality in children and adolescents, but early detection and timely treatment have greatly improved the survival rates of OS[4]. The key factors affecting the prognosis of OS are early diagnosis and radical resection of the tumor. Therefore, it is urgent for us to develop new biomarkers for the diagnosis of OS. In recent years, microRNAs (miRNAs) have received increasing attention due to their specific role in tumorigenesis of human cancers. It has been reported that miRNAs are involved in the pathogenesis of malignant tumors by mediating some biological processes, such as proliferation, apoptosis or differentiation. The functions of miRNAs had also been reported in OS. For example, miR-126 inhibited migration, invasion, proliferation and EMT in OS by modulating ZEB1[5]. Ma et al[6] proposed that miR-603 functioned as an oncogene by suppressing the translation of BRCC2 protein in OS. Now, the role of miR-765 in human disease and cancers has caused great concern. MiR-765 has been shown to be a non-invasive biomarker for the diagnosis of geriatric coronary artery disease patients[7]. In some human cancers, different effects of miR-765 have been found. MiR-765 showed an inhibitory effect on cell proliferation and invasion in tongue squamous cell carcinoma[8]. In contrast, miR-765 promoted cell proliferation through suppressing INPP4B in human hepatocellular carcinoma[9]. Further-

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more, miR-765 was found to enhance the anti-angiogenic effect of CDDP *via* APE1 in OS[10]. However, the function of miR-765 involved in tumorigenesis of OS remains unknown and needs to be investigated. MTUS1 (Microtubule-associated tumor suppressor 1) is a novel tumor suppressor gene located on chromosome 8p21.3-22[11]. And downregulation of MTUS1 had been identified in colon tumors[12] and bladder cancer[13]. Functionally, downregulation of the tumor suppressor MTUS1/ATIP promoted proliferation and predicted poor prognosis in oral tongue squamous cell carcinoma[14]. Moreover, MTUS1 had been found to be involved in the extracellular-signal-regulated kinase/ epithelial-mesenchymal transition (ERK/EMT) signaling pathway in ovarian carcinoma[15]. Previous studies have demonstrated that the mitogen-activated protein kinase (MAPK)/ERK signaling pathway plays an important role in human cancers. As one of the MAPK/ERK kinases, ERK1/2 was found to promote cell proliferation and inhibit cell apoptosis in human cervical cancer[16]. The interaction between MTUS1 and MAPK/ERK signaling pathway was investigated in this study. In the current study, the alternation of miR-765 expression was examined in the OS. More importantly, the function of miR-765 for cell survival and metastasis was explored in the OS. Finally, the molecular mechanisms of miR-765 and MTUS1 involved in MAPK/ERK were elucidated.

Patients and Methods

Clinical Tissues

A total of 54 human OS tissues and non-cancerous bone tissues were acquired from the Henan Provincial People's Hospital. These tissues were obtained from OS patients without treatment. All OS patients provided written informed consents. This study was approved by the Institutional Ethics Committee of Henan Provincial People's Hospital. Finally, these tissues were frozen in liquid nitrogen and then stored in a -80°C refrigerator.

Cell Lines Culture

Human normal osteoblastic cell line hFOB1.19 and U2OS, Saos-2, MG-63 cell lines were used for this experiment. These cell lines were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cell lines were then inoculated into Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). And they were cultured at 37°C with 5% CO_2.

Cell Transfection

MiR-765 mimics or inhibitor and negative control (NC) were obtained from RiboBio (Guangzhou, China). MTUS1 siRNA or negative control siRNA was purchased from Genechem (Shanghai, China). They were separately transferred into U2OS cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufactures' protocols.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA in the OS was extracted using TRIzol reagent (Promega, Madison, WI, USA). The synthesis of complementary deoxyribose nucleic acid (cDNA) was performed using a miScript reverse transcription kit (Qiagen, Hilden, Germany). We performed RT-qPCR through using SYBR Green Reagent (Applied Biosystems, Foster City, CA, USA) on 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control for miR-765 or MTUS1. And their expressions were calculated using the $2^{-\Delta\Delta ct}$ method. The primers used were: miR-765 GCCTGGAGGAGAAGGAA (F), GTGCAGGGTCCGAGGT (R); GAPDH TCAACGACCACTTTGTCAAGCTCAGCT (F), GGTGGTCCAGGGGTCTTACT (R); MTUS1 AGCTTCGGGACACTTACATT-3 (F), ATAGGCCTTCTTTAGCAATTC (R); U6 CTCGCTTCGGCAGCACA (F), AAC GCT TCA CGA ATT TGC GT (R).

Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 assay was used to assess cell viability in the OS. The U2OS cells (3×10^4) with aforementioned transfection were seeded in 96-well plates. Next, each well was added with 10 μL CCK-8 reagents for 2 h (Dojindo, Kumamoto, Japan) based on the manufacturer's instructions. Finally, they were detected using a microplate reader (Molecular Devices, Eugene, OR, USA) at an absorbance of 450 nm.

Transwell Assay

The migratory and invasive abilities of U2OS cells were assessed using transwell assay. Transwell chambers (8 μm pore size; Millipore, Bil-

lerica, MA, USA) were added in 24-well plates. U2OS cells at a density of 5×10^4 cells/well without serum were placed in the upper chamber on the non-coated membrane, and the lower chamber was filled with 20% fetal bovine serum (FBS) to induce U2OS cells to migrate through the membrane. For invasion assay, cells were placed in the upper chamber with the coated membrane. The migrated or invasive cells were then stained with 0.1% crystal violet. A microscope (Olympus, Tokyo, Japan) was used for counting migrated and invaded cells.

Dual Luciferase Reporter Assay

U2OS cells with pcDNA3.1 plasmid vector (Promega, Madison, WI, USA) containing Renilla luciferase were cultured for 24 h. Then, wild or mutant type of 3'-UTR of MTUS1 and miR-765 mimics was transfected into U2OS cells. Finally, luciferase activity was measured through dual luciferase assay system (Promega, Madison, WI, USA).

Western Blot Analysis

Protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein was then separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and incubated with 5% skim milk in polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at room temperature. Next, we incubated the membrane with EMT markers (E-cadherin, N-cadherin, Vimentin), ERK pathway marker (ERK), MTUS1 and GAPDH antibodies overnight at 4°C. After washing, they were incubated with corresponding secondary antibodies for 2 h at room temperature. Protein expression levels were then measured by enhanced chemiluminescence (ECL) (Pierce Biotechnology, Rockford, IL, USA).

Statistical Analysis

Data were shown as mean ± SD (Standard Deviation). The correlation between miR-765 and clinicopathological characteristics of OS patients was calculated through Chi-squared test. Differences between groups were calculated by one-way analysis of variance (ANOVA) with Bonferroni post-hoc test using Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Armonk, NY, USA). The overall survival rates and survival differences were detected by the univariate Kaplan-Meier method with log-rank test. Significant differences were defined as $p<0.05$.

Results

The Expression of miR-765 was Upregulated in OS Tissues

First, abnormal expressions of miR-765 were detected in OS tissues *via* RT-qPCR assay. The expression of miR-765 was increased in OS tissues compared to normal tissues ($p<0.01$, Figure 1A). Then, the correlation between miR-765 and clinicopathological characteristics of OS patients was analyzed. We found that high miR-765 expression was closely associated with distant

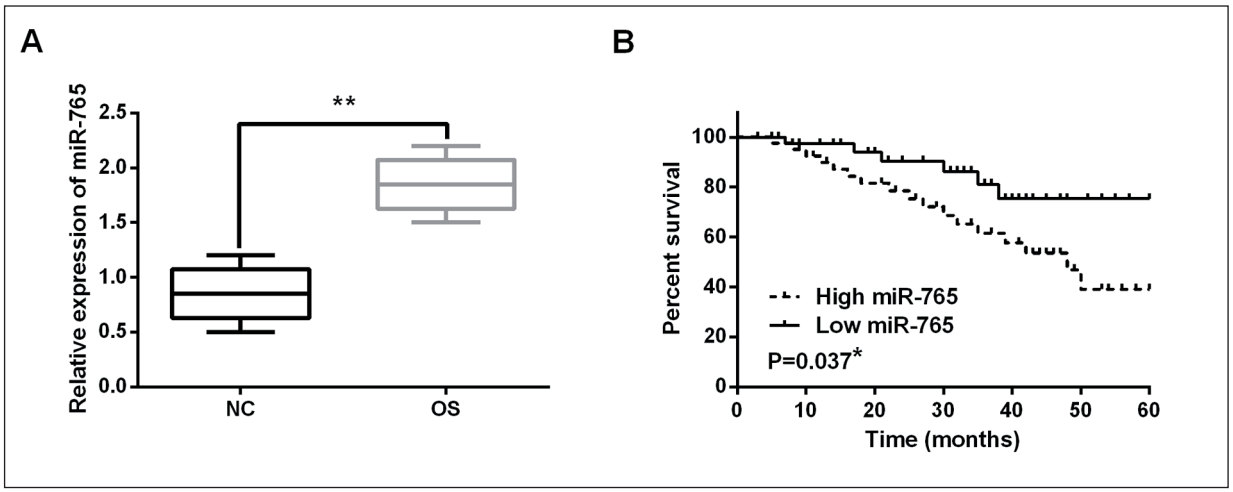

Figure 1. The expression of miR-765 was upregulated in OS tissues. ***A***, The expressions of miR-765 in OS tissues ***B***, High miR-765 expression was correlated with shorter overall survival in OS patients. *$p<0.05$, **$p<0.01$.

Table I. Relationship between miR-765 expression and their clinic-pathological characteristics of OS patients.

Characteristics	Cases	miR-765		*p*-value
		High	Low	
Age (years)				0.202
≥ 20	20	13	7	
< 20	34	20	14	
Gender				0.302
Male	32	24	8	
Female	22	15	7	
Tumor size (cm)				0.323
≤ 8	35	23	12	
> 8	19	11	8	
Distant metastasis				0.011*
Absent	38	28	10	
Present	16	12	4	
Clinical stage				0.049*
I-II	37	25	12	
III	17	12	5	

Statistical analyses were performed by the χ^2 test. *p<0.05 was considered significant.

metastasis (p=0.011) or clinical stage (p=0.049, Table I). Furthermore, high miR-765 expression was related to a shorter overall survival rates in OS patients, which predicted a poor prognosis (p=0.037, Figure 1B). These results suggest that miR-765 can be involved in tumorigenesis and prognosis of OS.

Overexpression of miR-765 Promoted Cell Proliferation, Migration and Invasion in OS

Next, the alternation of miR-765 expression was examined in U2OS, Saos-2, MG-63 and hFOB1.19 cell lines. Similarly, upregulation of miR-765 was measured in U2OS, Saos-2 and MG-63 cell lines compared with hFOB1.19 cells (p<0.05 or 0.01, Figure 2A). Then, miR-765 mimics or inhibitor was transfected into U2OS cells to investigate its function in OS. And the expression level of miR-765 was enhanced by miR-765 mimics and decreased by miR-765 inhibitor (p<0.01, Figure 2B). More importantly, CCK-8 assay showed that upregulation of miR-765 promoted proliferation of U2OS cells (p<0.05, Figure 2C). In contrast, downregulation of miR-765 inhibited cell proliferation in U2OS cells (p<0.01, Figure 2D). In addition, U2OS cell migration was promoted by upregulation of miR-765. And cell migration was suppressed by knockdown of miR-765 in U2OS cells (p<0.01, Figure 2E). Furthermore, the same effect of miR-765 on cell invasion was also identified (p<0.01, Figure 2F). Based on these results, miR-765 was considered to play an important role in carcinogenesis of OS.

MTUS1 was a Direct Target Gene of miR-765

Further, target genes of miR-765 were searched in the database of TargetScan (http://www.targetscan.org/). MTUS1 was found to have binding sites with miR-765 (Figure 3A). Luciferase reporter assay was then performed to confirm the relationship between miR-765 and MTUS1. As predicted, the miR-765 mimics significantly inhibited luciferase activity of Wt-MTUS (p<0.01, Figure 3B). However, the luciferase activity of Mut-MTUS1 was not affected by miR-765 mimics. Moreover, miR-765 was found to be negatively correlated with MTUS1 expression in OS tissues (p<0.0001, R^2=0.706; Figure 3C). Furthermore, the expression of MTUS1 was measured in U2OS cells with miR-765 mimics or inhibitor. Consistently, MTUS1 expression was reduced by miR-765 mimics (p<0.01, Figure 3D). And MTUS1 expression was promoted by miR-765 inhibitor (p<0.01, Figure 3E). MiR-765 was confirmed to directly target MTUS1, and there was a negative correlation between miR-765 and MTUS1.

The Knockdown of MTUS1 Promoted the Development of OS

Subsequently, abnormal expression of MTUS1 was detected in OS tissues and cell lines. The results

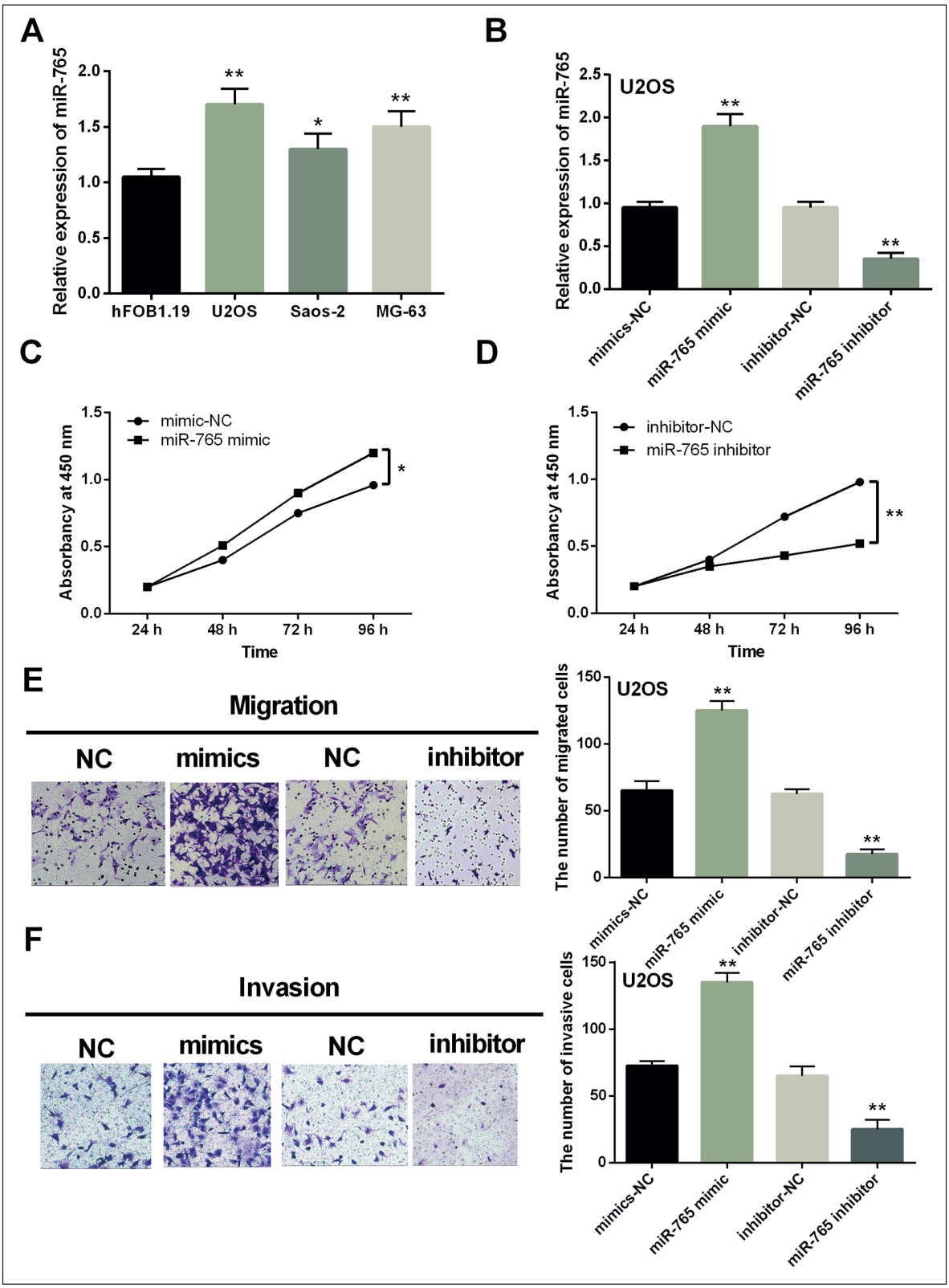

Figure 2. Overexpression of miR-765 promoted cell proliferation, migration and invasion in OS. ***A,*** The expressions of miR-765 in U2OS, Saos-2, MG-63 and hFOB1.19 cell lines ***B,*** The expression of miR-765 in U2OS cells with miR-765 mimics or inhibitor. ***C, D,*** Cell proliferation was measured in cells containing miR-765 mimics or inhibitor. ***E, F,*** Cell migration and invasion analysis in cells containing miR-765 mimics or inhibitor *p<0.05, **p<0.01.

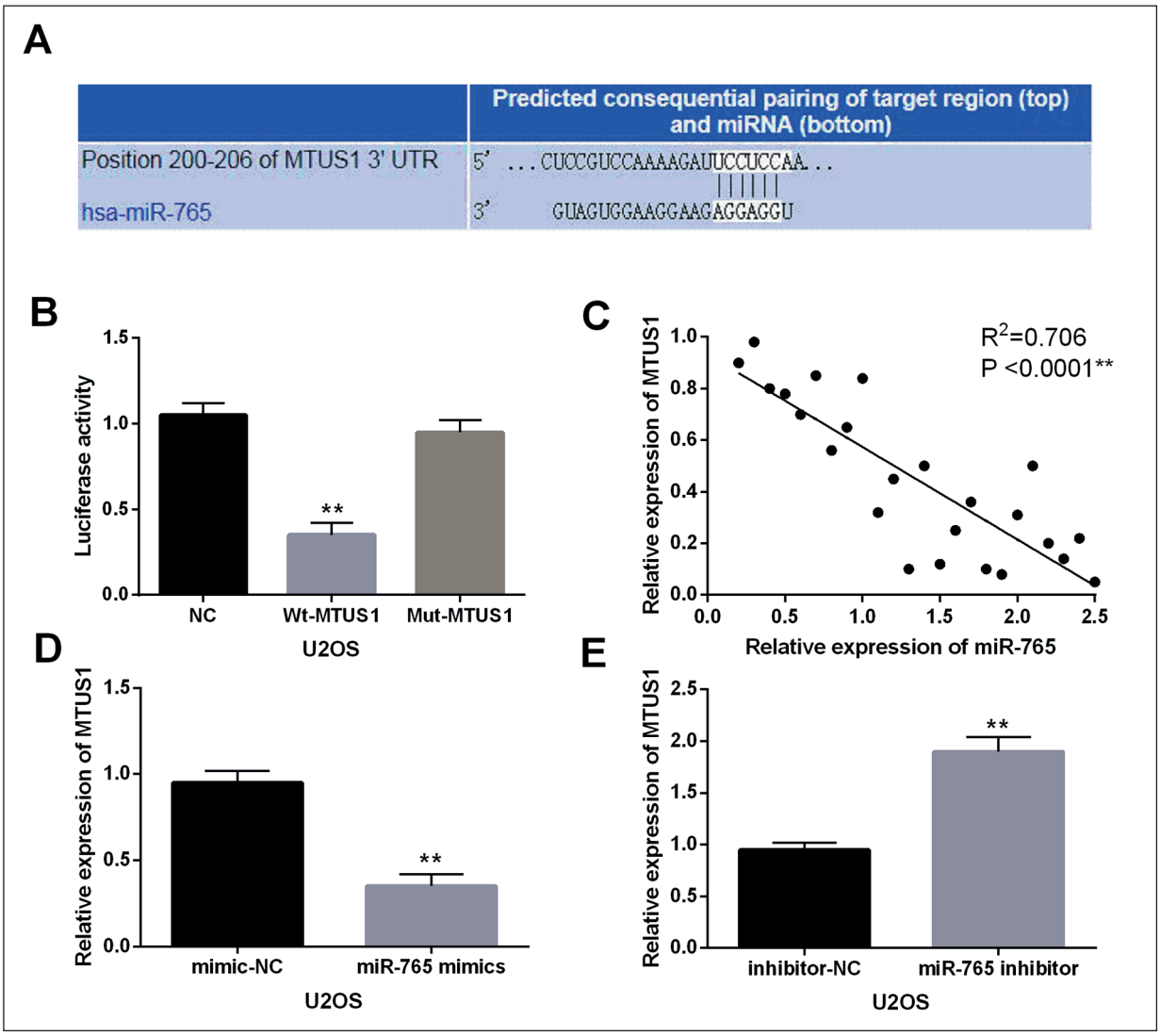

Figure 3. MTUS1 was a direct target gene of miR-765. ***A,*** The binding sites between MTUS1 and miR-765 ***B,*** Luciferase reporter assay ***C,*** MiR-765 had negative correlation with MTUS1. ***D, E,*** The expression of MTUS1 in U2OS cells containing miR-765 mimics or inhibitor **p<0.01.

of RT-qPCR showed that MTUS1 was downregulated in OS tissues compared with normal tissues (p<0.01, Figure 4A). Downregulation of MTUS1 was also identified in U2OS, Saos-2 and MG-63 cell lines compared to hFOB1.19 cells (p<0.05 or 0.01, Figure 4B). To investigate the role of MTUS1 in OS, MTUS1 siRNA was transfected into U2OS cells. MTUS1 siRNA obviously reduced MTUS1 expression (p<0.01, Figure 4C). Functionally, knockdown of MTUS1 promoted proliferation of U2OS cells (p<0.05, Figure 4D). Similarly, MTUS1 siRNA also promoted migration and invasion of U2OS cells (p<0.01, Figure 4E, 4F). It was suggested that MTUS1 was a tumor-suppressor gene in OS.

MiR-765 Promoted the Progression of OS Through Targeting MTUS1

To confirm the interaction between miR-765 and MTUS1, miR-765 mimics and the MTUS1 vector were co-transfected into U2OS cells. As expected, reduced MTUS1 expression induced by miR-765 mimics was recovered by MTUS1 vector in U2OS cells (p<0.01, Figure 5A). Similarly, the promoted effect of miR-765 on cell proliferation was impaired by MTUS1 vector (p<0.05, Figure 5B). Moreover, upregulation of MTUS1 also weakened the promoted effect of miR-765 on cell migration and invasion in OS (p<0.01, Figure 5C, 5D). These findings proved

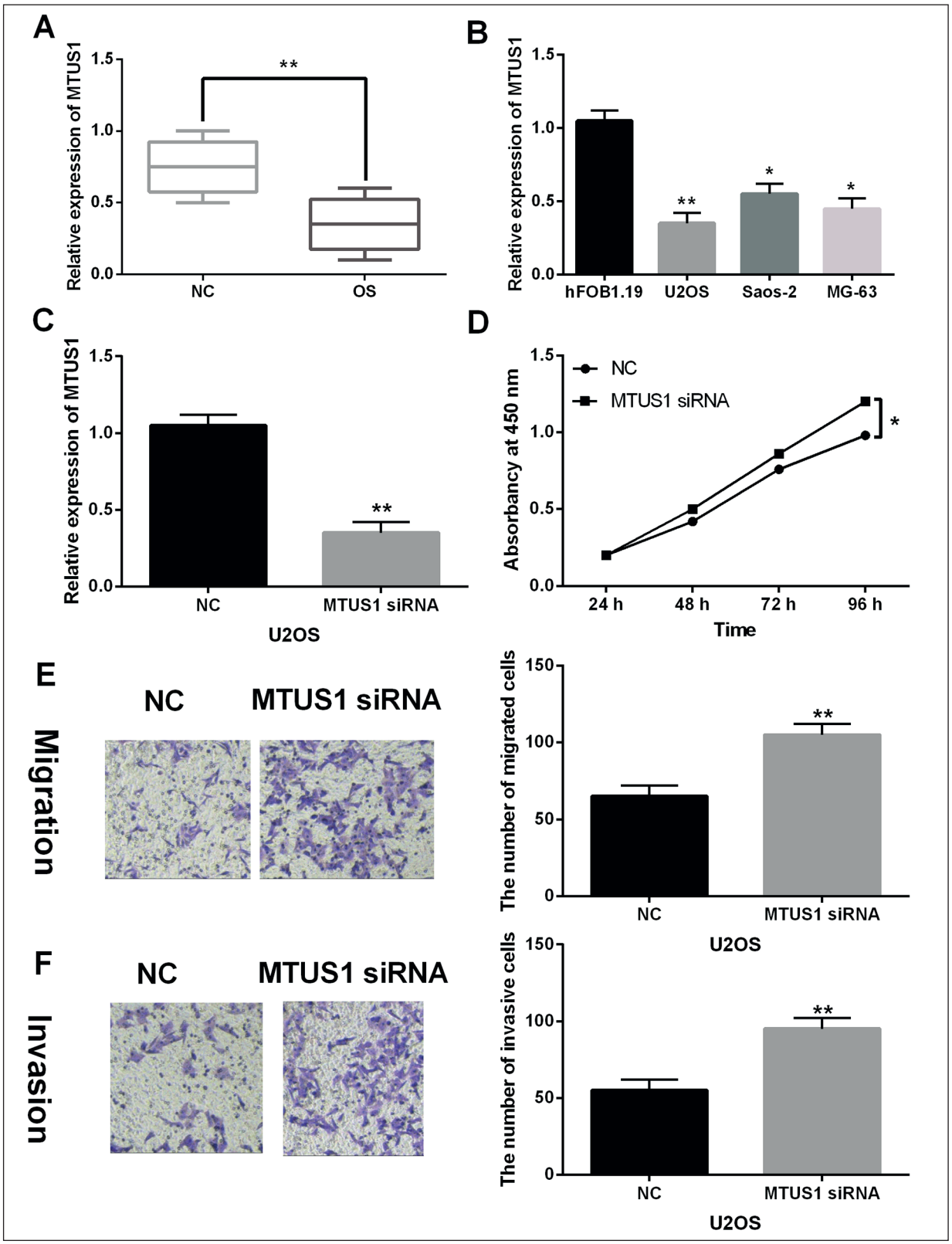

Figure 4. Knockdown of MTUS1 promoted the development of OS. ***A,*** The expressions of MTUS1 in OS tissues ***B,*** The MTUS1 expression in U2OS, Saos-2, MG-63 and hFOB1.19 cell lines ***C,*** The expression of MTUS1 in U2OS cells with MTUS1 siRNA ***D,*** Cell proliferation in cells containing MTUS1 siRNA. ***E, F,*** Cell migration and invasion analysis in cells containing MTUS1 siRNA *p<0.05, **p<0.01.

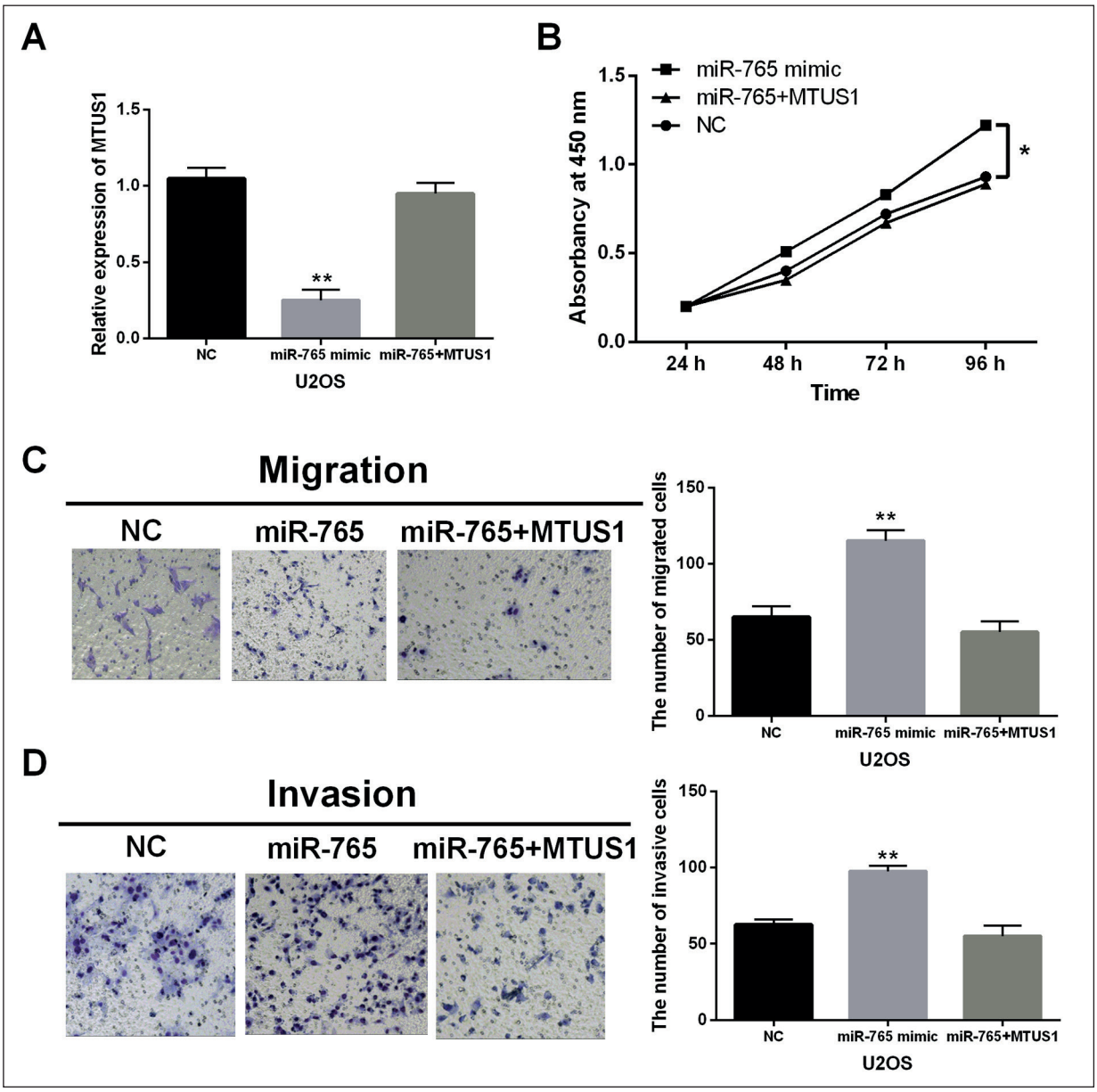

Figure 5. MiR-765 promoted the progression of OS through targeting MTUS1. ***A,*** The expression of MTUS1 in U2OS cells with MTUS1 vector and miR-765 mimics ***B,*** Cell proliferation in U2OS cells with MTUS1 vector and miR-765 mimics ***C, D,*** Cell migration and invasion in U2OS cells with MTUS1 vector and miR-765 mimics *p<0.05, **p<0.01.

that miR-765 promoted proliferation, migration and invasion of OS cells *via* targeting MTUS1.

MiR-765 was Involved in the Tumorigenesis of OS Through Activating ERK/EMT Pathway

Finally, the effect of miR-765 on the ERK/EMT pathway was investigated to further explore the molecular mechanism of miR-765 in OS. First, protein expressions of E-cadherin, N-cadherin and Vimentin contained in EMT were examined in U2OS cells with miR-765 mimics or inhibitor. MiR-765 mimics were found to decline protein level of E-cadherin and promote expressions of N-cadherin and Vimentin (Figure 6). In contrast, miR-765 inhibitor showed the opposite effect on the expressions of these three markers, as shown in Figure 6. The results indicated that miR-765 promoted EMT process in OS cells to regulate cell metastasis.

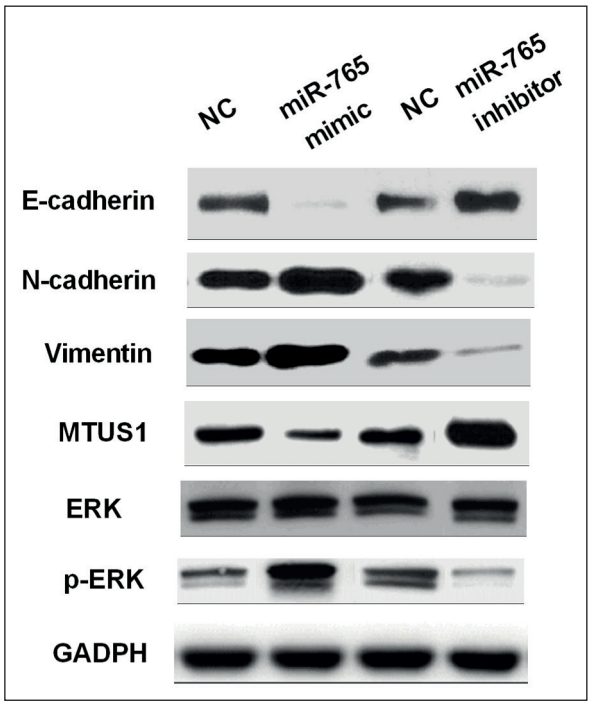

Figure 6. MiR-765 was involved in tumorigenesis of OS through activating ERK/EMT pathway. Western blot analysis of E-cadherin, N-cadherin, Vimentin, MTUS1, ERK and p-MTUS1 in U2OS cells with miR-765 mimics or inhibitor.

Previous studies have shown that the ERK pathway plays an important role in cell survival. We found that overexpression of miR-765 promoted the expression of p-ERK in OS cells, and downregulation of miR-765 had opposite results (Figure 6). However, no difference was detected in the expression of ERK. Taken together, miR-765 promoted cell survival and metastasis by activating the ERK/EMT pathway in OS.

Discussion

Various miRNAs have been shown to be involved in the pathogenesis of OS via regulating the expression of target genes, such as miR-206[17] and miR-127[18]. In this study, upregulation of miR-765 was identified in OS tissues, which was closely related to distant metastasis and clinical stage. In addition, high miR-765 expression predicted poor prognosis in OS patients. Jiang et al[19] reported that the high expression of miR-765 was associated with tumor stage, lymph nodes metastasis and clinical stage in patients with esophageal squamous cell carcinoma (ESCC). Same as our results, upregulation of miR-765 also predicted a poor prognosis in ESCC patients. Functionally, overexpression of miR-765 promoted cell proliferation, migration and invasion in OS. The effect of miR-765 on tumorigenesis of OS has not been reported previously. However, studies have reported that overexpression of miR-765 promoted cell proliferation and differentiation by modulating Hes1 expression in neural stem cells[20]. And miR-765 promoted the proliferation of human hepatocellular carcinoma cells[9]. These results validated our findings in this study. Moreover, Liang et al[10] proposed that the combination of miR-765 and cisplatin weakened the capability of HUVEC migration in OS. The difference may be due to the action of cisplatin. In addition, miR-765 promoted tumorigenesis of OS by activating the ERK/EMT pathway. As an important contributing factor, EMT can regulate the invasion and metastasis of human cancers and promote the aggressiveness of malignant tumors[21]. For example, the miR-135b-TAZ feedback loop has been revealed to promote EMT and tumorigenesis in OS[22]. Besides that, it has been shown that activation of the ERK/MAPK signaling pathway promotes tumor growth and cell proliferation in human cancers through enhancing the expression of p-ERK[23]. Furthermore, downregulation of MEK had been reported to suppress cell invasion and migration in ovarian cancers with activation of ERK1/2[24]. In addition, the interaction between miRNAs and the MAPK/ERK signaling pathway was also investigated. For example, miR-592 promoted proliferation, migration, and invasion through the MAPK/ERK signaling pathway in gastric cancer[25]. Here, miR-765 was found to promote proliferation, migration and invasion of OS cells through activating ERK pathway. Further, we found that MTUS1 was a direct target gene of miR-765. And knockdown of MTUS1 promoted proliferation of OS cells. Ding et al[14] reported that downregulation of MTUS1 promoted cell proliferation in oral tongue squamous cell carcinoma, which was consistent with our results. In addition, MTUS1 was downregulated in gastric cancer, and the loss of MTUS1 promoted cell metastasis in gastric cancer [26]. In OS, downregulation of MTUS1 was also identified, and knockdown of MTUS1 promoted migration and invasion of OS cells. Moreover, it had been demonstrated that oncogenic miR-19a and miR-19b together regulated the tumor suppressor MTUS1 to promote cell

proliferation and migration in lung cancer[27]. Here, miR-765 also promoted the progression of OS through targeting MTUS1.

Conclusions

We observed that the expression of miR-765 was significantly increased in OS tissues. Besides that, high miR-765 expression was associated with unfavorable clinical outcomes and poor prognosis in OS patients. These findings suggest that miR-765 may be used as a potential biomarker for the diagnosis and prognosis of OS patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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