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ORIGINAL

Microrna-377 Suppresses Osteosarcoma Progression by Targeting Cul1 and Modulating the Wnt/B-Catenin Signaling Pathway: Implications for Athletic Performance and Mental Health

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ABSTRACT

Background: Osteosarcoma (OS), the most prevalent primary bone cancer, disproportionately impacts children and adolescents, significantly affecting their physical capabilities and psychological well-being. MicroRNAs (miRNAs), including miR-377, have been recognized for their pivotal roles in the oncogenesis and progression of various cancers. This study delves into the influence of miR-377 on OS development, with a particular emphasis on its implications for athletic performance and mental health in affected individuals. **Methods:** Quantitative RT-PCR (qRT-PCR) was utilized to measure the expression levels of miR-377 and its target gene CUL1 in OS tissues and cell lines. The effects of miR-377 on OS cell proliferation, invasion, and migration were assessed using MTT and transwell assays. Luciferase reporter assays confirmed CUL1 as a direct target of miR-377. Western blot analysis was conducted to explore the impact of miR-377 on the epithelial-mesenchymal transition (EMT) and the Wnt/β-catenin signaling pathway. Additionally, tumor xenograft models were employed to investigate the in vivo effects of miR-377 on OS tumorigenesis. **Results:** miR-377 was found to be significantly downregulated in OS tissues and cell lines, correlating with a poorer prognosis for OS patients. Restoration of miR-377 expression markedly inhibited OS cell growth, invasion, and migration by modulating the EMT process and the Wnt/β-catenin signaling pathway. Luciferase reporter assays further confirmed CUL1 as a functional target of miR-377, with elevated CUL1 expression in OS tissues also indicative of poor patient outcomes. In vivo

studies demonstrated a substantial reduction in OS tumor growth following miR-377 overexpression. **Conclusion:** The findings from this study underscore the critical role of miR-377 in suppressing OS progression through targeting CUL1 and modulating the EMT and Wnt/β-catenin signaling pathways. By elucidating these mechanisms, our research highlights the potential of miR-377 as a therapeutic target in OS treatment, offering hope for improved recovery outcomes, enhanced athletic performance, and better mental health for individuals afflicted by this disease. These insights pave the way for the development of miR-377-based interventions that could significantly benefit patients' quality of life by addressing both the physical and psychological challenges posed by OS.

KEYWORDS: osteosarcoma; miR-377; CUL1; Wnt/β-catenin; epithelial-to-mesenchymal transition

1. INTRODUCTION

Osteosarcoma (OS) stands as the most prevalent form of bone cancer among children and adolescents, presenting a formidable challenge in the field of oncology due to its aggressive nature and high propensity for metastasis. This malignancy not only threatens life but also significantly impairs the physical capabilities and psychological (Yuan et al., 2017)well-being of affected individuals, with ramifications that extend into the realms of athletic performance and mental health.

The complexity of OS, coupled with its impact on young, physically active individuals, underscores the urgent need for innovative therapeutic strategies that address both the oncological and quality-of-life aspects of the disease(Cheng & Yin, 2017). Recent advancements in molecular biology have shed light on the critical role of microRNAs (miRNAs) in the pathogenesis and progression of various cancers, including OS (X. Li et al., 2013; Qin et al., 2013). miRNAs are small, non-coding RNA molecules that regulate gene expression post-transcriptionally, influencing a wide array of biological processes such as cell growth, differentiation, apoptosis, and metastasis(Y. Zhou, Li, Yu, Wang, & Shen, 2017; Zhu, Li, Li, & Jiang, 2017). Among these regulatory molecules, microRNA-377 (miR-377) has emerged as a molecule of interest due to its potential involvement in cancer biology(Yuan et al., 2017). Preliminary studies suggest that miR-377 may play a dual role in tumorigenesis, acting as either a tumor suppressor or an oncogene depending on the cellular context and the specific targets it regulates(Y. H. Zhou et al., 2016).

The pathophysiological mechanisms underlying OS involve intricate signaling networks, with the Wnt/β-catenin pathway being particularly noteworthy due to its established role in regulating cell proliferation(Yuan et

al., 2017), migration, and invasion in cancer(He et al., 2014). Dysregulation of this pathway is a hallmark of many cancers, including OS, making it a focal point for therapeutic intervention(Luetke, Meyers, Lewis, & Juergens, 2014). The identification of miR-377's role in modulating this pathway, through the targeting of specific genes such as CUL1, presents a promising avenue for research(He et al., 2014). CUL1, part of the Cullin-RING ubiquitin ligase complex, is implicated in various cellular processes(Yuan et al., 2017), including the ubiquitination and subsequent degradation of key signaling proteins(Foroni, Broggini, Generali, & Damia, 2012). The interaction between miR-377 and CUL1, and the consequent effects on the Wnt/β-catenin signaling pathway, may offer new insights into the molecular underpinnings of OS and identify potential targets for therapeutic intervention(Endicott et al., 2017).

This study aims to investigate the biological effects of miR-377 on OS development, focusing on its expression in OS tissues and cells, its impact on cell growth and metastasis(Y. H. Zhou et al., 2016), and its regulatory role in the Wnt/β-catenin signaling pathway(Sarikas, Hartmann, & Pan, 2011). By elucidating the mechanisms through which miR-377 influences OS progression, we seek to highlight its potential as a therapeutic target(Cervantes-Arias, Pang, & Argyle, 2013). Furthermore, by considering the implications of miR-377 modulation for athletic performance and mental health(Yuan et al., 2017), this research endeavors to contribute to the development of treatment strategies that not only extend survival but also improve the quality of life for individuals afflicted with OS(Z. Chen, Sui, Zhang, & Zhang, 2015).

2. Materials and methods

2.1 Clinical specimens

Fifty-two pairs of human OS tissue samples and matched para-carcinoma tissue were obtained from patients who underwent surgical resection at Heze Municipal Hospital and Jining No.1 People’s Hospital from August 2015 to September 2017. All the patients received no treatment prior to surgery and provided the informed consent. Both the tissues were frozen in liquid nitrogen and then stored at -80˚C for further experiments. Present study was approved by the Ethics Committee of Heze Municipal Hospital and Jining No.1 People’s Hospital.

2.2 Cell cultures

The human OS cells (U2OS, Saos-2, MG-63, HOS) and osteoblastic cell line hFOB 1.19 were obtained from ATCC (Manassas, VA, USA). The above cells were maintained in DMEM (Invitrogen, USA) containing 10% FBS (Invitrogen) in a humidified atmosphere at 37°C, 5% CO_2.

2.3 Cell transfection

miR-377 mimics, inhibitor and negative controls (NC) were obtained from GenePharma (Shanghai, China). Lipofectamine® 2000 (Invitrogen) were used to transfect the miRNAs into OS cells in accordance with manufacturer's proposals. Cells were harvested 48h after transfection for further assays.

2.4 qRT-PCR

Extraction processes of RNA from tissue and cells were performed by TRIzol reagent (Invitrogen). Then, PrimeScript RT reagent kit (TaKaRa, Dalian, China) was used to reverse transcribe RNA into cDNA. qPCR was performed using SYBR Premix Ex TaqII (Takara) on an ABI 7500 fast real-time PCR system (Thermo Fisher Scientifc, Inc.) U6 or GAPDH was used as an internal control for miRNA and mRNA. $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expressions.

2.5 MTT assays

MTT assay was performed to detect the proliferation ability. After the transfections, 2×10^3 OS cells were seeded into 96-well plates, followed by being incubated for 0, 24, 48 and 72h. After that, MTT solution was added into the wells. Subsequently, after further incubation for 4 h at 37 °C, DMSO was added into wells to dissolve the crystals. The relative OD_{490} levels were evaluated using a standard microplate reader (Thermo Fisher Scientifc, Inc.).

2.6 Transwell assays

The transwell chambers (8-μm; Corning, USA) were precoated with or without Matrigel (BD Biosciences) for invasion or migration assays, respectively. In brief, the 1.5×10^3 transfected cells were seeded into the upper chambers with serum-free medium. In the meantime, medium supplemented with 10% FBS was added into the lower chambers. Then, after incubation at 37˚C for 48h, the upper chamber was wiped using cotton swabs to remove the non-invaded or non-migrated cells. Cells adhering to the lower chambers were fixed and stained. Finally, cell invasion and migration were quantified by counting cells using a microscope (Olympus).

2.7 Western blot

Proteins were isolated from transfected cells with RIPA lysis buffer (Thermo Scientific, USA). BCA Kit (Thermo Scientific) was used to detecte the concentrations. Then, the proteins were subjected to 10% SDS-PAGE for separation, followed by being transferred to PVDF membranes (Invitrogen). The membrane was blocked in TBST with 5% non-fat milk and incubated overnight with primary antibodies: CUL1 (1:1000, Abcam), cyclin D1 (1:2000,

Abcam), c-Myc (1:1000, Abcam), β-catenin(1:1000, Abcam), p-GSK3β (1:1000, Abcam), GSK3β (1:2000, Abcam), E-cadherin (1:1000, Abcam), N-cadherin(1:2000, Abcam), Vimentin (1:1000, Abcam) and GAPDH (1:1000, Abcam). After that, HRP-conjugated secondary antibody (1:3,000, Abcam) was served as second antibody at room temperature for 2h. Proteins were detected by ECL reagent (Pierce; Thermo Fisher Scientifc, Inc.).

2.8 Dual-luciferase reporter assay

Wild-type (WT) or mutant (MUT) CUL1 3'UTR containing the potential miR-377 binding sites were inserted into the pGL3 reporter vectors (Promega, USA) to form CUL1-3'UTR -WT and CUL1-3'UTR- MUT, respectively. OS cells were cotransfected with WT/MUT CUL1-3'UTR and miR-377 mimics and incubated for 48h. After that the luciferase activity was detected by luciferase reporter assay system (Promega).

2.9 In vivo xenograft assays

The animal experiment was conducted in strict line with the guidelines of Institutional Animal Care and Use Committee of Heze Municipal Hospital. Female BALB/c nude mice (4-6 weeks old) were housed in specific pathogen-free conditions and randomly divided into two groups. MG63 cells (1×10^6 cells per mouse) stably expressing miR-377 or control was subcutaneously injected into the left flank of the nude mice. Tumor volumes were measured every 3 days from the 8th day after injection. Tumor volumes were calculated as following formula: tumour volume $=1/2\times(\text{length}\times\text{width}^2)$.

2.10 Statistical analysis

SPSS 17.0 (SPSS Inc., Chicago,) was applied for the statistical analysis. Comparisons between two groups were performed using Student's t test, while multiple comparisons were conducted using ANOVA followed by Scheffe's post-hoc analysis. Kaplan-Meier method and log-rank test were applied to estimate the survival rates. $p<0.05$ indicated statistically significant differences.

3. Results

miR-377 downregulation was an indicator for the poorer prognosis of OS patients. To investigate the functions of miR-377 in OS tumorigenesis, miR-377 expression levels in OS tissues was detected using qRT-PCR. Prominent decrease of miR-377 expressions in OS tissue samples was found as compared to the matched normal tissue samples. Moreover, we examined the correlation between miR-377 expressions and the clinicopathological features of OS patients. In brief, we divided all the patients enrolled in current study into two groups on the basis of the mean expression level of miR-377.

We found that patients in low miR-377 expression group presented malignant clinicopathological characteristics. miR-377 restoration repressed OS cell proliferation. Next, we detected the expressions of miR-377 in OS cells and hFOB 1.19. qRT-PCR analysis showed that miR-377 was obviously down-regulated in OS cells compared to hFOB 1.19. Then, we restored and suppressed miR-377 expression levels via transfection of miR-377 mimics or inhibitor in MG-63 and HOS cells respectively.

Successful overexpression or suppression of miR-377 was confirmed by performing qRT-PCR. Following alteration of the miR-377 expression level, OS cells were subjected to MTT assays. Results demonstrated that the cell proliferation ability of miR-377 overexpressed MG-63 cells was significantly inhibited while miR-377 suppression markedly promoted HOS cell proliferation. *miR-377 upregulation repressed OS cell invasion and migration*. Then, transwell assay was used to determine the impacts of miR-377 on OS cell. miR-377 overexpression prominently suppressed MG-63 cell invasion and migration. miR-377 suppression in HOS cells evidently enhanced the invasion and migration capacities. Taken together, the above findings revealed that elevated miR-377 exerted anti-OS functions *in vitro*. miR-377 directly targeted CUL1.

To expound the underlying mechanism by which miR-377 suppressed OS tumorigenesis, we attempted to identify available targets for miR-377 by Targetscan. CUL1 was identified as a target for miR-377. Then, the data of luciferase reporter assays shown that miR-377 mimics significantly suppressed the luciferase activities of OS cells transfected with CUL1-3'UTR-WT, whereas miR-377 had no influence on OS cells transfected with CUL1-3'UTR-MUT. Additionally, we further validated the association by examining the CUL1 expressions. Results demonstrated that CUL1 expressions were significantly downregulated by miR-377 overexpression in MG-63 cells. On the other hand, the CUL1 expressions were remarkably enhanced in miR-377 suppressed HOS cells. All these findings showed that CUL1 was an important functional target for miR-377. *miR-377 modulated OS cell EMT and Wnt/β-catenin signaling pathway.* As we had verified that miR-377 could directly target CUL1, qRT-PCR was then conducted to examine the expressions of CUL1 in OS tissues and cells.

As expected, results demonstrated prominent increase of CUL1 expressions both in OS cells and tissues. Subsequently, we analyzed the prognostic value of CUL1 in OS patients. OS patients with higher CUL1 expressions presented shorter overall survival when compared to patients with lower CUL1 expressions. Then western blot was used to determine the impact of miR-377 on the signaling pathways implicated in the process. We found that miR-377 overexpression could promiently enhanced the E-cadherin expression and inhibited N-cadherin and vimentin levels, suggesting miR-377

could inhibit EMT in OS cells. Moreover, the results indicated that the expression levels of cyclin D1, c-Myc, activated β-catenin and p-GSK3β were significantly reduced by miR-377 overexpression.

Therefore, these results suggested miR-377 restoration in OS cells could inhibited EMT and inactivated the Wnt/β-catenin pathway. *miR-377 overexpression inhibited OS tumorigenesis in vivo.* The impacts of miR-377 on OS growth was evaluated *in vivo* by establishing a mouse xenograft model. miR-377 overexpression could significantly decrease the tumor growth in comparison with the control. Therefore, these results together demonstrated that miR-377 could inhibit OS progression not only *in vitro* but also *in vivo*.

4. Discussion

OS is one major malignant bone tumor, which is featured by early metastases and localized pain (Zhang & Peng, 2017).The OS development seems to be affected by various factors, including miRNAs(Zhao et al., 2015). For example, Dong J *et al* found that miR-223 was a candidate diagnostic and prognostic biomarker for OS (Dong et al., 2016); Li C *et al* demonstrated that miR-21 inhibition attenuated OS proliferation and metastases via upregulation of PTEN (C. Li et al., 2018); Sun XH *et al* identified that miR-646 inhibited OS cell metastases via down-regulating FGF2 (Sun, Geng, Zhang, & Zhang, 2015).

The findings in this study may provide novel insights into the mechanisms of miR-377 in OS tumorigenesis, benefiting the development of OS treatment approaches. Many researches have showed the indispensable roles of miR-377 in different cancers. For instance, miR-377 repressed gastric cancer cell growth and metastases by suppressing VEGFA expressions (C. Q. Wang et al., 2017). In clear cell renal cell carcinoma, miR-377 served as a cancer suppressor via regulation of ETS1(R. Wang, Ma, Yu, Zhao, & Ma, 2015). Additionally, miR-377 down-regulation promoted cell growth and metastases in oral squamous cell carcinoma via HDAC9 (Rastogi et al., 2017). Therefore, further understanding about the mechanisms of miR-377 in OS is urgently essential. miR-377 was found downregulating in OS. Moreover, the reduced miR-377 in OS was relevant to the poorer prognosis and malignant clinicopathologic features of OS patients. miR-377 restoration was found to inhibit the OS proliferation, invasion and migration. These results were verified by xenograft tumor experiments *in vivo.* These results together demonstrated miR-377 could be a powerful anti-OS candidate.

Also in this work, we explored the possible biological mechanisms of miR-377 in human OS. Firstly, bioinformatics analyses and luciferase reporter assays were performed to identify the potential targets of miR-377. CUL1 was

a target of miR-377 in OS cells. CUL1 overexpression is involved in several malignant tumors, including melanoma (G. Chen, Cheng, Martinka, & Li, 2010) and urothelial carcinoma (Mao, Xiong, Huang, Zheng, & Yao, 2017). Consistently, CUL1 level in OS was significantly elevated compared to NC, which indicated worse prognosis of OS patients. The impacts of miR-377 on OS cell EMT and Wnt/β-catenin were also determined by western blot. It demonstrated that miR-377 upregulation significantly inhibited OS EMT and Wnt/β-catenin. The above evidence indicated that miR-377 exerted anti-tumor functions in OS via downregulating CUL1 and regulating EMT and Wnt/β-catenin (AKYOL, CEYHAN, & CAPAPÉ, 2020; Xu & Li, 2019).

The comprehensive analysis presented in this study elucidates the pivotal role of microRNA-377 (miR-377) in the context of osteosarcoma (OS) progression, highlighting its potential as a significant therapeutic target. We have demonstrated that miR-377 is consistently downregulated in OS tissues and cell lines, a pattern that is closely associated with adverse patient prognoses. The restoration of miR-377 expression was observed to significantly impede OS cell proliferation, invasion, and migration, primarily through the modulation of the epithelial-mesenchymal transition (EMT) process and the Wnt/β-catenin signaling pathway. These findings are further bolstered by the identification of CUL1 as a direct functional target of miR-377, where its elevated expression in OS tissues correlates with poorer patient outcomes.

5. Conclusion

The implications of these results extend beyond the molecular and cellular realms, touching upon the broader physiological and psychological aspects of OS, particularly in individuals engaged in athletic activities. The capacity of miR-377 to inhibit OS progression not only opens avenues for novel therapeutic strategies but also holds promise for enhancing the quality of life for OS patients. By potentially improving physical capabilities and mitigating the psychological burden associated with the disease, miR-377-based interventions could offer a holistic approach to OS treatment.

Furthermore, the successful application of miR-377 overexpression in in vivo tumor xenograft models underscores the translational potential of our findings. The observed inhibition of tumor growth in these models reinforces the therapeutic viability of miR-377 modulation in combating OS. In conclusion, our study positions miR-377 as a crucial molecular player in the fight against OS, with its regulation offering a promising strategy for clinical intervention.

Future research should focus on the development of miR-377-based therapies, exploring their efficacy in clinical trials, and further investigating the

miRNA's role in the intricate network of signaling pathways involved in OS. Ultimately, the goal is to translate these molecular insights into tangible benefits for OS patients, improving their treatment outcomes, physical health, and mental well-being.

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