Review Article MicroRNA-212-5p targeted xiap inhibits malignant behavior of osteosarcoma cells

Jian Guan¹, Hao Mo¹, Juliang He¹, Shian Liao¹, Xinli Zhan²

¹Department of Bone and Soft Tissue Surgery, Guangxi Medical University Cancer Hospital, No. 71 He Di Road, Nanning 530021, Guangxi, China; ²Department of Spine and Osteopathy Ward, The First Affiliated of Guangxi Medical University, No. 6 Shuangyong Road, Nanning 530021, Guangxi, China

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Abstract: Background: Osteosarcoma (OS) is a fatal primary bone cancer in humans. In recent years, it has been reported that microRNAs (miRNAs) are closely related to the emergence of OS. Moreover, miR-212-5p and X-linked apoptosis protein (XIAP) are abnormally expressed, according to cancer research. However, the function and mechanisms of the two in OS have not been clarified. Objective: The present study aimed to explore the effects and molecular mechanisms of miR-212-5p and X-linked apoptosis protein (XIAP) in osteosarcoma (OS). Methods: A total of 80 tissue samples of patients with OS, from May 2012 to May 2014, were obtained. Furthermore, miR-212-5p and XIAP levels in tissues and cells were detected. *In vitro* cell experiments were carried out, aiming to observe the roles of miR-212-5p and XIAP on the biological behavior of OS cells. Results: Results suggest that miR-212-5p was inhibited in OS tissues and cells, while XIAP was significantly overexpressed. Overexpression of miR-212-5p or silencing of XIAP may inhibit proliferation, invasion, and migration of OS cells, promoting cell apoptosis. However, these biological behaviors were the opposite in miR-212-5p knockdown or XIAP overexpression. XIAP could be used as a direct target of miR-212-5p through luciferase activity measurement. Overexpression of XIAP eliminated the biological behavior of OS cells. Moreover, miR-212-5p is related to OS and can directly target XIAP, inhibiting malignant behavior of OS cells. Moreover, miR-212-5p/XIAP can be a latent treatment target for OS.

Keywords: miR-212-5p, X-linked apoptotic protein, osteosarcoma, biological function

Introduction

Osteosarcoma (OS) is a familiar primary bone carcinoma in humans, commonly found in young adults and adolescents [1]. Although survival rates of OS patients have improved significantly in recent years, some patients still have poor response to chemotherapy. Even after radical resections or chemotherapy, OS may proliferate and metastasize, eventually leading to death [2, 3]. Molecular mechanisms of OS have received extensive attention in the past ten years, but have yet to be clarified [4]. Therefore, analysis of molecular mechanisms related to OS progress is crucial to the exploration of new treatment methods for OS.

MicroRNAs (miRNAs) are a kind of mini-type single-stranded non-coding RNA. They can interact with target genes to regulate gene expression [5]. Differences in miRNA expression in tumors or normal tissues act on occurrence and disease courses of various malignant tumors [6, 7]. Abnormal regulation of miRNAs can play a role in tumor initiation, progression, or therapeutic resistance by regulating multiple target genes [8]. Moreover, miR-212 shows an imbalance in human cancer and is located on chromosome 17p13.3 [9]. It plays an inhibitory role in thyroid cancer and lung cancer [10, 11]. However, some studies have shown that miR-212 is carcinogenic in pancreatic cancer [12]. Thus, miR-212 plays a specific role in tumors, perhaps caused by different tumor growth environments [13]. Importantly, the effects of miR-212-5p on OS remain unknown.

X-linked apoptosis protein (XIAP) is a powerful inhibitor of caspase. It acts on cell apoptosis and is a key barrier in inducing tumor cell apop-

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Gene	Forward	Reverse
miR-212-5p	5'-CCTCGACTGGGGGTGTAAACAT-3'	5'-GTGGAGTCGATTGCGTGTC-3'
XIAP	5'-CCGTGCGGTGCTTTAGTTGT-3'	5'-TTCCTCGGGTATATGGTGTCTGAT-3'
U6	5'-GCTTCGGCAGCACATATACTAAAAT-3'	5'-CGCTTCAGAATTTGCGTGTCAT-3'
GAPDH	5'-TGGTCACCAGGGCTGCTT-3'	5'-AGCTTCCCGTTCTCAGCCTT-3'

 Table 1. Primer sequences

tosis [14]. Previous studies have indicated that XIAP inhibited the malignant development of tumor cells and incubated the resistance of various cancers to chemotherapeutic drugs. In triple negative breast cancer, XIAP can be directly mediated by miR-429 to induce δ -tocotrienols, inducing tumor cell apoptosis [15]. It has also been reported that inhibition of XIAP could inhibit the development of OS cells and enhance the sensitivity of cisplatin and adriamycin in the treatment of OS [16]. These studies have confirmed that XIAP is feasible for gene therapy of tumors [17]. However, little is known about the interaction of miR-212-5p with XIAP in OS.

Therefore, the present study observed the roles of miR-212-5p on the biological behavior of OS cells by regulating XIAP, aiming to provide reference for targeted gene therapy of OS.

Data and methods

Data of the patients

A total of 80 patients with OS, from May 2012 to May 2014, were selected for the current study. OS tissues and adjacent normal tissues were obtained during surgery. All patients were diagnosed as OS via histopathological biopsies [18]. Biopsies were performed before radiotherapy and chemotherapy. Patients with other malignant tumors were excluded. The present research was approved by the Ethics Committee. All included patients provided informed consent.

Cell culturing

Osteosarcoma cell lines U2-OS, SAOS-2, and normal human osteoblast hFOB1.19 were purchased from the American Type Culture Collection. The cell line was placed in RPMI-1640 (Shanghai Yuduo Biotechnology Co., Ltd., China), containing 10% FBS. It was cultured in an incubator at 37°C with 5% CO₂.

Cell transfection

For cell transfection, miR-212-5p mimics, miR-212-5pm inhibitors, miR-NC, siRNA-XIAP, pc-DNA3.1-XIAP, and XIAP-NC were purchased from Thermo Fisher Scientific. When the cell fusion degree reached 60-70%, the cells were transfected using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Shanghai, China).

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted by TRIzol Reagent (Shanghai Yuduo Biotechnology Co., Ltd., China). RNA was reverse transcribed into cDNA using the reverse transcription kit (Takara). Quantitative PCR was applied on the 7900HT system (Applied Biosystems, USA) using the SYBR™ Green One-Step qPCR Kit (Invitrogen, USA). U6 was taken as an endogenous reference of miR and GAPDH was taken as an endogenous reference of mRNA. Data was tested using the 2^{-ΔΔCt} method. The primer sequence was purchased from Thermo Fisher, Shanghai, China, as shown in **Table 1**.

Cell proliferation testing

A CCK8 kit (Shanghai Yeasen BioTechnologies Co., Ltd., China) was used for transfection. After 12 hours of transfection, cells with 5×10³ cells/ well were inoculated into 96-well plates. The culture media was then removed and 10 µl of CCK8 was put into each well. Incubation was continued for 4 hours. OD values were detected at 450 nm using a Multiskan[™] FC enzyme analyzer (Thermo Fisher Scientific, Shanghai, China).

Scratch testing

When the cell fusion degree reached 90%, starvation was carried out for 12 hours using RPMI-1640 medium (serum-free). Glass slides were used as a support, then scratches and photographs were taken on the central axis of each well. The cells were incubated in a cell incubator and photographed every 24 hours.

Transwell invasion testing

Cell concentrations were improved to 1×10^5 cells/mL, then the chamber was placed in a 24-well plate of RPMI-1640 medium with 500 µl/well. After incubation for 24 hours, the chamber was taken out, rinsed twice with PBS solution, and fixed with 95% ethanol for 15 minutes. Cotton swabs were used to gently wipe away non-invasive cells. The chamber was then dyed with crystal violet for 20 minutes. Ten fields of view were randomly selected using an inverted microscope, aiming to observe membrane penetration of the cells.

Apoptosis testing

Transfected U2-OS and SAOS-2 cells were suspended in 500 μ l buffer. Annexin V, labeled with propidium iodide/fluorescein isothiocyanate, was added for staining and placed at room temperature for 15 minutes. Apoptotic cells were measured using Attune NxT (Thermo Fisher Scientific).

Double luciferase reporter gene testing

DNA fragments of specific sites of XIAP-WT and mutant (XIAP-MUT) XIAP 3'UTR were composed and inserted into psiCHECK2 vector (GenePharma, Shanghai, China). Logarithmic growth phase cells were taken out. In accordance with manufacturer instructions, miR-212-5p mimics, miR-212-5p inhibitors, miR-NC, and psiCHECK2 vectors were co-transfected into the cells. After 48 hours of transfection, luciferase activity levels were detected.

Western blotting

Cells were obtained at 48 hours after transfection and lysed with RIPA buffer (Thermo Scientific, USA). Total proteins were then extracted. The BCA method was used for protein quantification. Subsequently, 20 µg of protein was segregated by 10% SDS-PAGE gel electrophoresis and sealed on PVDF with 5% skim milk for 1 hour. XIAP primary (1:1000) and anti-GAPDH mouse monoclonal antibodies (1:1000) were added and incubated overnight. After washing with PBS, a secondary antibody was added and incubated together. ECL was developed and the gray value was analyzed using the Gel-Pro analyzer. The above antibody reagents were purchased from Shanghai Beyotime Biotechnology Co., Ltd. (China).

Statistical analysis

GraphPad 6.0 (GraphPad Software, La Jolla, CA, USA) was applied for statistical analysis. Data are expressed as mean ± SD. Student's t-tests were applied, exploring differences between the groups. ANOVA was applied for analysis of multiple groups. LSD-t tests were used for post-event pairwise comparisons, while Bonferroni's tests were used for pairwise comparisons after multiple time points. Pearson's testing was also applied, exploring correlation levels of miR-212-5p with XIAP. P<0.05 indicates statistical significance.

Results

miR-212-5p and XIAP in OS tissues and cells

The current study detected miR-212-5p and XIAP in OS tissues and adjacent tissues using qRT-PCR. It was found that miR-212-5p was evidently downregulated in OS tissues (Figure 1A), while XIAP was evidently enhanced (Figure 1B). In addition, miR-212-5p showed a negative correlation with XIAP (Figure 1C). Similarly, compared with hFOB1.19 cells, miR-212-5p in U2-OS and SAOS-2 cells was inhibited (Figure 1D), while XIAP was evidently enhanced in U2-OS and SAOS-2 cells (Figure 1E).

Overexpression of miR-212-5p enhanced malignant phenotype of OS cells

U2-OS and SAOS-2 were applied for subsequent experiments, while miR-212-5p mimics were applied to overexpress miR-212-5p in U2-OS cells. Moreover, miR-212-5p inhibitors were used to restrain miR-212-5p in SAOS-2 cells (Figure 2A). Observing the roles of miR-212-5p, CCK8 method results indicated that overexpression of miR-212-5p inhibited U2-OS cell proliferation. Conversely, after miR-212-5p was knocked down, SAOS-2 cell proliferation ability was significantly enhanced (Figure 2B). Transwell invasion and migration tests indicated that overexpression of miR-212-5p might attenuate the invasion ability of U2-OS cells, while the mobility of U2-OS cells decreased significantly. After miR-212-5p was knocked-down,



Figure 1. Expression of miR-212-5p and XIAP in OS tissues and cells. A. Expression of miR-212-5p in OS tissues detected by qRT-PCR. B. Expression of XIAP in OS tissues detected by qRT-PCR. C. Pearson's test analysis showed a negative correlation between miR-212-5p and XIAP expression in OS tissues. D. Expression of miR-212-5p in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. E. Expression of XIAP in hFOB1.19, U2-OS, and SAOS-2 cells detected by qRT-PCR. Note: **indicates P<0.001.

invasion and mobility levels of SAOS-2 cells were obviously enhanced (**Figure 2C**, **2D**). Flow cytometry was applied to explore the roles of miR-212-5p on cell apoptosis. Overexpression of miR-212-5p was shown to induce U2-OS cell apoptosis, while knock-down of miR-212-5p was shown to inhibit SAOS-2 cell apoptosis (**Figure 2E**). In short, miR-212-5p acted on the malignant phenotype of OS cells.

XIAP was the direct target of miR-212-5p

Present results confirmed the potential target gene of miR-212-5p on OS cells. Targetscan and the miRDB database were applied to predict the targets of miR-212-5p. As shown in Figure 3A, XIAP was a potential target for miR-212-5p. Results further showed that miR-212-5p mimics that co-transfected with XIAP-WT inhibited luciferase activity. In contrast, restriction of miR-212-5p significantly increased luciferase activity, while luciferase activity of XIAP-MUT was not restricted (Figure 3B). Western blotting was applied to detect XIAP in OS cells after miR-212-5p was overexpressed or inhibited. As shown in Figure 3C, XIAP protein in U2-OS cells was significantly decreased after overexpression of miR-212-5p, while XIAP protein in SAOS-2 was evidently upregulated after knock-down of miR-212-5p.

XIAP silencing improved malignant phenotype of OS cells

The current study transfected U2-OS with pcDNA3.1-XIAP to achieve overexpression. The aim was to determine the roles of XIAP on the biological phenotype of OS cells. Thus, siRNA-XIAP was used to knock-down XIAP in SAOS-2. Results showed that XIAP in U2-OS increased significantly after transfection of pcDNA3.1-XIAP, while XIAP in SAOS-2 cells decreased significantly after transfection of siRNA-XIAP (Figure 4A). The roles of XIAP on proliferation, invasion, migration, and apoptosis were observed. Results showed that, after XIAP was over-expressed, proliferation, invasion, and migration were increased, while apoptosis rates were inhibited. After XIAP was silenced, the opposite results were obtained (Figure 4B-E).

Overexpression of XIAP eliminated the biological behavior of miR-212-5p on OS

Aiming to observe whether XIAP can eliminate the biological behavior of miR-212-5p,



Figure 2. Overexpression of miR-212-5p ameliorated malignant phenotype of OS cells. A. qRT-PCR was used to verify the efficiency of miR-212-5p mimics and miR-212-5p1 hibitor transfecting U2-OS and SAOS-2 cells, respectively. B. CCK8 testing was used to determine the proliferation ability of U2-OS and SAOS-2 cells. C. Transwell invasion testing was used to determine the invasion ability of U2-OS and SAOS-2 cells. D. Scratch testing was used to determine the migration ability of U2-OS and SAOS-2 cells. D. Scratch testing was used to determine the migration ability of U2-OS and SAOS-2 cells. E. Flow cytometry was used to determine the apoptosis ability and apoptosis figure of U2-OS and SAOS-2 cells. Note: *indicates P<0.05, **indicates P<0.01, ***indicates P<0.001.

pcDNA3.1-XIAP and miR-212-5p mimics were transfected into U2-OS and SAOS-2. Results showed that, compared with transfected miR-212-5pmics, the co-transfection of PC DNA 3.1-XIAP+miR-212-5p mimics could promote the proliferation of U2-OS and SAOS-2. Moreover, cell invasion and migration were enhanced, while cell apoptosis ability was weakened. Co-transfection of PcDNA3. 1-XIAP+ miR-212-5p mimics could reverse the effects of miR-212-5p over-expression on U2-OS and SAOS-2 cell biological phenotype. Western blotting results showed that transfection of pcDNA3.1-XIAP+miR-212-5p mimics could turn the role of overexpression of miR-212-5p on XIAP protein. See Figure 5.

Discussion

More and more studies have shown that the exploration of miRNAs may provide new therapeutic targets for OS [19, 20]. In this experiment, it was revealed that miR-212-5p acted as an inhibitor in OS, while XIAP was highly expressed. Further basic experiments indicated that overexpression of miR-212-5p or silencing of XIAP could inhibit the malignant phenotype. Conversely, miR-212-5p knockdown and XIAP overexpression may promote the malignant phenotype, while XIAP overexpression could turn the impact of miR-212-5p knockdown. These studies revealed that miR-212-5p/XIAP may be a new approach to OS therapy.



Figure 3. XIAP was the direct target of miR-212-5p. A. XIAP was a potential target of miR-212-5p. B. Luciferase activity of XIAP-WT or XIAP-MUT reporter gene and miR-212-5p mimics or miR-212-5p1 hibitors and corresponding miR-NC transfected OS cells. C. Expression of XIAP protein in OS cells was determined by Western blotting. Note: **indicates P<0.01, ***indicates P<0.001.

In recent years, considerable progress has been made in the pathogenesis of OS [21]. However, understanding of the molecular mechanisms of the disease, tumor occurrence, disease progression, or metastasis and relapse remains unclear [22]. Recent studies have confirmed that miRNAs can play a role in tumor progression by regulating their targets [23]. Interestingly, single miRNAs often play a dual role in carcinogenesis or cancer suppression in different cancers [24]. In triple negative mammary cancer (TNBC), miR-212-5p in tumor tissues was significantly downregulated. This was closely related to vascular invasion, lymph node metastasis, and tumor size, inhibiting the progression of TNBC by downregulating Prrx2 [25]. However, miR-212-5p was overexpressed in non-small cell lung carcinoma (NSCL). Patients with high miR-212-5p had a short overall survival and could participate in disease progression through Id3-mediated PI3K/Akt pathways [26]. In the present research, miR-212-5p was

low expressed in OS tissues through gRT-PCR detection, suggesting that miR-212-5p acted as a tumor suppressor gene in OS. Next, this study further conducted in vitro experiments, aiming to observe the biological function of miR-212-5p. It was found that overexpression of miR-212-5p could inhibit the malignant phenotype of OS cells, while miR-212-5p knock-down could accelerate the malignant phenotype of OS cells. Moreover, miR-212-5p was likely to have special significance for OS cell movement. In the study of Wu et al., miR-212-3p in bladder carcinoma was evidently downregulated and nuclear factor IA was its target. After miR-212-3p over-expression or NFIA knock-down, the number of cell apoptosis might increase [27]. In lung adenocarcinoma and acute myeloid leukemia, miR-212-5p was found to have low expression. Regarding in vitro experiments, miR-212-5p could directly tar-

get gene curl receptor 5 and further affect the biological behavior of acute myeloid leukemia cells [28]. Present results suggest that miR-212-5p acts on proliferation, invasion, migration, and apoptosis of OS cells. However, the deeper mechanisms of miR-212-5p in OS remain unknown.

Further observing the action mechanisms of miR-212-5p in OS, the current study predicted the potential target of miR-212-5p using the database. Results showed that XIAP was associated with miR-212-5p. Moreover, overexpression of miR-212-5p restricted XIAP protein, while knock-down of miR-212-5p significantly increased XIAP protein expression. XIAP was also found to be significantly upregulated and negatively correlated with miR-212-5p by detecting OS tissues. Results showed the target roles of miR-212-5p of XIAP. It was found that XIAP played a role of a carcinogen in various malignant tumors and could inhibit apopto-



Figure 4. Silencing of XIAP improved malignant phenotype of OS cells. A. qRT-PCR was used to verify the efficiency of pcDNA3.1-XIAP and siRNA-XIAP transfecting U2-OS and SAOS-2 cells, respectively. B. CCK8 testing was used to determine the proliferation ability of U2-OS and SAOS-2 cells. C. Transwell invasion testing was used to determine the invasion ability of U2-OS and SAOS-2 cells. D. Scratch testing was used to determine the migration ability of U2-OS and SAOS-2 cells. E. Flow cytometry was used to determine the apoptosis ability and apoptosis figure of U2-OS and SAOS-2 cells. Note: *indicates P<0.01, ***indicates P<0.001.

sis of tumor cells [29, 30]. For example, in lung cancer, XIAP expression was shown to upregulated and could be directly regulated by miR-CHA1 to affect the proliferation and apoptosis [31]. Sun et al. found that the reduction of XIAP greatly weakened proliferation, migration, and invasion levels of gastric carcinoma cells [32]. The current study showed that XIAP could realize proliferation, invasion, and mobility after overexpression and could inhibit cell apoptosis. However, there were opposite results after XIAP silencing. Similar to previous reports, XIAP might be involved in the growth of OS. Finally, it was concluded that overexpression of XIAP eliminated the biological behavior of miR-212-5p overexpression. Current results suggest that miR-212-5p could regulate XIAP and further affect the progress of OS cells. In Hou et al., it was believed that XIAP played a key role in controlling cell death in mitosis, suggesting that phosphorylation of XIAP reduced the threshold of cell death in mitosis [33]. Regulation of cell death is essential for cancer cell response to drug therapy, leading to mitotic arrest [34]. Future research should focus on the roles of miR-212-5p/XIAP on cell apoptosis.

In conclusion, miR-212-5p has been proven to be related to OS. It can directly target XIAP to inhibit malignant behavior of OS cells. Therefore, miR-212-5p/XIAP should be considered a latent treatment target for OS.

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Figure 5. Overexpression of XIAP eliminated the biological behavior of miR-212-5p on OS. A. CCK8 testing was used to determine the proliferation ability of U2-OS and SAOS-2 cells transfected with miR-NC, miR-212-5p mimics, and pcDNA3.1-xiap+miR-212-5p mimics. B. Transwell invasion testing was used to determine the invasion ability of U2-OS and SAOS-2 cells transfected with miR-NC, miR-212-5p mimics. C. Scratch testing was used to determine the migration ability of U2-OS and SAOS-2 cells transfected with miR-NC, miR-212-5p mimics, and pcDNA3.1-XIAP+miR-212-5p mimics. D. Flow cytometry was used to determine the apoptosis ability and apoptosis figure of U2-OS and SAOS-2 cells transfected with miR-NC, miR-212-5p mimics. E. Western blotting was used to detect XIAP protein expression and protein bands in U2-OS and SAOS-2 cells transfected with miR-NC, miR-212-5p mimics. Note: *indicates P<0.05, **indicates P<0.01, ***indicates P<0.001.

Disclosure of conflict of interest

None.

Address correspondence to: Xinli Zhan, Department of Spine and Osteopathy Ward, The First Affiliated of Guangxi Medical University, No. 6 Shuangyong Road, Nanning 530021, Guangxi, China. Tel: +86-13907864389; E-mail: buzhuangxianlan995@163. com

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