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Loss of miR-217 promotes osteosarcoma cell proliferation through targeting SETD8

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Recently, many kinds of microRNAs (miRNAs) have been found to play a critical role in progression of osteosarcoma (OS). miR-217 was reported to function as a tumor suppressor in a number of human cancers but its precise mechanism to exert the suppressive role remains to be investigated. In this study, we found that miR-217 was downregulated in OS tissues and its downregulation predicts poor overall survival of OS patients. Importantly, we found that a lower expressed miR-217 in OS cell lines inhibited the cell proliferation and invasion *in vitro*. By bioinformatic analysis, we found that miR-217 targeted the SET Domain-Containing Protein 8 (SETD8), and there was a negative correlation between them in OS tissues. Furthermore, we found that miR-217 abolished the stimulation effect of SETD8 on cell proliferation and invasion. Taken together, our data provide solid evidence that miR-217 functions as tumor suppressor in OS, and its tumor-suppressive effect is exerted through interaction with SETD8.

1. Introduction

Osteosarcoma (OS) is a highly malignant cancer type mainly prevalent in children and young adults (Ward et al. 2014). Despite advances in therapeutic strategies including neoadjuvant and adjuvant chemotherapy have been made, the overall survival rates of OS patients remain unsatisfactory (Anderson et al. 2016). The main obstacle is that we did not fully understand the mechanisms responsible for the development and progression of OS (Misaghi et al. 2018). To address this issue, extensive effects have been put on the elucidating the molecules that control OS progression.

MicroRNAs (miRNAs) are non-coding RNAs (17-25 nucleotides) with diverse functions in disease progression (Pillai et al. 2007; Lu et al. 2005). To date, over 1,000 miRNAs have been identified to be associated with tumorigenesis (Zhou et al. 2013; Di Leva et al. 2014). MiRNAs play important roles in various biological processes by binding to the 3'-untranslated region (3'-UTR) of target genes (Bartel et al. 2004), while one miRNA is capable to interact with multiple target genes and one gene can be regulated by multiple miRNAs (Bartel et al. 2004; Hammond et al. 2015). Many studies have shown that miRNAs are abnormally expressed in human cancer cells and play critical roles in tumor initiation and progression (Shen et al. 2014; Wei et al. 2015; Chen et al. 2017; Jiang et al. 2017; Hong et al. 2017). For example, miR-217 was found to be responsible for to dysregulation in various human cancers including pancreatic adenocarcinoma, hepatocellular carcinoma, OS, renal cell carcinoma (Shen et al. 2014; Wei et al. 2015; Chen et al. 2017; Jiang et al. 2017; Hong et al. 2017). It was also reported that miR-217 could target Wnt5a in OS to regulate various cell behaviors including proliferation, apoptosis, migration, and invasion (Wei et al. 2015). In addition, WASF3 was identified as target for miR-217 and is involved in the tumor growth and metastasis regulation processes mediated by miR-217 (Shen et al. 2014). However, the authors did not further investigate the molecules involved in the miR-217 mediated OS progression process.

In this report, we focus our attention on the detailed regulatory mechanism of miR-217 in the development and progression of OS. First, we determined miR-217 expression levels in human OS cell lines and tissues. Next, we investigated the effects of miR-217 on OS cell growth and invasion *in vitro*. Finally, we determined the downstream target molecules of miR-217 to elucidate the regulatory mechanism of miR-217 in the development and progression of OS.

2. Investigations and results

2.1. Downregulation of miR-217 in OS tissues and cell lines

Expression of miR-217 was detected by RT-qPCR in OS tissues and matched noncancerous tissues. We revealed that miR-217 expression was significantly reduced in OS tissues compared with the matched noncancerous tissues (Fig. 1A). Subsequently, we detected miR-217 expression in OS cell lines. The results demonstrated that miR-217 expression was also downregulated in OS cell lines compared with the hFOB1.19 cell line (Fig. 1B). We found miR-217 expression to be the lowest in MG-63 cell line, and therefore it was used in the following experiments (Fig. 1B).

2.2. Clinical significance of miR-217 expression in OS

To assess the correlation between miR-217 expression and clinicopathological features, we divided 52 enrolled patients into two groups based on the median value of miR-217 expression. The patients with miR-217 expression levels higher than the median value were classified into a high miR-217 group (n=24). The others formed a low miR-217 group (n=28). The correlation analysis showed that low miR-217 expression was significantly correlated with larger tumor size (P=0.034) and advanced tumor stage (P=0.018). However, we did not find a significant correlation between miR-217 expression and age and gender (Table). Kaplan-Meier survival analysis showed that low miR-217 expression was correlated with shorter overall survival (P=0.023, Fig. 1C)

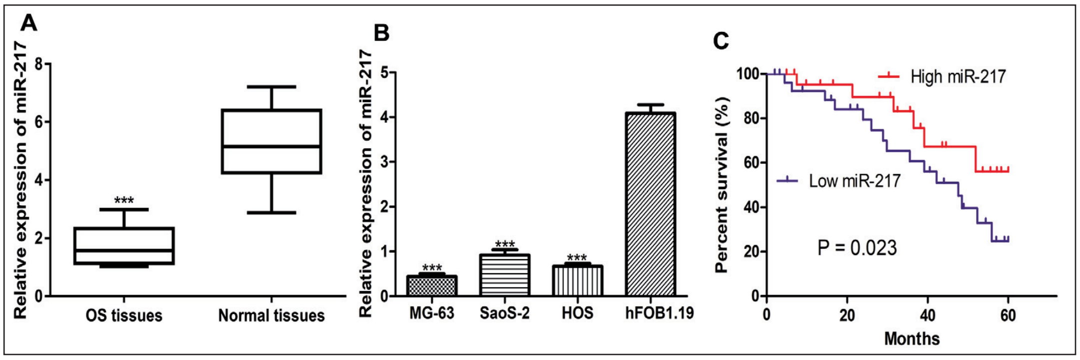

Fig. 1: MiR-217 expression was downregulated in OS tissues and cell lines. A, miR-217 expression was significantly downregulated in OS tissues compared with matched normal tissues. B, miR-217 expression was significantly downregulated in OS cell lines MG-63, SaoS-2 and HOS compared with the human normal osteoblastic hFOB1.19. C, Kaplan–Meier survival curves revealed patients with low miR-217 had significantly poorer prognosis than those with high miR-217. (***P<0.001) OS: osteosarcoma; miR-217: microRNA-217.

Table: Correlations of miR-217 expression and clinicopathological features of osteosarcoma

Features	No.	miR-217 expression Low (n=28)	High (n=24)	P value*
Age (years)				
≥ 60	25	15	10	0.228
< 60	27	13	14	
Gender				
Male	28	16	12	0.248
Female	24	12	12	
Tumor size (cm)				
≥ 5	32	17	15	0.034
< 5	20	11	9	
Tumor stage				
I-II	20	9	11	0.018
III	32	19	13	

*Chi-square test. miR-217: microRNA-217.

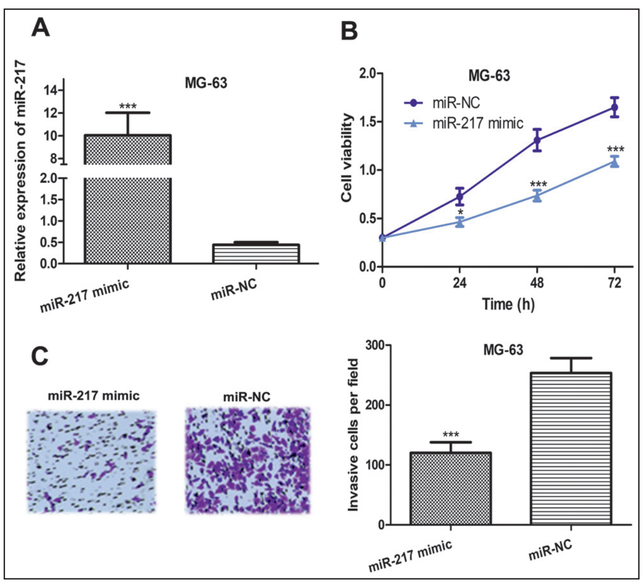

Fig. 2: Overexpression of miR-217 inhibits MG-63 cell proliferation and invasion. A, miR-217 expression in MG-63 cell line after synthetic miRNAs transfection. B, CCK-8 assay to detect cell proliferation in MG-63 cell line after synthetic miRNAs transfection. C, Transwell invasion assay to detect cell invasion in MG-63 cell line after synthetic miRNAs transfection. (***P<0.001, *P<0.05) miR-217: microRNA-217; CCK-8: cell counting kit-8; miR-NC: negative control miRNA.

2.3. MiR-217 inhibited OS cell proliferation and invasion

We further measured the biological role of miR-217 in OS progression. The synthetic miRNAs were transfected to OS cells and RT-qPCR was performed to evaluate the transfection efficiency. As shown in Fig. 2A, miR-217 mimic significantly increased the levels of miR-217 in MG-63 cells. CCK-8 assay showed that cell proliferation was significantly reduced by miR-217 mimic (Fig. 2B). Transwell invasion assay revealed that overexpression of miR-217 impeded cell invasion compared with miR-NC group (Fig. 2C).

2.4. MiR-217 targeted SETD8 and inhibited the expression of p21

We further detected potential targets of miR-217 to fully understand the role of miR-217 in OS. SETD8 was predicted as a target of miR-217 by TargetScan (Fig. 3A). The luciferase activity assay revealed that miR-217 mimic significantly reduced the luciferase activity of SETD8-WT, but not SETD8-MUT (Fig. 3B). Further, we

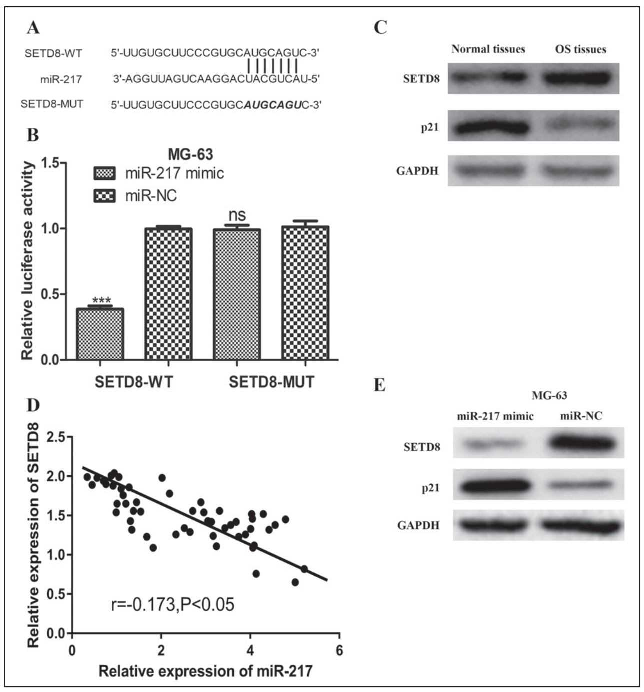

Fig. 3: SETD8 was a direct target of miR-217 in OS. A, Predicted binding site for miR-217 in SETD8 3'-UTR. B, Relative luciferase activities of MG-63 cells with SETD8-WT or SETD8-MUT and miR-217 mimic or miR-NC co-transfection. C, SETD8 or p21 protein expression in OS tissues. D, Inversely correlation between miR-217 and SETD8 in OS tissues. E, SETD8 or p21 protein expression in MG-63 cell line with synthetic miRNAs transfection. (***P<0.001, ns: not significant) miR-217: microRNA-217; OS: osteosarcoma; miR-NC: negative control miRNA; WT: wild-type; MUT: mutant.

detected the SETD8 expression was upregulated, while p21 expression was downregulated in OS tissues compared with the adjacent normal tissues (Fig. 3C). Moreover, we found an inverse correlation between miR-217 and SETD8 expression in OS tissues (Fig. 3D). Overexpression miR-217 in MG-63 cell line led to decreased SETD8 expression but increased p21 expression (Fig. 3E).

2.5. *Overexpression of SETD8 impaired the miR-217 mimic-induced inhibition of proliferation and invasion in OS cells*

To investigate whether the role of miR-217 in OS was mediated by SETD8, we overexpressed SETD8 in the MG-63 cell line. We found that SET8 was remarkably increased while p21 was decreased by overexpression of SETD8 (Fig. 4A). CCK-8 and wound-healing assays revealed that overexpression of SETD8 abolished the effects of miR-217 mimic on cell proliferation and invasion in MG-63 cell line, indicating miR-217 exert its role in OS through regulating SETD8/p21 (Figs. 4B,C).

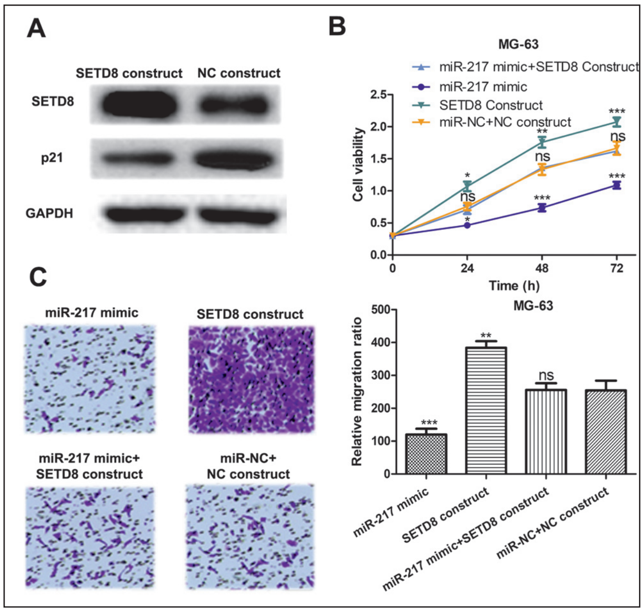

Fig. 4: Overexpression of SETD8 impairs miR-217 mimic induced OS cell proliferation and invasion inhibition. A, SETD8 or p21 expression in MG-63 cell line with SETD8 construct or miR-217 mimic transfection. B, CCK-8 assay to detect cell proliferation in MG-63 cell line after synthetic miRNAs or SETD8 construct transfection. C, Transwell invasion assay to detect cell invasion in MG-63 cell line after synthetic miRNAs or SETD8 construct transfection. (***P<0.001, *P<0.05) miR-217: microRNA-217; CCK-8: cell counting kit-8; miR-NC: negative control miRNA.

3. Discussion

Many studies have shown that miR-217 is involved in the human cancer progression process by targeting multiple genes (Shen et al. 2014; Wei et al. 2015; Chen et al. 2017; Jiang et al. 2017; Hong et al. 2017). It was reported that miR-217 can function as either tumor suppressor or oncogene in a cell-type context (Shen et al. 2014; Wei et al. 2015; Chen et al. 2017; Jiang et al. 2017; Hong et al. 2017). For instance, Wang et al. (2017) reported that miR-217 could promote glioblastoma cell malignancy both *in vitro* and *in vivo* through regulating the expression of YWHAG (Wang et al. 2017). On the contrary, numerous studies revealed that miR-217 functions as tumor suppressor in OS through targeting different genes including Wnt5a and WASF3 (Shen et al. 2014; Wei et al. 2015). These findings advanced our understanding regarding the biological role of miR-217 in OS. However, the detailed regulatory mechanisms of miR-217 in OS remain to be explored. In the present study we determined miR-217 expression levels in OS and normal tissues and revealed that miR-217 expression was reduced in OS tissues, which was in line with previous reports (Shen et al. 2014; Wei et al. 2015; Hong et al. 2017). Also, we found low miR-217 expression associated with large tumor size, advanced tumor stage, and poor overall survival. We also found that miR-217 overexpression inhibited OS cell proliferation and invasion *in vitro*, suggesting a tumor suppressive role of miR-217 in the progression of OS.

SETD8 belongs to the SET domain containing family and is capable to catalyze histone H4 Lys20 monomethylation (Wu et al. 2011). Moreover, it was reported that the progression of cancer is accompanied with an abnormal status of protein methylation (Copeland et al. 2013). SETD8 is an important oncogene in tumors including gastric cancer, breast cancer, and OS (Yu et al. 2013; Shi et al. 2015; Zhang et al. 2016). High SETD8 expression is associated with poor survival time of gastric cancer (Shi et al. 2015). Also, it was reported that miR-7 could downregulate SETD8 expression to suppress metastasis of breast cancer cells (Yu et al. 2013). However, it was not previously reported whether miR-217 could regulate SETD8 expression. In this present study, SETD8 3'-UTR was found has a putative miR-217 binding site. Luciferase reporter assay and western blot further confirmed SETD8 as a direct target of miR-217. The expression correlation analysis revealed that miR-217 and SETD8 expression was inversely correlated in OS tissues. Functional assays revealed that SETD8 was the mediator for the tumor suppressive role of miR-217 in OS. A recent study revealed that SETD8 could regulate the H4K20me1 levels at p21 promoter to inversely regulate the expression of p21 in tumors (Shih et al. 2017). Therefore, to perfect the miR-217/SETD8 regulatory axis, we also detected whether p21 expression could be regulated by miR-217/SETD8. Our data show that p21 expression was inversely correlated with SETD8. Collectively, these results demonstrate that the tumor suppressive role of miR-217 was exerted through regulating SETD8 and p21.

Our study for the first time revealed the connections between miR-217 and SETD8 and p21 in OS. We also demonstrated that miR-217 could inhibit OS cell proliferation and invasion by targeting SETD8 and p21. Therefore, the miR-217/SETD8/p21 axis may be used as potential therapeutic targets for the treatment of OS.

4. Experimental

4.1. *Ethics statement*

Study protocol was approved by The affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College and was conducted following the Declaration of Helsinki. We have obtained written informed consent from the enrolled patients.

4.2. *Human tissues*

Human OS tissues and adjacent non-cancerous tissues were obtained from 52 patients who received treatment at the The affiliated Yantai Yuhuangding Hospital of Qingdao University Medical college between May 2011 and November 2012. Patients who have ever received anti-cancer treatments were excluded from this study. All tissues collected were immediately frozen in liquid nitrogen and stored at -80 °C for further usage.

4.3. *Cell culture*

Human OS cell lines (MG-63, SaoS-2 and HOS) and the human normal osteoblastic hFOB1.19 cell line was purchased from ATCC (Manassas, VA, USA). Cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) in a humidified 37°C incubator containing 5% CO_2.

4.4. *Cell transfection*

miR-217 mimic and negative control (miR-NC) were synthesized by GenePharma (Shanghai, China). ORF construct for SETD8 (SETD8 construct) and NC were purchased from GenScript (Nanjing, China). (Guangzhou, China). Cells were seeded in 6-well plates (1 x 10^5 cells/well) and cultured until about 70 % confluence. Then, these cells were transfected with synthetic miRNAs or expression constructs using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. After transfection for 48 h, these cells were collected for following assays.

4.5. *Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

miR-217 expression levels were measured using RT-qPCR. TRIzol regent (Thermo Fisher Scientifc, Inc.) was used to isolate total RNA from tissues and cells according to the manufacturer's protocol. U6 snRNA was used as internal control for the analysis of miR-217 expression levels. PrimeScript™ RT reagent kit (Takara, Dalian, China) was used to synthesize cDNA. RT-qPCR was performed using SYBR Premix Ex Taq™ II (TaKaRa) according to the provided instructions at ABI 7500 equipment (Applied Biosystems) with the following procedures: 1 cycle of 95 ˚C for 5 min; 40 cycles of denaturation at 95 ˚C for 15 s; and annealing/extension at 60 ˚C for 60 s. The primers used were listed as follows: miR-217 forward, forward: 5'-TACTCAACTCACTACTGCATCAGGA-3' and reverse: 5'-TATGGTTGTTCTGCTCTCTGTGTC-3'; U6 snRNA forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3'; The $2^{-\Delta\Delta Ct}$ method was utilized to calculate the expression level of miR-217.

4.6. Cell Counting Kit-8 (CCK-8) assay

CCK-8 kit (Takara) was used to analyze OS cell proliferation. After seeding these cells in 96-well plate, 10 µl CCK-8 reagent was added to each well at indicated points (0, 24, 48 and 72 h) and incubated for additional 2 h. Absorbance was determined at 450 nm using a microplate reader.

4.7. Transwell invasion assay

Transwell invasion assay was conducted to analyze OS cell invasion. The transwell chamber (Corning Incorporated, Corning, NY, USA) was coated with Matrigel (BD Biosciences, San Jose, CA, USA). The lower chambers were filled with DMEM containing FBS. The cells were seeded in the upper chamber and incubated for 48 h. The non-invasive cells were gently removed by cotton swabs. Invasive cells were fixed with 4 % paraformaldehyde and stained with 0.5 % crystal violet. Cell numbers from five fields were counted under a microscope (Olympus IX83; Olympus Corporation, Tokyo, Japan).

4.8. Bioinformatics predication and luciferase reporter assay

In silico prediction algorithm TargetScan (http://www.targetscan.org/) was conducted to predict the potential targets of miR-217. The wild-type 3'-UTR of the SETD8 (SETD8-WT) and mutant SETD8 (SETD8-MUT) was cloned into the pMIR-REPORT vector (Promega, Madison, WI, USA). To detect luciferase activity, SETD8-WT or SETD8-MUT, together with miR-217 mimic or NC was transfected into cells with Lipofectamine 2000 (Invitrogen). Dual-luciferase reporter assay kit (Promega) was used to detect the firefly and luciferase activities after transfection for 48 h. Relative luciferase activity was normalized to firefly activity.

4.9. Western blot analysis

RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China) with protease inhibitor was used to extract total protein from tissues and cell lines. Protein concentration was determined by bicinchoninic acid protein assay (Beyotime). Protein samples (30 µg) were isolated using on 10% SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Beyotime). 5% fat-free milk was employed to block the membranes at room temperature for 1 h. Then, the membranes were incubated with primary antibodies (anti-SETD8: ab3798; anti-p21: ab80633; anti-GAPDH: ab8245; all from Abcam, Cambridge, MA, USA) at 4 ˚C for overnight. After washing with TBST, the membranes were cultured with goat anti-mouse horseradish peroxidase-conjugated IgG (ab6789; Abcam) at room temperature for 1 h. Finally, the blots were developed using enhanced chemiluminescence kit (Beyotime).

4.10. Statistical analysis

Data analysis were conducted in SPSS 17.0 (SPSS Inc., Chicago, IL, USA) were presented as the mean±standard deviation. Differences between groups were evaluated using Student's t-test (two groups) or one-way analysis of variance and. Tukey post hoc test (three or above groups). Chi-square test was conducted to calculate the associations between miR-217 and clinicopathological factors. Spearman's correlation analysis was performed to analyze the association between miR-217 and SETD8 expression in OS tissues. Kaplan-Meier curve and log-rank test was employed to investigate the effect of miR-217 expression on OS overall survival. P value less than 0.05 was considered to indicate a statistically significant.

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