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Original Article

Up-regulation of miRNA-19b is associated with unfavorable prognosis in patients with osteosarcoma

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Abstract: Osteosarcoma is the most common malignant bone tumor with high morbidity, the molecular mechanism of metastatic dissemination remains unclear. Our previous studies have found that miR-19b play an important roles in osteosarcoma carcinogenesis and progression, however the relationship between the miR-19b expression and clinicopathological features in osteosarcoma remains unclear. Thus, the aim of this study was to investigate the clinical significance of miR-19b in osteosarcoma. Our study shows that the expression of miR-19b in osteosarcoma tissues was significantly higher than that in normal adjacent bone tissues. Up-regulation of miR-19b was significantly correlated with the status of distant metastasis and TNM stage of osteosarcoma patients. Moreover, high miR-19b expression was associated with overall survival and progression-free survival. Additionally, western-blot analysis revealed that the expression of Mfn1 protein negatively correlated with the expression of miR-19b in osteosarcoma tissues. In conclusions, our data showed that the miR-19b may contribute to the progression of osteosarcoma and it may serve as a promising marker for further risk stratification in the treatment of osteosarcoma.

Keywords: miR-19b, osteosarcoma, Mfn1, overall survival, progression-free survival

Introduction

Osteosarcoma is the most primary malignant tumor of bone mostly affecting the metaphyses of long bones in children and young adults [1]. One disreputable features of this tumor is its high rate of systemic metastasis [2]. The 5-year survival rate of osteosarcomas patients has been remarkably improved due to the advances in therapeutic strategies including radical surgery and adjuvant polychemotherapy [1]. However, outcome remains dissatisfactory and most of them died of distant metastasis, especially pulmonary metastasis [3]. Therefore, identification of effective prognostic markers responsible for understanding the molecular mechanisms involved in osteosarcoma carcinogenesis is of great clinical significance, which for predicting novel targeted therapeutic strategies to treat this disease.

MicroRNAs (miRNAs) are a family of endogenous, single stranded and small non-coding RNA molecules, which ranging from 21 to 25 nucleotides in length [4]. MiRNAs are widely expressed in cells from plants to animals and play an essential role in the regulation of their target gene expression post-transcriptionally, resulting in the degradation of the target mRNA or post-transcriptional repression [5]. Recently, several studies have pointed out that aberrant miRNA expression is related with the development and metastatic progression of cancers, several miRNAs which can function either as oncogene or tumor suppressor have been identified [6]. Previous studies have demonstrated that abnormal expression of miR-26a, miR-183 and miR-214 may play an important role in osteosarcoma carcinogenesis and progression [7].

miR-19b belongs to the miR-17-92 cluster family and is located on chromosome 13q31 [8], a region frequently amplified in colon cancer [9], breast cancer [10] and gastric cancer [11]. In our previous studies, we have found that the relative expression of miR-17-92 cluster in osteosarcoma tissues was significantly higher

Table 1. Correlation between miR-19b expression and clinicopathological features in 72 patients with osteosarcoma

Clinical features	No.	miR-19b expression (n, %)		x^2	P
		High (n=39)	Low (n=33)		
Gender				0.039	0.844
Male	38	21 (55.26%)	17 (44.74%)		
Female	34	18 (52.94%)	16 (47.06%)		
Age				0.076	0.783
<30	49	26 (53.06%)	23 (46.94%)		
≥30	23	13 (54.17%)	10 (45.83%)		
Locations				0.873	0.646
Femur	34	18 (52.94%)	16 (47.06%)		
Tibia	21	13 (61.90%)	8 (38.10%)		
Humerus	17	8 (47.06%)	9 (52.94%)		
Pathological Type				0.532	0.766
Telangiectatic Os	15	9 (60.00%)	6 (40.00%)		
Small Cell Os	21	12 (57.14%)	9 (42.86%)		
Other	36	18 (50.00%)	18 (50.00%)		
Tumor size				3.384	0.066
<8 cm	39	25 (64.10%)	14 (35.90%)		
≥8 cm	33	14 (42.42%)	19 (57.58%)		
TNM stage				8.311	0.016
I	19	5 (26.32%)	14 (73.68%)		
II	31	19 (61.29%)	12 (38.71%)		
III	22	15 (68.18%)	7 (31.82%)		
Metastasis				7.903	0.005
Present	23	18 (78.26%)	5 (21.74%)		
Absent	49	21 (42.86%)	28 (57.14%)		

than those in adjacent normal tissues and miR-19b can regulate osteosarcoma cells cycle, apoptosis, invasion, and proliferation through regulating of the tumor suppressor gene Mfn1 [12, 13]. However, the relationship between the miR-19b expression and clinicopathological parameters and prognosis in osteosarcoma was not fully elucidated. Therefore, the aim of this study was to investigate the clinical significance of miR-19b in patients with osteosarcomas.

Materials and methods

Patients and tissue samples

Our study was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University. The written informed consent was obtained from all of the patients prior to sample collection. All samples were treated anonymous on the basis of the ethical and legal standards.

Seventy-two pairs of osteosarcoma tumor tissues and paired normal adjacent bone tissues were collected between 2004 and 2008 from the First Affiliated Hospital of Fujian Medical University. All tissue samples were immediately snap frozen in liquid nitrogen after surgery and stored at -80°C until the extraction of total RNA. None of the patients received chemotherapy or radiotherapy prior to the surgery. All patients investigated in the study were independently reviewed and diagnosed by two pathologists. All of the patients included in the study were classified according to the TNM classification [14]. The clinicopathologic features of the patients with osteosarcoma are shown in **Table 1**.

To investigate the overall survival and progression-free survival, overall survival was calculated as the time interval from the date of surgery to either the day of the last follow-up or cancer-related death. Progression-free survival was defined as the time from the date of surgery to the first of either recurrence or relapse, second cancer, or death. The patients who died from diseases other than osteosarcoma or from unexpected events were excluded.

Real-time quantitative RT-PCR for miRNA

MiR-19b expression in osteosarcoma tissues and normal adjacent bone tissues was performed by real-time quantitative RT-PCR. Briefly, microRNAs were isolated from the frozen osteosarcoma tissues using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A TaqMan microRNA assays and the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were used to detect the expression of mature miR-19b after reverse transcription. Primers for miR-19b: (F) 5'-ACACTCCAGCTGGGTGTGCAAATCCATGCAAA-3', and the primers for Mfn1 were refer to our previous study (14). RNU6B served as an endogenous control for normalization. All TaqMan PCRs were performed in triplicate.

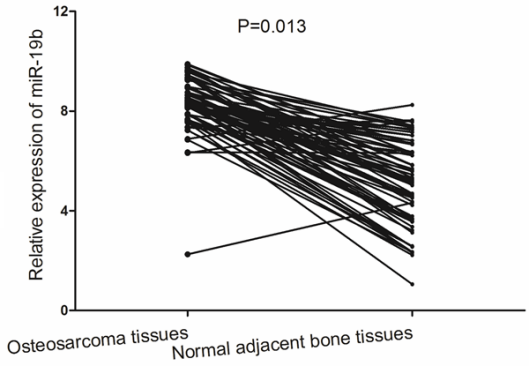

Figure 1. Expression of miR-19b was up-regulated in osteosarcoma samples detected by real-time quantitative RT-PCR.

Western blotting

Total proteins were isolated from osteosarcoma samples and normal adjacent bone tissues. The concentrations of Protein were determined by a Micro BCA protein assay kit (Pierce, USA). Then, proteins were resolved by 10% SDS-PAGE gel electrophoresis and transferred to the nitrocellulose membrane. After blocking in 5% non-fat milk, the membranes were incubated with rabbit anti-Mfn1 monoclonal antibody (1:500; Cell Signaling Technology) or rabbit anti-GAPDH (1:1000; Novus Biologicals) in 5% non-fat milk overnight at 4°C. Protein bands were visualized by the West Femto system (Pierce).

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software (SPSS Inc, USA). Values were presented as the mean ± standard deviation. The correlation between miR-19b expression and clinicopathological features were analyzed using Fisher's exact test, χ^2 test and Student *t* test. The patients who had a 1.5-fold increase of miR-19b expression compared to normal adjacent bone tissues were placed in the high expression group, and the rest were placed in the low expression group [6]. The association of miR-19b expression with overall survival and progression-free survival was analyzed using Kaplan-Meier Survival Analysis, and the log-rank test was used to compare the resulting curves. The Cox proportional hazards model was used for multivariate analysis. Differences were assumed statistically significant when *P* value was less than 0.05.

Results

Up-regulation of miR-19b in clinical osteosarcoma samples

Real-time quantitative RT-PCR was used to detect the expression levels of miR-19b in 72 pairs of osteosarcoma tissues and normal adjacent normal bone tissues normalized to RNU6B. As shown in **Figure 1**, the expression levels of miR-19b were found to be distinctly increased in osteosarcoma tissues compared to normal adjacent bone tissues. The mean level of miR-19b expression in osteosarcoma tissues was 8.33±1.09, which was significantly higher than that in normal adjacent bone tissues (5.08±1.69, P<0.001, **Figure 1**). While, the average level of miR-19b expression was 8.62±1.37 in 23 patients who had a distant metastasis.

Association between miR-19b up-regulation and clinicopathological features of osteosarcoma

To analyse the association of miRNA-19b expression with clinicopathological features, all patients were divided into two groups using an optimal cut-off value calculated as described in the Methods section which was used throughout the study. Thus, thirty-nine cases were placed in the high expression group and 33 cases in the low expression group. And the results are showed in **Table 1**. Expression of miR-19b significantly up-regulated in patients with metastasis compared with patients without metastasis (P=0.005) (**Table 1**). Moreover, high level miR-19b expression was also statistical associated with advanced TNM stage in osteosarcoma (P=0.016) (**Table 1**). No correlation was observed between miR-19b expression and other clinical parameters such as gender, age, differentiation, pathological type and size (**Table 1**).

Prognostic value of miR-19b in osteosarcoma patients

In order to investigate the prognostic value of miR-19b expression in osteosarcoma patients, we defined a time point of 60 months. During the time study, 23 (31.94%) patients presented a distant metastasis and 49 (68.06%) did not. In patients who had a distant metastasis, 18 (78.26%) had high miR-19b expression. By

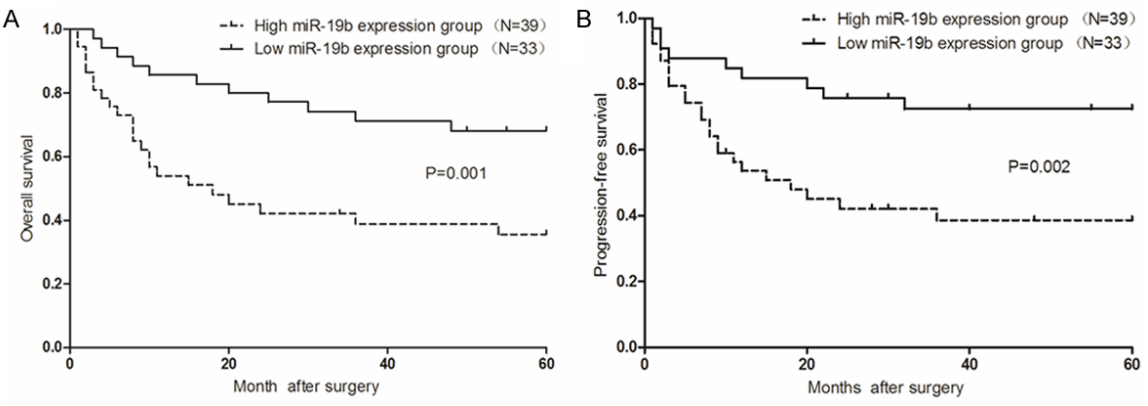

Figure 2. Correlation between miR-19b expression and survival rates in 72 patients with osteosarcoma. A. Overall survival. B. Progression-free survival.

Table 2. Univariate analysis of the associations of prognosis with various clinicopathologic parameters and miR-19b expression in osteosarcoma

Clinical features	Overall survival			Progression-free survival		
	HR	95% CI	P	HR	95% CI	P
Gender	1.192	0.601-2.367	0.615	1.112	0.667-1.854	0.684
Age at diagnosis	1.002	0.976-1.028	0.908	1.013	0.980-1.046	0.459
Locations	0.803	0.536-1.204	0.289	0.823	0.474-1.429	0.489
Pathological Type	1.022	0.744-1.404	0.893	1.018	0.986-1.052	0.263
Tumor size	0.503	0.243-1.038	0.063	0.913	0.610-1.366	0.659
TNM stage	2.077	1.215-3.552	0.008	1.952	1.027-3.710	0.041
Metastasis	1.871	1.107-3.163	0.019	1.990	1.056-3.749	0.033
miR-19b expression	2.436	1.400-4.241	0.002	2.152	1.148-4.036	0.017

Table 3. Multivariate analysis of the associations of prognosis with various clinicopathologic parameters and miR-19b expression in osteosarcoma

Clinical features	Overall survival			Progression-free survival		
	HR	95% CI	P	HR	95% CI	P
TNM stage	1.893	1.105-3.245	0.020	2.092	1.148-3.812	0.016
Metastasis	1.923	1.136-3.253	0.015	1.843	1.083-3.136	0.024
miR-19b expression	1.843	1.083-3.136	0.024	1.750	1.045-2.930	0.033

means of the Kaplan-Meier method and log-rank test, osteosarcoma patients with high miR-19b expression were correlated with shorter overall survival and shorter progression-free survival (**Figure 2A**, **2B**; P=0.001 and P=0.002, respectively) compared with those with low miR-19b expression.

In univariate analysis, high miR-19b expression (P=0.002 and P=0.017, respectively), advanced TNM stage (P=0.008 and P=0.041, respectively) and distant metastasis (P=0.019 and 0.033, respectively) were found to be a significant predictor for poor overall survival and progression-free survival of patients with osteosarcoma (**Table 2**). Furthermore, multivariate cox proportional hazard regression analysis using all three variables was performed and revealed that high miR-19b expression (for overall survival: Hazard Ratio (HR)=1.843, P=0.024; for progression-free survival, HR=1.750, P=0.033), TNM stage (for overall survival: HR=1.893, P=0.020; for progression-free survival, HR=2.092, P=0.016) and distant metastasis (for overall survival: HR=1.923, P=0.015; for progression-free survival, HR=1.843, P=0.024) were both independent predictors for overall survival and progression-free survival of patients with osteosarcoma (**Table 3**).

Mfn1 and miR-19b are inversely expressed in osteosarcoma tumor tissues

Our previous study have showed that Mfn1 is a direct target of miR-19b in MG-63 cells, to analysis the relation between Mfn1 and miR-19b in tumor specimens, we detected the expression

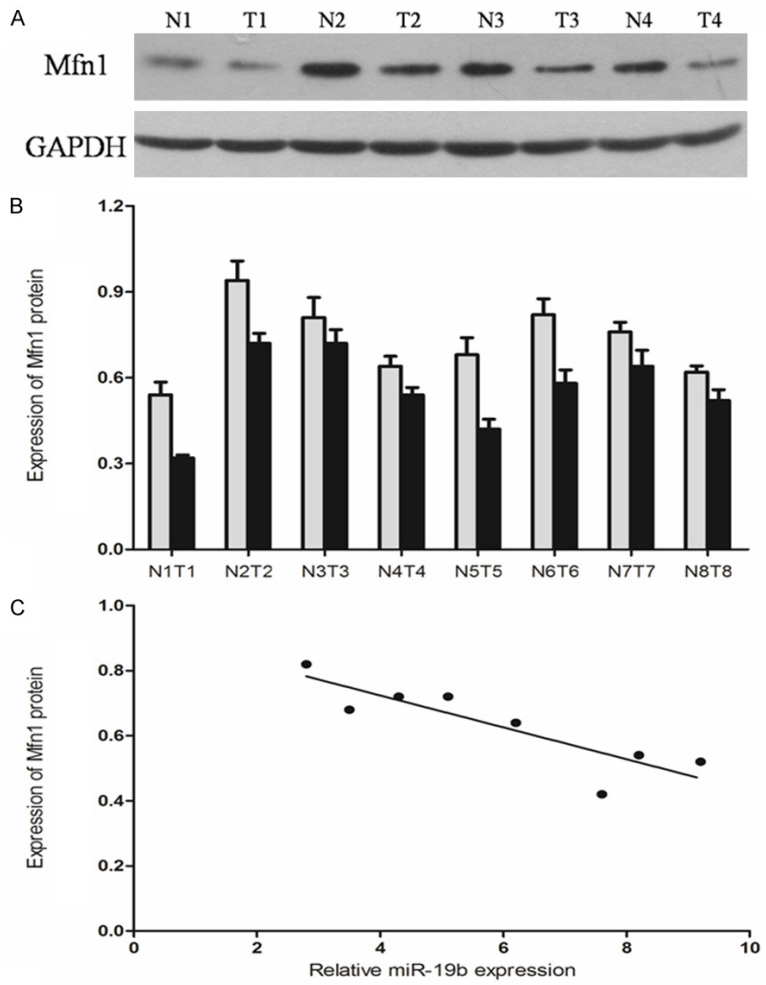

Figure 3. Expression of Mfn1 protein in osteosarcoma and normal bone tissues, as detected by western blotting. N, normal bone tissues; T, tumor tissues. A. Expression of Mfn1 protein detected by western blotting. Four representative cases are shown. B. Quantification of Mfn1 protein level in eight pairs of matched osteosarcoma and normal bone tissues. C. A negative correlation between miR-19b and Mfn1 in eight matched tumor specimens.

level of Mfn1 and miR-19b in eight pairs of matched osteosarcoma specimens using western blotting and real-time quantitative RT-PCR respectively. As expected, the mean level expression of Mfn1 mRNA in tumor tissues is 4.35±1.69, which was significantly higher than that in normal adjacent bone tissues (1.25±0.25), and lower level of Mfn1 protein was found in tumor tissues (**Figure 3A**, **3B**; $P<0.05$). Statistical analysis indicates that there is an obvious inverse correlation between Mfn1 protein and miR-19b in these tumor tissue specimens by using the Pearson's method (**Figure 3C**; $P<0.05$), with a correlation coefficient of -0.876.

Discussion

Osteosarcoma is a fatal malignancy characterized by frequent recurrences and poor clinical outcome and is hard to cure by the current treatment [15]. Hence, it is inevitable to identify novel therapeutic targets and biomarkers of treatment response for osteosarcoma patients. Studies have indicated that miRNAs serve as biomarkers and therapeutic targets for osteosarcoma [16, 17]. We recently reported that miR-19b is high expression in human osteosarcoma cells and can regulate osteosarcoma cells apoptosis and proliferation through regulating of the tumor suppressor gene mitofusins 1 [13], In this study, we investigate the clinical significance of miR-19b in osteosarcoma. To the best of our knowledge, there are currently no systematic studies in osteosarcoma that evaluate miR-19b expression and its association with patient clinical outcome.

The tumor promotor functions of miR-19b have been substantiated in cervical carcinoma, in which miR-19b is frequently upregulated, promoted cell growth and invasion [18]. In another study, high expression of the miR-19b was strongly associated with an adverse prognosis in non-small cell lung cancer [19]. In the present study, we reveal that the expression of miR-19b in osteosarcoma tissues were significantly higher than those in normal adjacent bone tissues; 39 (54.17%) cases of osteosarcoma samples had higher miR-19b expression. Additionally, high expression of miR-19b is significantly had distant metastasis and advanced TNM stage of osteosarcoma patients, suggesting that miR-19b have an important role in the development or pathogenesis of osteosarcoma, these results were consistent with the previous studies which showed

that miR-19b involved in tumor pathogenesis and the development of cervical cancer [18].

Furthermore, the influence of miR-19b expression on clinical outcome was evaluated by univariate and multivariate analyses, which revealed that miR-19b expression was a statistically significant risk factor affecting overall survival and progression-free survival of patients with osteosarcoma, suggesting that miR-19b expression could be a useful marker to predict the prognosis of this disease, which is also consistent with the previous studies in non-small cell lung cancer and esophageal squamous cell carcinoma [20]. Interestingly, we found that Mfn1 and miR-19b are inversely expressed in osteosarcoma tumor tissues, which consistent with the results in osteosarcoma cells in our previous study [20].

According to the results of our study, miR-19b may acts as a potential biomarkers and therapeutic target of treatment for the findings as following, the first is that the relative expression of miR-19b in osteosarcoma tissues were significantly higher than those in normal adjacent bone tissues. Second, high miR-19b expression is significantly correlated with metastasis and TNM stage of osteosarcoma patients. Finally, higher miR-19b expression was associated with shorter overall survival and progression-free survival in osteosarcoma patients.

In conclusion, our data demonstrate for the first time that miR-19b may acts as a potent prognostic marker for overall survival and progression-free survival of osteosarcoma patients, and combination with our in vitro study, which suggesting that miR-19b might be a prospective therapeutic target for osteosarcoma.

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Disclosure of conflict of interest

None.

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