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Original Article

Expression and diagnostic value of miR-586 and miR-223 in the peripheral blood of patients with osteosarcoma

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Abstract: Purpose: To investigate the diagnostic value of miR-586 and miR-223 in osteosarcoma and provide a theoretical basis for the application of these indices in osteosarcoma diagnosis. Method: Fifty-three patients with osteosarcoma (osteosarcoma group) were included in this study. The normal group comprised 30 healthy volunteers. Results: The serum miR-586 expression level in patients with osteosarcoma was significantly higher than that in normal controls ($t=8.226$, $P < 0.001$), while the serum miR-223 expression level in patients with osteosarcoma was significantly lower than that in normal controls ($t=8.739$, $P < 0.001$). The serum miR-586 level in patients with metastatic osteosarcoma was higher than that in patients without metastatic osteosarcoma ($P < 0.05$), while the serum miR-223 level in patients with metastatic osteosarcoma was lower than that in patients without metastatic osteosarcoma ($P < 0.05$). The use of serum miR-586 combined with miR-223 as osteosarcoma diagnostic criteria for patients with osteosarcoma revealed a higher area under the curve than the use of miR-586 or miR-223 alone. There were statistically significant differences in the sensitivity of the three methods for osteosarcoma diagnoses. The sensitivity of miR-586 combined with miR-223 in the diagnosis of osteosarcoma was higher than that of miR-586 and miR-223 individually (all $P < 0.05$). Conclusion: In the serum of patients with osteosarcoma, miR-586 is highly expressed, while miR-223 is lowly expressed. Both these biomarkers are associated with osteosarcoma clinical stage and tumor metastasis and have a potential value in osteosarcoma diagnoses.

Keywords: miR-586, miR-223, osteosarcoma, peripheral blood, diagnostic value

Introduction

Osteosarcoma is the most common primary malignant bone tumor and has a male predominance. The incidence of osteosarcoma is about 30% across all malignant bone tumors, and 2-3 of 1 million people are diagnosed with osteosarcoma every year [1, 2]. The prognosis is poor, and the five-year survival rate is only about 50% [3, 4]. Therefore, the diagnosis of osteosarcoma has extremely important significance in the treatment and prognosis of patients.

Recently, many scholars reported single-stranded non-coding microribonucleic acids (miRNAs) which are widely expressed in eukaryotic cells and regulate the biological behavior of cells by modifying the transcription and translation of downstream RNAs. Similarly, abnormal changes in miRNA biosynthesis are involved in a variety of pathophysiological processes [5, 6]. Many studies have reported that miRNAs are closely related to the occurrence and development of tumors and the proliferation, invasion, and other behaviors of tumor cells [7, 8]. Only a few miRNAs have been reported to be associated with osteosarcoma, such as recently reported miR-586 and miR-223. Down-regulation of miR-586 can inhibit the proliferation and invasion of osteosarcoma cells, while up-regulation of miR-223 can inhibit osteosarcoma metastasis [9, 10]. However, the diagnostic value of miR-586 and miR-223 in osteosarcoma has rarely been reported.

Currently, the diagnosis of osteosarcoma mostly depends on pathological diagnosis and ultrasonic imaging. However, these procedures are invasive and subjective factors have great influences. Serological examinations, especial-

Table 1. Primer sequences

	Upstream	Downstream
miR-586		
miR-223	5'-AGC CGT GTCAGTTTG TCA AAT-3'	5'-GTGCAGGGTCCGAGG TC-3'
GAPDH	5'-CGGAGTCAACGGATTTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

ly serum miRNA examinations, allows tumor screening and early diagnosis. Serum miRNA examinations have high sensitivity, high specificity, small trauma, good repeatability, and high patient compliance. These procedures avoid the disadvantages of large traumas, complicated methods, and time consuming procedures that are often used during pathological examinations [11, 12].

Therefore, this study analyzed the miR-586 and miR-223 expression levels in the serum of patients with osteosarcoma and their diagnostic value in order to further understand the mechanism of osteosarcoma development and provide references for the diagnosis of osteosarcoma.

Materials and methods

Subjects

Fifty-three patients with osteosarcoma (osteosarcoma group) were admitted to Jingzhou Central Hospital. This study included 33 males and 20 females aged 15-30 years. Serum samples from 30 healthy volunteers were used as the normal group. All patients were diagnosed by our department of pathology, and none of the patients had a Cain's physical condition of < 70 points. There were no distant metastases or ≥ 3-month life expectancies according to radiographic diagnoses. Patients with one of the following were excluded from this study: pregnant or breastfeeding women, patients who received any anti-tumor therapy before surgery, patients with large tumors, previous history of other tumors, family genetic diseases, autoimmune disease history, heart, liver, kidney or other organ dysfunction, abnormal hemorrhagic or coagulation disorders, alcohol abuse, and history of taking nitroglycerin. All healthy volunteers had no organ diseases, neurological disorders, abnormal blood system function, etc. This study was approved by the ethics committee of Jingzhou Central Hospital, and informed consent was signed by the patient or his family.

Extraction of total miRNA in cells

The morning fasting venous blood of all patients was collected by our hospital nurses and sent for examination within one hour. TRIzol reagent (Shanghai Enzyme Biotech Co., Ltd.) was used for the extraction of total RNA from patient serum. The volume ratio of TRIzol reagent to serum was 3:1. The detailed procedure followed the instructions in the kit. The concentration and purity of the extracted total RNA were analyzed using the micro UV spectrophotometer DanoProp 1000 (Tomorgan Biotech Co., Ltd.), and A260/A280 values between 1.45 and 1.60 were considered to meet the experimental requirements. Furthermore, 1% agarose gel electrophoresis (Shanghai Lianmai Biological Engineering Co., Ltd.) was performed to analyze RNA integrity.

RT-PCR reaction

Reverse transcription synthesis of cDNA from the extracted RNA was performed according to the instructions of the TaqMan® MicroRNA Reverse Transcription Kit (Thermo Scientific (China) Co., Ltd.). The reverse transcription reaction was performed as follows: 37°C for 60 min and 85°C for 5 min. The total cDNA amplification reaction volume was 25 μL, containing 5.0 μL of 5 * Reaction Buffer, 2 μL of 2 μg 2.5 U/μL RNA polymerase, 1 μL of RTase Mix, and ribonuclease distilled water to 25 μL. PCR amplification was performed as follows: after denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 60°C for 20 s, extension at 72°C for 34 s for 35 cycles, and extension at 72°C for 5 min after completion of the cycle. The reaction system contained 20 μL volume, which was composed of 2 μL of cDNA template, 12 μL of SYBR Green Mix, 2 μL of each upstream and downstream primer, and double distilled water was added to 20 μL. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as the reaction reference. Each sample was tested in triplicate, and the results were analyzed using the $2^{-\Delta\Delta ct}$ method (**Table 1**).

Table 2. General information

	Osteosarcoma (n=53)	Normal (n=30)	X^2	*p*-valued
Sex [n (%)]				0.494
Male	33 (62.3)	16 (53.3)		
Female	20 (37.7)	14 (46.7)		
Age (years)	19.3±6.8	21.5±8.9	1.264	0.210
Diameter of tumor [n (%)]				
≥ 8 cm	24 (45.3)			
< 8 cm	29 (54.7)			
Tumor type [n (%)]				
Osteoblast type	23 (43.4)			
Chondroblast type	15 (28.3)			
Fibroblast type	13 (24.5)			
Mixed type	2 (3.8)			
Tumor site [n (%)]				
Thighbone	38 (71.7)			
Tibiofibula	8 (15.1)			
Eelse	7 (13.2)			
Clinical stages [n (%)]				
I-IIA	22 (41.5)			
IIB-III	31 (58.5)			
Transfer [n (%)]				
Yes	21 (39.6)			
No	32 (60.4)			

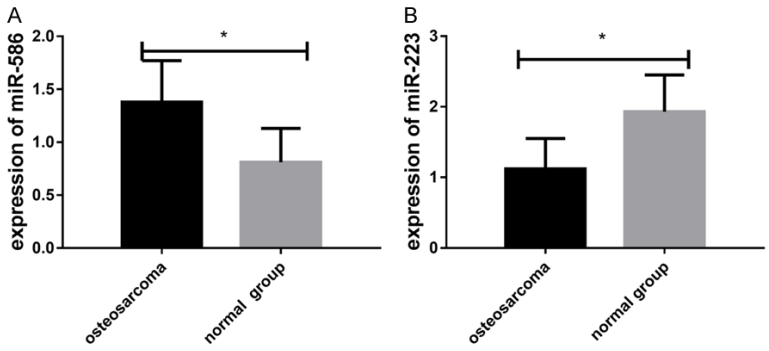

Figure 1. A. The expression of miR-586 in the serum of patients with osteosarcoma. *, is P < 0.05. B. The expression of miR-223 in the serum of patients with osteosarcoma. *, is P < 0.05.

Statistical analysis

SPSS 19.0 (Asia Analytics Formerly SPSS China) was used for statistical analyses. The counted data are presented in rates, and the rates were compared using the X^2 test. Measured data are expressed as x ± sd, and t-tests were used to compare the data. The receiver operating characteristic (ROC) curve was used to analyze the diagnostic value of miR-586 and miR-223 in patients with osteosarcoma, where *P* values of < 0.05 were considered statistically significant.

Results

General information

Fifty-three patients with osteosarcoma were included in this study, with an age range of 15-30 years and an average age of 19.3±6.8 years. This cohort included 33 males and 20 females. Twenty-four patients (45.3%) had tumor diameters of > 8 cm, and 29 patients (54.7%) had tumor diameters of < 8 cm. There were 23 patients (43.4%) with osteoblast-type osteosarcoma, 15 (28.3%) with chondrocyte osteosarcoma, 13 (24.5%) with fibroblast-type osteosarcoma, and two (3.8%) with mixed-type osteosarcoma. Most of the osteosarcoma sites were concentrated in the femur (38 patients, 71.7%). Osteosarcomas also occurred in the patella of eight patients (15.1%) and in other parts of seven patients (13.2%). Twenty-two patients (41.5%) had stage I-IIA osteosarcoma and 31 (58.5%) had stage IIB-III. Twenty-one patients (39.6%) had tumor metastases. There were no statistically significant differences in sex and age between the two groups (P > 0.05) (**Table 2**).

miR-586 and miR-223 expression levels in the serum of subjects in the two groups

We used qRT-PCR to analyze the miR-586 and miR-223 expressions in the serum of 53 patients with osteosarcoma and 30 normal controls. The results showed that the serum miR-586 expression level in patients with osteosarcoma was 1.38±0.39 and that in normal controls was 0.81±0.32. The serum miR-

Table 3. Relationship between serum miR-586 and miR-223 expression levels and clinical characteristics in patients with osteosarcoma

	Number	miR-586	t	*p*-valued	miR-223	t	*p*-valued
Sex [n (%)]			1.053	0.295		1.435	0.154
Male	33	1.34±0.42			1.18±0.45		
Female	20	1.42±0.36			1.06±0.41		
Age (years)			0.446	0.657		1.414	0.259
≤ 18	33	1.36±0.38			1.21±0.42		
> 18	20	1.31±0.42			1.08±0.37		
Diameter of tumor [n (%)]		1.40±0.38			1.15±0.44		
≥ 8 cm	24	1.36±0.40			1.09±0.42		
< 8 cm	29		1.826	0.071		1.665	0.099
Tumor type [n (%)]		1.31±0.33			1.05±0.38		
Osteoblast type	23	1.45±0.45			1.19±0.48		
Chondroblast type	15		0.487	0.692		0.841	0.473
Fibroblast type	12	1.33±0.36			1.13±0.36		
Mixed type	2	1.38±0.39			1.18±0.41		
Tumor site [n (%)]		1.42±0.41			1.06±0.37		
Thighbone	38	1.39±0.40			1.12±0.42		
Tibiofibula	8		1.219	0.298		0.489	0.615
Else	7	1.45±0.42			1.07±0.45		
Clinical stages [n (%)]		1.36±0.45			1.13±0.41		
I-IIA	22	1.33±0.36			1.15±0.44		
IIB-III	31		2.395	0.019		2.567	0.012
Transfer [n (%)]		1.28±0.36			1.22±0.45		
Yes	21	1.48±0.49			1.01±0.39		
No	32		2.109	0.037		2.258	0.026
Sex [n (%)]		1.42±0.37			1.04±0.34		
Male		1.26±0.41			1.21±0.43		

586 expression level in patients with osteosarcoma was significantly higher than that in normal controls (t=8.226, P < 0.001). The serum miR-223 expression level in patients with osteosarcoma was 1.12±0.43 and that in normal controls was 1.93±0.52. The serum miR-223 expression level in patients with osteosarcoma was significantly lower than that in normal controls (t=8.739, P < 0.001) (**Figure 1**).

Relationship between serum miR-586 and miR-223 expression levels and clinical characteristics of patients with osteosarcoma

Across patients with osteosarcoma, the differences in serum miR-586 and miR-223 expression levels with respect to sex, age, tumor size, tumor type, and tumor site were not statistically significant (P > 0.05). However, the differences in serum miR-586 and miR-223 expression levels in patients with osteosarcoma were statistically significant between clinical stages and the presence of metastases. The serum miR-586 expression level in patients with stage I-IIA osteosarcoma was lower than that in patients with stage IIB-III (P < 0.05). The miR-223 expression level in patients with stage I-IIA osteosarcoma was higher than that in patients with stage IIB-III (P < 0.05). Furthermore, the serum miR-586 expression level in patients with metastatic osteosarcoma was higher than that in patients without metastatic osteosarcoma (P < 0.05), while the serum miR-223 expression level in patients with osteosarcoma with metastasis was lower than that in patients with osteosarcoma without metastasis (P < 0.05) (**Table 3**).

Diagnostic value of miR-586 and miR-223 in osteosarcoma

Our investigation of serum miR-586 combined with miR-223 as the criteria for the diagnosis of

Table 4. Diagnostic value of miR-586 and miR-223 in osteosarcoma

	miR-586	miR-223	miR-586 combine miR-223	X^2	*p*-valued
AUC	0.743	0.747	0.813		
95% confidence Interval	0.637-0.849	0.616-0.879	0.709-0.918		
Diagnostic level	1.132	1.425			
Sensitivity	64.2%	73.6%	88.7%*,#	11.242	0.004
Specificity	70.0%	60.0%	63.4%	0.679	0.712

Note: *indicates the sensitivity compared with miR-586 (P < 0.05); #indicates the sensitivity compared with miR-223.

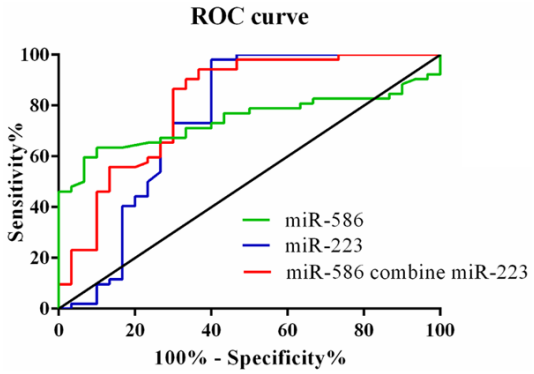

Figure 2. ROC curve of miR-223 and miR-586 in the diagnosis of osteosarcoma. AUC (miR-586)=0.743, AUC (miR-223)=0.747, AUC (miR-586 combine miR-223)=0.813. The black line is a baseline. For every point in the line, (Sensitivity)/(1-Specificity)=1. The closer the curve comes to the 45-degree diagonal of the ROC space, the less accurate the test.

osteosarcoma revealed that the area under the curve (AUC) was higher for the combined use than that for miR-586 and miR-223 alone. The diagnostic level of miR-586 for osteosarcoma was 1.132, and the diagnostic level of miR-223 was 1.425. There was a statistically significant difference in the sensitivity of the three methods for the diagnosis of osteosarcoma. The sensitivity of miR-586 combined with miR-223 in the diagnosis of osteosarcoma was higher than that of miR-586 and miR-223 individually (all P < 0.05). The sensitivity of miR-586 in the diagnosis of osteosarcoma was not statistically different from that of miR-223 (P > 0.05), and the specificity of the three methods in the diagnosis of osteosarcoma was not statistically different (P > 0.05) (**Table 4**; **Figure 2**).

Discussion

Osteosarcoma is a primary malignant tumor with high heterogeneity that is mainly characterized by vascular invasion and local soft tissue infiltration [13]. The detection of tumor-associated miRNAs as diagnostic markers of malignancy is currently a highly researched topic [14, 15]. miR-586 and miR-223 are two miRNAs that have been reported to be involved in biological behaviors, including the proliferation and metastasis of osteosarcoma [9, 10].

In this study, the results of the patients with osteosarcoma were compared with 30 healthy patients at the same time. All serum samples were analyzed by qRT-PCR, and the results showed that the serum miR-586 expression level in patients with osteosarcoma was significantly higher than that in normal people. Furthermore, the serum miR-223 expression level in patients with osteosarcoma was significantly lower than that in normal people, which is consistent with results of recent relevant reports [16, 17]. We then analyzed the relationship among miR-586, miR-223, and clinical characteristics of patients with osteosarcoma. The results showed that the serum miR-586 and miR-223 expression levels in patients with osteosarcoma did not significantly differ with sex, age, tumor diameter, tumor type, and tumor location. However, the serum miR-586 expression level in patients with stage I-IIA osteosarcoma was lower than that in patients with stage IIB-III osteosarcoma, and the serum miR-223 expression level in patients with stage I-IIA osteosarcoma was higher than that in patients with stage IIB-III. The miR-586 expression level in patients with metastatic osteosarcoma was higher than that in patients without metastases, while the miR-223 expression level in patients with metastatic osteosarcoma was lower than that in patients with non-metastatic osteosarcoma.

Zhang et al. [18] independently reported that the high expression of miR-223 closely associated with decreased TNM stage and tumor metastasis, which is similar to our results. However, few reports have investigated the

relationship between miR-586 and clinical characteristics of patients with osteosarcoma [19], Therefore, our results need to be verified with more clinical data.

We analyzed the diagnostic value of serum miR-586 and miRNA-223 in patients with osteosarcoma and found that serum miR-586 combined with miR-223 provides sufficient criteria for the diagnosis of osteosarcoma. The AUC and sensitivity of these biomarkers are significantly higher than that of miR-586 or miR-223 as single biomarkers [20, 21]. The three osteosarcoma diagnostic methods achieved AUCs of >0.7, which suggested that miR-586 and miRNA-223 have potential diagnostic value in osteosarcoma, especially the combination of miR-586 and miRNA-223 [22].

Dong et al. [23] reported that the AUC of miR-223 in the diagnosis of osteosarcoma was 0.956, the specificity was 86.4%, and the sensitivity was 97.2%. This is far from our results and may be related to the inadequacy of our included subjects. Dong et al. included 112 patients with osteosarcoma, while we included 53 patients with osteosarcoma and 30 healthy individuals. This makes the accidental errors of the qRT-PCR detection results larger than those of Dong et al. The internal parameters we used also differed from those of Dong et al., and this may also be a reason for the discrepancy. In future studies, we will increase the sample size and analyze different internal parameters. There have been very few reports on the diagnosis of miR-586 and osteosarcoma, but in one report, miR-586 was one of the most significant differences among osteosarcoma-associated miRNAs [24]. This observation, to some extent, can reflect its potential value in the diagnosis of osteosarcoma.

In summary, miR-586 is highly expressed in the serum of patients with osteosarcoma, while miR-223 is expressed at a low level. Both of these miRNAs are associated with osteosarcoma clinical stage and tumor metastasis and have potential value in the diagnosis of osteosarcoma.

Disclosure of conflict of interest

None.

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