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miR-218 expression in osteosarcoma tissues and its effect on cell growth in osteosarcoma cells

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ABSTRACT

Objective: To investigate the expression of miR-218 and its clinical significance in osteosarcoma tissues and explore its effect on proliferation and apoptosis in osteosarcoma cells. **Methods:** miR-218 expression was detected in 76 samples of surgically resected osteosarcoma and matched normal tumor-adjacent tissues using quantitative reverse transcription polymerase chain reaction (qRT-PCR). MiR-218 was over-expressed by exogenous miR-218 plasmids in Saos-2 cells, and then BrdU cell proliferation assay and flow cytometry were used to determine cell proliferation and apoptosis. **Results:** The expression of miR-218 in osteosarcoma tissues was significantly lower than those in normal tumor-adjacent tissues (t=8.735, P<0.001). MiR-218 expression in tumor tissues was significantly correlated with tumor size (χ^2=5.380, P=0.020), clinical stage (χ^2=6.692, P=0.010) and distant metastasis (χ^2=4.180, P=0.041). MiR-218 was obviously over-expressed by exogenous miR-218 plasmids (t=19.42, P<0.001), and miR-218 overexpression significantly reduced cell proliferation (t=9.045, P<0.001) and induced apoptosis (t=12.38, P<0.001) in Saos-2 cells. **Conclusions:** The low-expression of miR-218 is correlated with the poor clinicopathological features in osteosarcoma. Moreover, miR-218 overexpression reduces cancer cell proliferation and induces apoptosis in Saos-2 cells, suggesting that miR-218 may play a key role in the progression of human osteosarcoma.

1. Introduction

Osteosarcoma is the most common primary malignant cancer in bone[1]. It has been reported that the incidence of osteosarcoma is obviously correlated with the age of patient[2]. The annual incidence of osteosarcoma is 0.017‰ in children under 10 years old, while it is 0.082‰ in children among 10 to 19 years old[2]. The emerging evidence showed that major advances in diagnosis and treatment have improved the outlook for osteosarcoma patients, but it has not yet achieved satisfactory curative effect. Therefore, it is important to explore new biomarkers for predicting the prognosis and improving the outcome of osteosarcoma patients.

MicroRNAs (miRNAs) are an abundant group of endogenous non-coding single strand RNAs of ~22 nucleotides[3]. They regulate gene expression at post-transcriptional level by translational repression or degradation of target mRNA. In this manner, they participate in various biological process including cell development[4,5], cell cycle[6], and stem cell renewal[7]. Aberrant expression of miRNAs plays a key role in cancer development and progression through modulating oncogenic and tumor suppressor pathways. Using microarray or real-time PCR, miRNA expressing profiles

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which can differentiate cancer and normal tissues have been identified[8]. Moreover, different types of cancers have varied expression pattern of miRNAs. Due to its high stability in serum, miRNAs are becoming promising diagnostic and prognostic biomarkers for patients with malignancy[9,10].

MiR-218 is proposed as a novel tumor-related miRNA and has been found to be significantly deregulated in tumors[11-17]. It was down-regulated in cervical[11], gastric[12], lung[13], colon[14], prostate[15], bladder cancer[16] and hepatocellular carcinoma[17]. Furthermore, low miR-218 expression was correlated with a significant worse survival of HCC patients[17]. Recently, Jin et al reported that reduced miR-218 expression was observed in osteosarcoma tissues[18]. Otherwise, miR-218 inhibited osteosarcoma cell migration and invasion by downregulating T-cell lymphoma invasion and metastasis 1 (TIAM1), matrix metalloproteinase2 (MMP2) and MMP9[18]. However, the clinical significance of miR-218 in osteosarcoma tissues and its role in cell growth remain poorly understood.

In this study, we detected miR-218 expression in human osteosarcoma and matched normal tumor-adjacent tissues using qRT-PCR. The correlation between miR-218 expression and clinicopathologic features of osteosarcoma were systemically analyzed. Furthermore, we explored the role of miR-218 in the proliferation and apoptosis of osteosarcoma cells to confirm the effect of miR-218 on the initiation and development of human osteosarcoma.

2. Materials and methods

2.1. Clinical samples

68 osteosarcoma samples were collected from patients including 40 males and 28 females, who underwent the resection of their primary osteosarcoma in the Department of Orthopaedics, PLA 180 Hospital during Jan 2004 to Dec 2008. All samples were used after obtaining informed consent. The demographic features and clinicopathologic date are shown in Table 1. All the patients did not receive preoperative blood transfusion, radiation therapy and chemotherapy. The Union for International Cancer Control (UICC) tumor-node-metastases (TNM) Staging Classification (6th ed.) was used for the staging of the tumor. The Fujian Medical University Ethics Committee approved all protocols according to the Helsinki Declaration (as revised in Edinburgh 2000).

2.2. Real time quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated using TRIzol® (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The PCR amplification for the quantification of the miR-218 and U6 was performed using TaqMan miRNA Reverse Transcription Kit (Applied Biosystms, Foster City, CA, USA) and TaqMan Human MiRNA Assay Kit (Applied Biosystems). The relative expression of miR-218 was shown as fold difference relative to U6.

2.3. Cell line and transfection

The human osteosarcoma cell line, Saos-2 (ATCC, Manassas, VA, USA), were cultured in complete Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco) with 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma, St-Louis, MO, USA) in a humidified containing of 5% CO2 incubator at 37 ℃.

MiRNA vectors, including miR-218 and the negative control for the miR-218 were purchased from Genecopoeia (Guangzhou, China). Cells were transfected with the vectors mentioned above using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

2.4. BrdU cell proliferation assay

Saos-2 cells that were transfected with miR-218 or control plasmids were seeded into 96-well plates at 5 000 cells per well for 24 hours and detected using a Cell Proliferation ELISA, BrdU (5-bromodeoxyuridine) (chemiluminescent) (Roche, Indianapolis, IN, USA), as previously described[19].

2.5. Cell apoptosis assay

Briefly, Saos-2 cells were transfected with miR-218 or control plasmids and 106 treated cells were collected for flow cytometry. The flow cytometry for the quantification of the cell apoptosis was performed using a BD FACS Canto II Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and an Annexin-V-FLUOS Staining Kit (Roche), as previous reported[20].

2.6. Statistical analysis

Results are expressed as Mean±SEM. Significance was established, with GraphPad Prism 5 software (GraphPad

Software, Inc, San Diego, CA, USA), using a Pearson *Chi*-squared test and a student's *t*-test when appropriate. Difference were considered significant when $P< 0.05$.

3. Results

3.1. Levels of miR-218 expression in osteosarcoma and matched noncancerous tissues

To determine the expression of miR-218 in osteosarcoma specimens, we detected the levels of miR-218 expression in a retrospective cohort of 76 osteosarcoma and matched normal tumor-adjacent tissues using qRT-PCR. Quantitative analysis indicated that the level of miR-218 expression in osteosarcoma tissues was significantly lower as compared with that in matched nontumor tissues (0.31±0.04 *vs.* 0.63±0.06, t=8.735, P<0.001).

3.2. Clinical significance of miR-218 in osteosarcoma cases

Since reduced miR-218 expression correlates with poor clinicopathologic features of human cancers[11-17]. To investigate the clinical significance of miR-218 in osteosarcoma, we analyzed the correlation between miR-218 expression and different clinicopathological parameters of osteosarcoma. The level of miR-218 was considered as either high (T/NT≥1) or low (T/NT<1) expression. As shown in Table1, Clinical association analysis using a Pearson chi-squared test indicated that low miR-218 expression was evidently correlated with large tumor size (χ^2=5.380, P=0.020), advanced clinical stage (χ^2=6.692, P=0.010) and distant metastasis (χ^2=4.180, P=0.041). Taken together, our data indicate that the level of miR-218 expression is down-regulated in osteosarcoma and reduced miR-218 level is associated with poor clinicopathological characteristics.

3.3. Upregulation of miR-218 inhibits cell proliferation and induces apoptosis in Saos-2 cells

Recent researches suggested that miR-218 acts as a tumor suppressor by inhibiting cancer cell proliferation and inducing apoptosis[11,14,17]. To confirm the role of miR-218 in osteosarcoma, Saos-2 cells that were transduced with miR-218 or miR-control plasmids were subjected to BrdU assay and flow cytometry for cell proliferation and apoptosis. As measured by qRT-PCR, the level of miR-218 expression was significantly upregulated by exogenous miR-218 in Saos-2 cells (0.97±0.04 *vs.* 16.86±0.77, t=19.42, P<0.001). BrdU assays were performed to determine the effect of altering miR-218 levels on tumor cell proliferation. We found that upregulation of miR-218 resulted in a significant reduction of cell proliferation in Saos-2 cells (133.70±6.27 *vs.* 60.83±5.06, t=9.045, P<0.001). Furthermore, as determined by flow cytometer, the number of apoptotic Saos-2 cells was prominently increased after upregulation of miR-218 (5.67

Table 1
Correlation between the clinicopathological characteristics and expression of miR-218 in the osteosarcoma patients (n=76).

Clinicopathologic features		n	miR-218 expression		χ^2	P
			High	Low		
Age (years)	<15	32	12	20	1.966	0.161
	≥15	44	10	34		
Gender	Male	46	14	32	0.125	0.723
	Female	30	8	22		
Tumor size (cm)	<8	36	15	21	5.380	0.020*
	≥8	40	7	33		
Site	Tibia/Femur	47	12	35	0.699	0.403
	Other	29	10	19		
Serum LDH (U/L)	Elevated	41	11	30	0.194	0.659
	Normal	35	11	24		
Serum ALP (U/L)	Elevated	49	17	32	2.214	0.137
	Normal	27	5	22		
Clinical stage	IIA	31	14	17	6.692	0.010*
	IIB/III	45	8	37		
Distant metastasis	Absent	57	13	44	4.180	0.041*
	Present	19	9	10		
Chemotherapy response	Well	31	12	19	2.426	0.119
	Poor	45	10	35		

LDH, Lactic dehydrogenase; ALP, Alkaline phosphatase, * Statistically significant.

±0.76% *vs.* 30.20±1.89%, t=12.38, P<0.001; Figure 1). Thus, miR-218 may exert an anti-growth effect by inhibiting cell proliferation and inducing apoptosis in osteosarcoma.

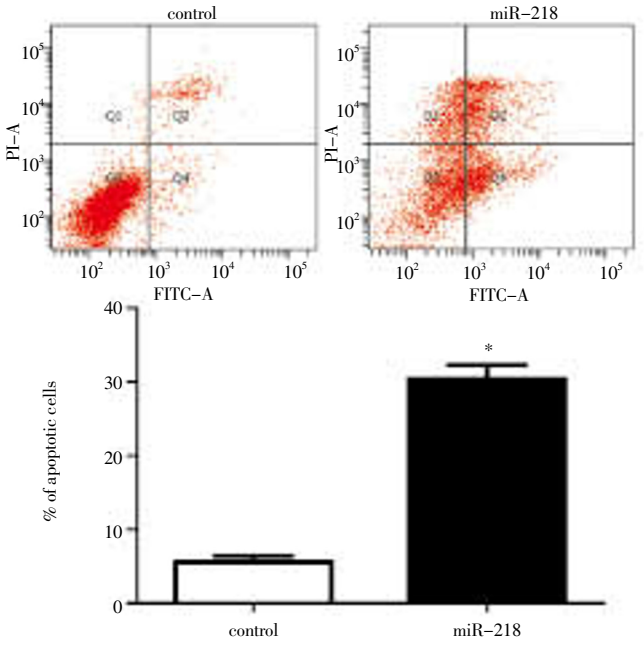

Figure 1. Upregulation of miR-218 increased apoptotic osteosarcoma cells.

Saos-2 cells that were transfected with miR-218 or miR-control were subjected to flow cytometry for cell apoptosis. Quantitative data revealed that upregulation of miR-218 led to a significant elevated number of apoptotic Saos-2 cells. n=6, * P<0.05 by t test.

4. Discussion

Recently, low miR-218 expression has been observed in various human tumor cells and tissues[11-17]. The level of miR-218 expression is down-regulated in cervical cancer tissues, and human papilloma virus 16 (HPV 16) lead to reduction of miR-218 in cervical cancer cells[11, 21]. In colon cancer, the expression of miR-218 in the cancer tissues is significantly lower than that in the matched noncancerous tissues[14]. The level of miR-218 is correlated with TNM stage, lymph node metastasis and histological differentiation. Furthermore, low miR-218 expression confers a worse survival of colon cancer patients[14]. Tu et al has reported that the expression of miR-218 is impaired in hepatocellular carcinoma tissues[17]. MiR-218 is expressed at significant lower level in patients with large tumor size and advanced TNM tumor stage[17]. In this study, the levels of miR-218 expression were detected in 76 pairs of osteosarcoma and matched nontumor tissues. We found that miR-218 expression in the osteosarcoma tissues was prominently lower than that in the matched noncancerous tissues, which was consistent with previous report[18]. Moreover, we showed that miR-218 expression was obviously correlated with tumor size, clinical stage and distant metastasis in osteosarcoma. Taken together, our results indicate that miR-218 may be a potential tumor suppressor and act as a prognostic biomarker for predicting survival of osteosarcoma patients.

Prior study reported that miR-218 inhibited cell cycle progression and promoted apoptosis through downregulating B lymphoma Mo-MLV insertion region 1 homolog (BMI-1) in colon cancer[14]. Venkataraman et al reported that miR-218 acted as a tumor suppressor by targeting several cancer-related genes including cyclin dependent kinase (CDK6), Rictor and cathepsin B (CTSB) in medulloblastoma[22]. Researchers found that tumor suppressive microRNA-218 inhibits cancer cell migration and invasion through targeting laminin-332 in head and neck squamous cell carcinoma[23]. The data from Liu ea al indicated that miR-218 inhibited oncogenic transcription factor lymphoid enhancer factor 1 (LEF1) and subsequently reversed high invasiveness of glioblastoma cells[24]. Recently, Alajez et al approved that miR-218 inhibited nasopharyngeal cancer progression by inversely regulating of survivin and the SLIT2-ROBO1 pathway[25]. All the researches above suggest that miR-218 functions as a potent tumor suppressor in multiple human cancers by targeting different genes. MiR-218 in osteosarcoma has been reported previously, Jie et al suggest that miR-218 inhibits osteosarcoma cell migration and invasion by down-regulating TIAM1, MMP2 and MMP9 expression[18]. However, the role of miR-218 in osteosarcoma cell proliferation and apoptosis remain poorly undetermined. In our study, miR-218 was over-expressed in Saos-2 cells via exogenous expression plasmids transfection. BrdU cell proliferation assays found that miR-218 overexpression led to a significant reduction of cell proliferation in Saos-2 cells. Otherwise, flow cytometer detection showed that miR-218 overexpression increased the percentage of apoptotic Saos-2 cells. Our data indicate that miR-218 may suppress tumor progression by inhibiting cell proliferation and inducing apoptosis in osteosarcoma.

In conclusion, we find that the expression of miR-218 is down-regulated in osteosarcoma tissues and its low expression is correlated with tumor size, distant metastasis and clinical stage. Furthermore, miR-218 overexpression leads to growth arrest and apoptosis in Saos-2 cells, suggesting that miR-218 may be a potential valuable biomarker and therapeutic target for human osteosarcoma.

Conflict of interest statement

We declare that we have no conflict of interest.

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