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miR-21 predicts poor prognosis in patients with osteosarcoma

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ABSTRACT

Background and aims: miR-21 has been demonstrated to play an important role in tumour progression. The aim of the present study was to analyse the correlation between miR-21 expression level and clinicopathologic features, as well as to assess the prognostic significance of miR-21 in osteosarcoma.

Methods: Eighty-four pairs of osteosarcoma and corresponding non-cancerous bone tissues were obtained, and miR-21 expression levels were detected using quantitative real-time PCR (qRT-PCR). A χ^2 test was used to assess the relationship between miR-21 expression and clinicopathological features. Overall survival (OS) and disease-free survival (DFS) rates were determined by the Kaplan–Meier method and analysed by the log-rank test. The Cox proportional hazards model was used for multivariate analysis.

Results: qRT-PCR indicated that miR-21 expression in tumour tissues was strongly elevated compared with the adjacent corresponding non-cancerous bone tissue (7.88 ± 1.04 vs. 1.12 ± 0.37, respectively; $P < 0.001$). High miR-21 expression levels were linked to advanced clinical stage ($P = 0.001$), distant metastasis ($P = 0.001$), high tumour grade ($P = 0.032$) and large-sized tumours ($P = 0.013$). A higher miR-21 expression was significantly linked to shorter OS and DFS (both $P < 0.001$). Furthermore, a multivariate analysis confirmed that miR-21 was an independent and significant prognostic factor to predict poor OS and DFS (both $P < 0.001$).

Conclusions: Upregulation of miR-21 was associated with poor clinicopathological characteristics. It is used as a marker of poor prognosis in patients with osteosarcoma.

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Introduction

Osteosarcoma is the most frequent primary malignant sarcoma of the bone in adolescents and young adults and derives from primitive bone-forming mesenchyme. [1] The development of multiple therapeutic strategies for osteosarcoma including wide tumour excision, adjuvant chemotherapy and radiotherapy has significantly improved the prognosis of patients with a malignancy. [2] However, 30% of those diagnosed with osteosarcoma do not survive for more than 5 years, and approximately 80% of patients eventually develop metastatic disease after surgical treatment; for patients, pulmonary metastasis of osteosarcoma is a major cause of feral outcome. [3,4]

MicroRNAs (miRNAs), a class of small non-coding RNA molecules, play critical roles in a variety of biological events including development, metastasis, cell proliferation and cell differentiation.[5,6] Some miRNAs are differentially expressed in osteosarcoma tissues, cell lines and sera and have been shown to correlate with the malignant phenotype and prognosis.[7–13] These altered miRNAs function as oncogenes or tumour suppressor genes in this process.

Recently, miR-21 has been reported to be upregulated in some cancer types such as non-small-cell lung cancer,[14] oesophageal cancer,[15] breast cancer,[16] thyroid cancer [17] and colorectal cancer.[18] In these malignancies, forced expression of miR-21 has a poor prognosis in these patients. miR-21 has also been found to be overexpressed in osteosarcoma tissues, and knockdown of miR-21 greatly decreased the cellular invasion and migration of MG-63.[19] On the contrary, miR-21 overexpression in MG63 caused a significant increase in cell proliferation and invasion, and a significant reduction in cell apoptosis. [20] However, whether tissue miR-21 may be a potential biomarker for the prediction of prognosis and survival in patients with osteosarcoma is still unclear.

Herein, the level of miR-21 was detected using qRT-PCR in osteosarcoma tissues, and correlations with clinicopathological features were assessed. The study also investigated whether miR-21 might be a useful molecular biomarker for predicting prognosis in osteosarcoma.

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Table 1. Correlation of miR-21 expression with clinicopathological features in osteosarcoma.

Clinicopathological features		Number of patients	miR-21 expression		*P*-value
			High	Low	
Gender	Male	48	29(60.4%)	19(39.6%)	0.543
	Female	36	22(61.1%)	14(38.9%)	
Age (years)	<25	53	33(62.3%)	20(37.7%)	0.427
	≥25	31	18(58%)	13(41.9%)	
Tumour diameter (cm)	<8	50	22(44%)	28(56%)	0.013
	≥8	34	29(85.3%)	5(14.7%)	
Anatomic location	Tibia/femur	58	37(63.8%)	11(36.2%)	0.263
	Elsewhere	26	14(53.8%)	12(46.2%)	
Clinical stage	I+IIA	36	13(36%)	23(64%)	0.001
	IIB/III	48	38(79.2%)	10(20.8%)	
Distant metastasis	Yes	21	16(76.2%)	5(23.8%)	0.001
	No	63	35(55.6%)	28(44.4%)	
Tumour grade	Low	35	15(42.9%)	20(57.1%)	0.032
	High	49	36(73.5%)	13(26.5%)	
Histological type	Osteoblastic	37	21(56.7%)	16(43.3%)	0.572
	Others	47	30(63.8%)	17(36.2%)	

Materials and methods

Patients and tissue samples

Eighty-four primary osteosarcoma and corresponding non-cancerous bone tissue (located >3 cm away from a tumour) samples from the same specimens were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. All 84 patients with osteosarcoma obtained follow-up. The median follow-up was 86 months (range: 8.3–141 months). During the follow-up period, 29 patients (29/84, 34.5%) died from the disease. Distant metastases developed in 21 patients at a mean of 11.4 months (range 3–39 months) after the original diagnosis. Of these patients, six had bone metastases and 15 had lung metastases (two patients had both bone and lung metastases). In this study, the median overall survival (OS) and disease-free survival (DFS) was 29 months (95% confidence interval [CI], 28.4–41.3 months) and 23 months (95% CI, 22.6–33.7 months), respectively.

All specimens were collected from the Department of Orthopedics, People's Hospital of Weifang, China, from January 2008 to March 2012. No patient was administered a blood transfusion, radiotherapy or chemotherapy before surgery. Patients with evidence of other malignancies were excluded from this study. For these patients with osteosarcoma, the clinical stage was classified according to the sixth edition of the tumour-node-metastases (TNM) classification of the International Union against Cancer (UICC). Clinicopathological information for the patients is shown in Table 1.

All patients or the patients' parents, if the patient was a child, agreed to participate in the study and provided written informed consent. This study and the consent was approved by the ethical board of the Institute of The People's Hospital of Weifang and complied with Declaration of Helsinki.

TaqMan RT-PCR for miRNA expression

Total RNA was extracted from the tissues using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). MicroRNAs were quantified using real-time PCR with a TaqMan MicroRNA assay (Invitrogen, USA). First-strand complementary DNA (cDNA) synthesis was carried out from 1 μg of total RNA in a 12-μl final volume containing a 2-M stem-loop primer and 10-mM dNTP mix (Invitrogen, USA). The mix was plated at 65 °C for 5 min and then mixed with 5 × RT buffer, 0.1 M DTT, 200 U/μl of MultiScribe reverse transcriptase, and 40 U/μl of RNase inhibitor (Invitrogen, USA). The mix was plated at 37 °C for 55 min and 70 °C for 15 min and then held at −20 °C. Real-time PCR was performed using a standard TaqMan PCR protocol. The 20-μl PCR reactions included 1 μl of RT product, 1 Universal TaqMan Master Mix, and 1 × TaqMan probe/primer mix (Invitrogen, USA). Quantitative PCR was performed with specific primers as follows: miR-21, 5′-CTGGACGGTGCCAGGT-3′ and 5′-CGGGTCTTTCGTTAGCCTGC-3′; U6, 5′-CTCGCTTCGG CAGCACA-3′ and 5′-AACGCTTCACGAATTTGCGT-3′. The RT reactions including no-template controls were run in triplicate. All mRNA quantification data were normalised to U6. The cycle threshold (C_T) was calculated. The $2^{-\Delta CT}$ ($\Delta C_T = C_{T\text{miR-21}} - C_{T\text{U6 RNA}}$) method was used to quantify the relative amount of miR-21.

All statistical analyses were performed with SPSS 18.0 (SPSS Inc, Chicago, IL, USA). A χ^2 test was used to assess the relationship between miR-21 expression and clinicopathological features. A receiver operating characteristic (ROC) curve was drawn to evaluate the diagnostic value of miR-21 levels. DFS was calculated from the time of surgery to the date of progression (local and/or distal tumour recurrence), death or last follow-up. DFS was calculated using a Kaplan–Meier method and compared using a log-rank test. Cox regression (proportional hazard model) was adopted for a multivariate analysis of prognostic factors. Differences were considered significant with a P of < 0.05.

Results

miR-21 is overexpressed in osteosarcoma tissues

We detected the expression of miR-21 in 84 pairs of osteosarcoma tissues and matched normal tissues with a TaqMan RT-PCR analysis. As demonstrated using quantitative real-time PCR, the miR-21 expression was significantly increased in clinical osteosarcoma tissues (median relative expression level ± SD: 7.88 ± 1.04) compared with adjacent normal bone tissues (median relative expression level ± SD: 1.12 ± 0.37, $P < 0.001$) (Figure 1).

Correlation between miR-21 expression and clinicopathologic features in patients with osteosarcoma

The median miR-21 expression level was used as a cut-off value to divide all 84 patients into two groups: high miR-21 expression group ($n = 51$) and low miR-21 expression group ($n = 33$). As shown in Table 1, the upregulation of *miR-21* frequently occurred in patients with osteosarcoma who had distant metastases, larger sized tumours, high tumour grade and advanced tumour stage. However, there was no significance between miR-21 expression and other clinicopathological factors such as age, gender, anatomic location and histological type.

Expression of miR-21 and its prognostic potential in osteosarcoma

miR-21 was analysed for its prognostic significance and survival outcome in osteosarcoma. Prognostic significance was elucidated using ROC curves. To use an ROC curve analysis, the clinical and tumour characteristics were made binary and therefore dichotomised. Stage was dichotomised as I+IIA or IIB/III; tumour grade as low or high; distant metastasis as yes or no; tumour size of ≥8 cm or <8 cm; and survival as death due to osteosarcoma or other (censored/alive). As shown in Table 2, the miR-21 expression and clinical stage yielded a significant AUC of 0.853 with a sensitivity of 63.8% and specificity of 67.4%. ROC curve for distant metastasis yielded an AUC of 0.878 with a sensitivity and specificity of 92.3 and 68.4%, respectively; an AUC of 0.659 was obtained in an ROC curve with respect to tumour grade, with a sensitivity of 62.4% and specificity of 72.8%. An AUC of 0.712 was obtained with respect to *tumour size*, and the sensitivity was 65.2%, while the specificity was 69.2%. An ROC curve for survival yielded an AUC of 0.705, with a sensitivity of 69.4% and specificity of 80.3%.

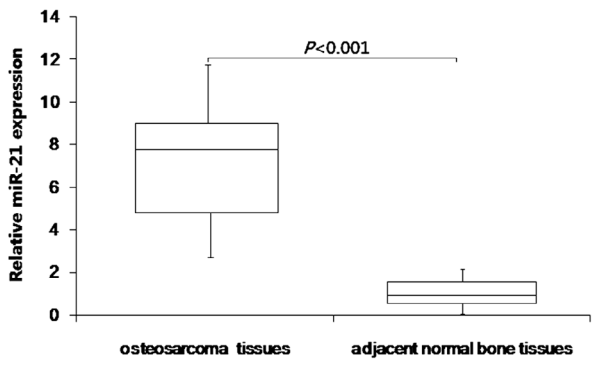

Figure 1. The relative expression levels of miR-21 in osteosarcoma specimens and matched adjacent non-cancerous bone tissues.

Upregulation of miR-21 was associated with poor prognosis in patients with osteosarcoma

Using a Kaplan–Meier method and log-rank test, the OS (Figure 2(A), $P < 0.001$) and DFS (Figure 2(B), $P < 0.001$) for osteosarcoma tissues with high miR-21 levels were significantly shorter than those with low miR-21 levels ($P < 0.001$). However, the survival benefits were also found in those patients with a smaller tumour size ($P = 0.024$ and $P = 0.03$), no distant metastases ($P = 0.002$ and $P = 0.001$) and high clinical stage ($P = 0.016$ and $P = 0.01$) for OS and DFS, respectively.

A multivariate Cox regression analysis using the above-mentioned significant parameters revealed that miR-21 expression, clinical stage and distant metastasis status were independent prognostic markers for OS in patients with osteosarcoma (Table 3). As for DFS, miR-21 expression, clinical stage and metastasis status were also independent prognostic markers for DFS in patients with osteosarcoma (Table 3).

Discussion

Cancer biomarkers must be sensitive enough to identify individuals with disease and specific enough to exclude healthy individuals. However, as no currently used biomarker is 100% specific and sensitive, there is a need for use of more than one, or perhaps a panel of biomarkers, in the molecular screening for diseases.[21] miRNAs are small single-stranded non-coding RNAs about 20–22 nucleotides in length that play an important role in the post-transcriptional regulation of mRNA.[5,6] They bind to the targeting mRNA sequence within a complex of specific proteins, called an RNA-induced silencing complex (RISC), and induce cleavage or repression of the target gene.[6] Therefore, miRNAs play an important role

Table 2. AUC for the ROC curve corresponding to the diagnostic value of miR-21 in osteosarcoma.

Parameter	AUC	Standard error	95% CI
Distant metastasis	0.878	0.016	0.782–0.936
Clinical stage	0.853	0.028	0.764–0.902
Tumour size	0.712	0.054	0.643–0.845
Tumour grade	0.659	0.063	0.587–0.803
Survival	0.705	0.045	0.636–0.843

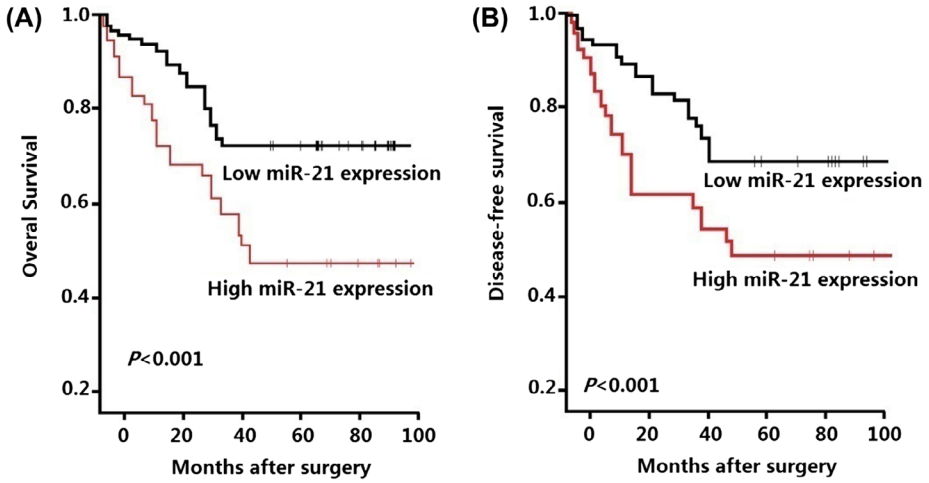

Figure 2. Kaplan–Meier curves for patients with osteosarcoma.

Table 3. Multivariate survival analysis for OS and DFS in 84 patients with osteosarcoma.

	OS			DFS		
Variables	RR	95% CI	*P*	RR	95% CI	*P*
miR-21 level	6.3	1.8–12.4	<0.001	5.8	1.7–13.5	<0.001
Distant metastasis	3.1	1.8–6.7	0.017	2.5	1.3–7.9	0.028
Clinical stage	3.6	2.1–10.6	0.01	2.4	1.5–9.3	0.02

OS = overall survival, DFS = distance free survival.

in gene expression and influence many physiological and pathophysiological processes. These qualities make them promising candidates for biomarkers in tumour specimens and liquid biopsies.

For miRNAs to be used as cancer biomarkers, they have to meet general requirements such as being measureable, reliable for assessing the progression of disease and patient prognosis, and capable of predicting drug efficacy.[22]

It has been reported that an miRNA signature composed of 12 miRNA probes is associated with DFS in patients with acute myeloid leukaemia (AML) [23]; a low expression of miR-181a and miR-181b has been associated with shorter OS and treatment-free survival in patients with chronic lymphocytic leukaemia.[24] High miR-326/miR-130a levels and low miR-323/miR-329/miR-155/miR-210 levels are associated with increased OS in patients with glioblastomas, and high miR-326/miR-130a expression and low miR-155/miR-210 expression are associated with increased progression-free survival.[25] MiR-375 is frequently downregulated in patients with oesophageal squamous cell carcinomas and is associated with advanced clinical stage, metastasis, and poorer outcomes.[26] Likewise, a high miR-155 expression and low let-7a-2 expression correlate with poor survival in patients with lung adenocarcinomas.[27]

Overexpression of miR-21 is common in many solid and haematological malignancies.[28] A higher miR-21 expression is associated with an increase in stage in renal cancer and shorter survival for these patients.[29] A high miR-21 expression correlates with lower OS in patients with breast cancer.[30]

This study focused on the potential relationship between miR-21 expression levels and various clinicopathological characteristics in patients with osteosarcoma, as well as DFS and OS. We found that the miR-21 expression was significantly increased in clinical osteosarcoma tissues compared with the adjacent normal bone tissues, which could identify individuals with OS to exclude healthy individuals. In addition, high levels of miR-21 appear to be significantly correlated with tumour size, clinical stage, distant metastasis and poor prognosis in patients with osteosarcoma. miR-21 was upregulated in patients presenting with metastases, suggesting that its upregulation was acquired in the course of tumour progression and in particular, during the acquisition of metastatic potential.

After dividing the patients using the cut-off method, a multivariate Cox proportional hazard regression analysis revealed that miR-21 overexpression had a significantly worse prognostic impact on the OS and DFS in patients with osteosarcoma. These results indicate that as an independent risk factor, miR-21 could serve as a prognostic marker for the survival of patients. To date, several studies have revealed the prognostic significance of miR-21 overexpression in various carcinomas.[14–19] To the best of our knowledge, our research may be the first report to evaluate the prognostic value of miR-21 in osteosarcoma.

The results of our study indicate that the expression of miR-21 is strongly linked to distant metastasis, tumour size, clinical stages and OS in patients with osteosarcoma, providing evidence that the upregulation of miR-21 might play an important role in the progression of the disease.

Summary

What this study adds
- miR-21 was overexpressed in tissues in osteosarcoma.
- miR-21 levels could discriminate osteosarcoma from normal controls.
- Patients with osteosarcoma had a poor prognosis with high miR-21 expression.

What is known about this subject
- A tissue biomarker is needed for diagnosis and discrimination in osteosarcoma.
- miR-21 was overexpressed in tissues in osteosarcoma.
- Whether tissue miR-21 could be a diagnostic and prognostic marker for osteosarcoma is unknown.

Disclosure statement

No potential conflict of interest was reported by the authors.

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