MicroRNA-375 as a potential serum biomarker for the diagnosis, prognosis, and chemosensitivity prediction of osteosarcoma

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Abstract

Objective: This study was performed to examine serum microRNA-375 (miR-375) expression in patients with osteosarcoma and determine its diagnostic and prognostic value.

Methods: Serum samples were obtained from 95 patients with osteosarcoma and 95 healthy individuals. miR-375 expression was detected by real-time polymerase chain reaction. The associations of serum miR-375 expression with the patients' clinicopathological characteristics and prognosis were then evaluated. Receiver operating characteristic curve analysis was performed to obtain the potential value of serum miR-375 as a biomarker for osteosarcoma diagnosis and chemosensitivity prediction.

Results: Serum miR-375 expression was significantly lower in patients with osteosarcoma than in healthy individuals. Low serum miR-375 levels were associated with advanced clinical stages, large tumor size, positive distant metastasis, and poor tumor response to preoperative chemotherapy. Receiver operating characteristic curve analysis illustrated that serum miR-375 could distinguish patients with osteosarcoma from healthy individuals and distinguish patients with a good pathologic response from those with a poor response. Multivariate analysis confirmed low serum miR-375 expression as a statistically significant independent unfavorable prognostic factor.

Conclusions: Serum miR-375 expression was downregulated in patients with osteosarcoma and might serve as a biomarker for its diagnosis, prognosis, and chemosensitivity prediction.

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Introduction

Osteosarcoma, accounting for approximately 60% of pediatric bone tumors, is an aggressive disease that occurs predominantly in adolescents and young adults.[1] Despite recent developments in surgical techniques and combinational chemotherapy, the long-term survival of patients with osteosarcoma remains unsatisfactory, mainly because of delayed diagnosis, distant metastasis, and chemoresistance.[2] Thus, there is an ongoing need for a better understanding of the molecular mechanisms underlining osteosarcoma and identification of novel and efficient biomarkers for its diagnosis, prognosis, and chemosensitivity prediction.

MicroRNAs (miRs) are a novel class of short, endogenous, noncoding, and highly conserved RNA molecules that play a critical role in regulating post-transcriptional gene expression.[3] Emerging evidence has shown that miRs are essential regulators in a wide range of fundamental physiological processes, and dysregulated miR expression contributes to many human diseases, including the development of malignant tumors.[4] Recently, miRs were shown to be stably expressed in plasma, serum, and other body fluids.[5] More importantly, circulating miRs have distinct expression signatures in different diseases, allowing the application of circulating miRs as non-invasive biomarkers for cancer and other diseases.[6] For example, plasma miR-145, miR-20a, and miR-223 could serve as novel biomarkers for screening early-stage non-small cell lung cancer (NSCLC).[7] Increased serum miR-21 expression predicted poor overall survival in patients with breast cancer,[8] colorectal cancer (CRC),[9] and NSCLC.[10] High serum miR-200c expression in patients with esophageal squamous cell carcinoma (ESCC) was significantly associated with advanced clinical stages and a poor tumor response to platinum-based chemotherapy.[11]

As a potential tumor-suppressor gene, miR-375 has been corroborated to be downregulated in a variety of human malignancies including breast cancer,[12] ESCC,[13] lung cancer,[14] CRC,[15] prostate cancer,[16] gastric cancer,[17] hepatocellular carcinoma,[18] and osteosarcoma.[19] Shi et al.[19] showed decreased miR-375 expression in osteosarcoma tissues and cell lines. In vitro functional experiments also confirmed the tumor-suppressive ability of miR-375 in osteosarcoma.[19,20] More importantly, several recent studies have reported low miR-375 expression in the serum/plasma of patients with prostate cancer,[21] ESCC,[22] gastric cancer,[17] CRC,[23] and NSCLC[24] and confirmed its utility as an miR-biomarker. However, no data regarding the clinical significance of circulating miR-375 in patients with osteosarcoma have yet been reported in the literature. Therefore, the aim of this study was to determine the clinicopathological, diagnostic, and prognostic value of serum miR-375 in human osteosarcoma.

Materials and methods

Patients and samples

Patients with primary osteosarcoma treated in Tianjin Baodi Hospital and Tianjin Medical University General Hospital from January 2005 to December 2012 were

retrospectively enrolled in this study. All patients received neoadjuvant chemotherapy (combination of doxorubicin and methotrexate) and underwent surgical resection. An equal number of age- and sex-matched healthy individuals was recruited as the control group. For each participant, 5 mL of venous blood was collected before any treatment and centrifuged immediately at $1500 \times g$ for 15 min at 4°C. The serum was then stored in 1.5-mL RNase-free tubes at -80°C. The tumor response to chemotherapy was assessed according to the Huvos grading system.[25] Tumor necrosis of $\geq 90\%$ in response to neoadjuvant chemotherapy was classified as a good pathologic response, while tumor necrosis of $<90\%$ was defined as a poor pathologic response. Patients were followed up after surgical treatment at 3- to 6-month intervals, and overall survival was defined as the interval from surgery to death or last follow-up. This study was approved by the Research Ethics Committee of Tianjin Baodi Hospital and Tianjin Medical University General Hospital. All participants provided written informed consent.

RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from 400 μL of serum using an mirVana PARIS Kit (Ambion, Austin, TX, USA). Reverse transcription was performed with 100 ng of total RNA using a TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a 15-μL reaction system. Real-time polymerase chain reaction (PCR) was performed with a TaqMan microRNA Assay Kit (Applied Biosystems) on a 7500 Sequence Detection System (Applied Biosystems). The reaction system was 20 μL, containing 8 μL of nuclease-free water, 1 μL of reverse transcriptase product, 10 μL of $2 \times$ Universal

PCR Master Mix, and 1 μL of the TaqMan microRNA Assay. U6 RNA was used as the reference control for normalization, and relative miR-375 expression was calculated by the $2^{-\Delta\Delta Ct}$ method.[22] The PCR primers used in this study were as follows: miR-375, 5′-AGC CGT TTG TTC GTT CGG CT-3′ (forward) and 5′-GTG CAG GGT CCG AGG T-3′ (reverse); U6 RNA, 5′-CGC TTC GGC AGC ACA TAT AC-3′ (forward) and 5′-TTC ACG AAT TTG CGT GTC AT-3′ (reverse).

Statistics

Statistical analysis was carried out with the SPSS 20.0 statistical package (IBM Corp., Armonk, NY, USA), and the significance was set at $P < 0.05$. The serum miR-375 levels between patients with osteosarcoma and healthy controls were compared by the Mann–Whitney U test. Correlations between serum miR-375 expression and clinicopathological parameters were analyzed by the chi-square test. Receiver operating characteristic (ROC) analysis was applied to determine the diagnostic utility of serum miR-375. The cumulative overall survival rates were calculated using the Kaplan–Meier method, and a Cox regression model was used to test the independence of risk factors.

Results

Downregulation of serum miR-375 in patients with osteosarcoma and its diagnostic value

In total, 95 patients with primary osteosarcoma were included in this study, and 69 of them were under the age of 20 years (Table 1). This is consistent with previous reports showing that the majority of patients with osteosarcoma are under the age of 25 years and that most develop

Table 1. Association of serum miR-375 expression with clinicopathological features of osteosarcoma

Clinicopathological features	Number of patients	Serum miR-375 expression		P value
		Low, n (%)	High, n (%)	
Age				
<20 years	69	33 (47.8)	36 (52.2)	0.650
≥20 years	26	14 (53.8)	12 (46.2)	
Sex				
Male	63	29 (46.0)	34 (54.0)	0.390
Female	32	18 (56.2)	14 (43.8)	
Tumor size				
>8 cm	34	24 (70.6)	10 (29.4)	0.003
≤8 cm	61	23 (37.7)	38 (62.3)	
Tumor location				
Tibia/femur	66	35 (53.0)	31 (47.0)	0.374
Elsewhere	29	12 (41.4)	17 (58.6)	
Clinical stage				
IIA	51	19 (37.3)	32 (62.7)	0.014
IIB/III	44	28 (63.6)	16 (36.4)	
Distant metastasis				
Absent	58	21 (36.2)	37 (63.8)	0.002
Present	37	26 (70.3)	11 (29.7)	
Response to chemotherapy				
Good	50	15 (30.0)	35 (70.0)	<0.001
Poor	45	32 (71.1)	13 (28.9)	

miR-375, microRNA-375.

osteosarcoma in their second decade of life (<20 years).[1] The clinicopathological data of all patients with osteosarcoma in this study are summarized in Table 1. Serum miR-375 expression was detected in all 95 patients and in 95 healthy controls by quantitative real-time PCR. The results showed that miR-375 expression in the serum of patients with osteosarcoma was significantly lower than that of controls ($P < 0.01$) (Figure 1(a)). ROC curve analysis demonstrated that serum miR-375 was a potential biomarker for a diagnosis of osteosarcoma with an area under the ROC curve of 0.89 (95% confidence interval, 0.84–0.93) (Figure 1(b)). Using a cut-off value of 0.42, the sensitivity and specificity were 82.1% and 74.7%, respectively.

Association of serum miR-375 expression with clinicopathological features and ability to predict chemosensitivity and patient's prognosis

We divided the patients with osteosarcoma into a high miR-375 expression group and low miR-375 expression group using the median value of serum miR-375 expression in all patients as a cut-off point. As summarized in Table 1, a low serum miR-375 level was significantly associated with an advanced clinical stage ($P = 0.014$), large tumor size ($P = 0.003$), distant metastasis ($P = 0.002$), and poor tumor response to chemotherapy ($P < 0.001$). ROC curve analysis showed that the serum miR-375 level could distinguish a good from poor
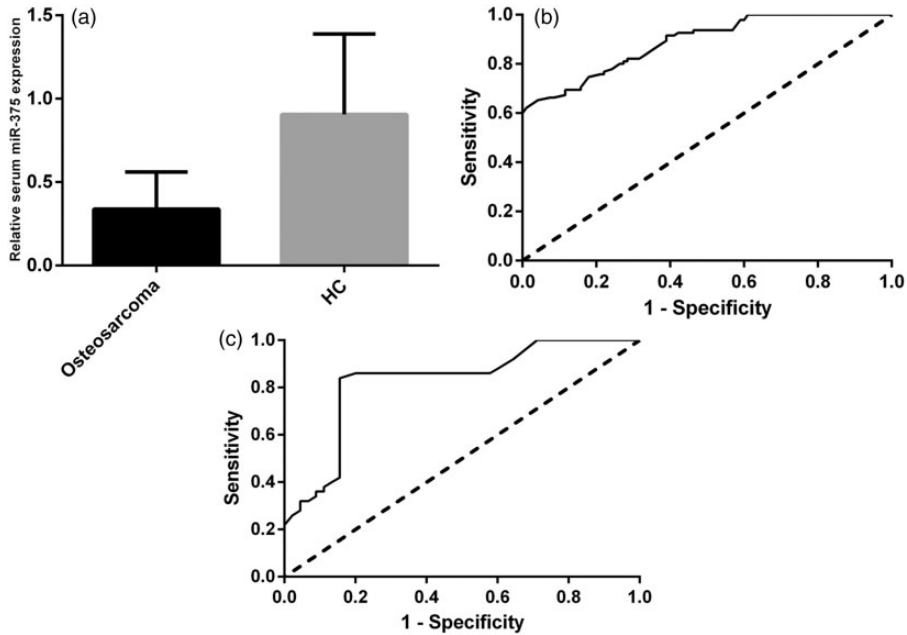
Figure 1. Serum microRNA-375 (miR-375) expression in patients with osteosarcoma and its potential value for osteosarcoma diagnosis and chemosensitivity prediction.
(a) miR-375 levels in serum from patients with osteosarcoma were significantly lower than those in healthy controls (HC) (P < 0.01).
(b) Receiver operating characteristic curve analysis illustrated that serum miR-375 was a reliable biomarker for discriminating patients with osteosarcoma from healthy controls.
(c) Receiver operating characteristic curve analysis illustrated that serum miR-375 could differentiate a good from poor pathologic response.

pathologic response with an area under the ROC curve of 0.83 (95% confidence interval, 0.74–0.91) (Figure 1c). At the optimal cut-off point (relative expression of 0.26), serum miRN-375 had a sensitivity of 84.0% and specificity of 84.4%.

According to the results of the Kaplan–Meier analysis and log-rank test, patients with osteosarcoma with low serum miR-375 levels had shorter overall survival than those with high serum miRNA-375 levels (P = 0.006) (Figure 2). Univariate analysis indicated that tumor size, clinical stage, distant metastasis, and response to chemotherapy were also risk factors affecting the patient's overall survival (all P < 0.05) (Table 2). Multivariate analysis confirmed that serum miR-375 expression

was an independent prognostic factor for osteosarcoma (P = 0.008) (Table 2).

Discussion

Exploration of reliable biomarkers with high sensitivity and specificity is of great clinical significance for early detection and therapeutic outcome prediction of osteosarcoma. Emerging studies have indicated that circulating miRs may serve as promising biomarkers in human diseases, including malignancies.[6] In the present study, we found decreased serum miR-375 expression in patients with osteosarcoma and observed that this decreased expression was correlated with tumor size, clinical stage, distant metastasis, and response to chemotherapy.
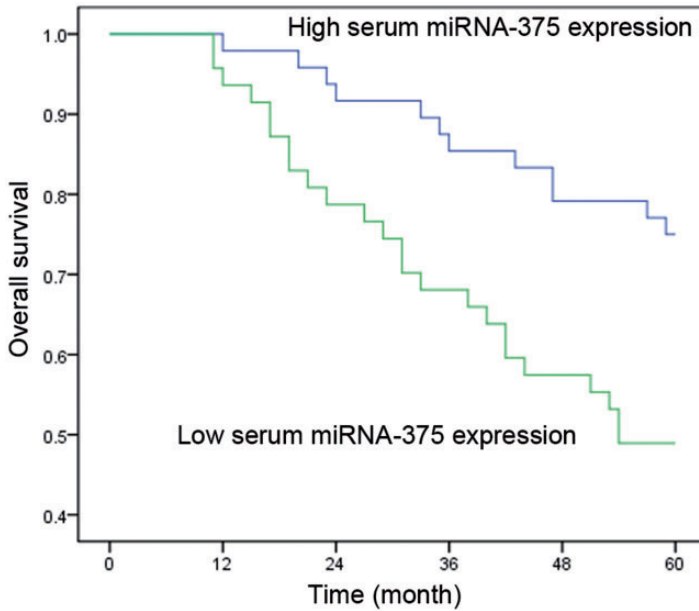
Figure 2. Prognostic value of serum microRNA-375 (miR-375) for patients with osteosarcoma. Patients with low serum miR-375 expression had significantly worse 5-year overall survival than those with high serum miR-375 expression (P = 0.006).

Table 2. Univariate and multivariate survival analyses of overall survival in 95 patients with osteosarcoma

Variables	Univariate analysis		Multivariate analysis	
	HR	P value	HR	P value
Age	0.86	0.672	——	——
Sex	1.14	0.584	——	——
Tumor size	2.19	0.028	2.04	0.032
Clinical stage	4.21	0.001	3.79	0.005
Distant metastasis status	3.92	0.003	4.03	0.002
Response to chemotherapy	2.87	0.015	2.45	0.021
Serum miR-375 expression	3.65	0.006	3.27	0.008

HR, hazard ratio; miR-375, microRNA-375.

A low serum miR-375 level was an independent and unfavorable prognostic factor for osteosarcoma. Furthermore, ROC analyses confirmed the value of serum miR-375 as a biomarker for osteosarcoma diagnosis and chemosensitivity prediction. To the best of our knowledge, this is the first study to evaluate serum miR-375 expression and its clinical significance in patients with osteosarcoma.

Previous research has revealed the tumor-suppressive function of miR-375 in many malignancies, including osteosarcoma. miR-375 was downregulated in osteosarcoma tumor samples and cell lines.[19,20] Overexpression of miR-375 markedly

suppressed osteosarcoma cell proliferation in vitro, and inhibition of miR-375 promoted osteosarcoma growth.[19] Mechanistic studies showed that PIK3CA was a potential target of miR-375 and that miR-375 might suppress osteosarcoma growth through mediation of the PI3K/Akt pathway.[19] As a potential cancer biomarker, aberrant circulating miR-375 expression has been reported in several human cancers. Plasma miR-375 was significantly downregulated in patients with prostate cancer compared with benign prostatic hyperplasia samples and showed superior diagnostic accuracy compared with the traditional biomarker prostate-specific antigen.[21] Downregulation of plasma miR-375 expression in NSCLC was associated with cancer metastasis.[24] Plasma miR-375 was a useful biomarker for the diagnosis of gastric cancer and CRC.[17,26] A low serum miR-375 level predicted poor survival of patients with ESCC.[22] Taken together, these findings may help to understand the diagnostic and prognostic value of serum miR-375 in patients with osteosarcoma. The expression and clinical significance of circulating miR-375 in other cancers is worthy of an in-depth study.

Chemoresistance remains a substantial problem in osteosarcoma treatment. Therefore, predictive markers in multimodality therapy of osteosarcoma are required. Some studies have indicated that miR-375 is involved in chemosensitivity regulation in several cancers. miR-375 overexpression could increase the cisplatin sensitivity of human gastric cancer cells by regulating ERBB2.[27] Lipid-coated cisplatin nanoparticles co-loaded with miR-375 enhanced the antitumor effect of cisplatin in chemotherapy-resistant hepatocellular carcinoma cells.[28] Upregulation of miR-375 in medullary thyroid carcinomas was associated with increased sensitivity to vandetanib.[29] In the present study, we observed a close relationship between the serum miR-375

level and osteosarcoma response to preoperative chemotherapy. ROC curve analysis confirmed serum miR-375 as a potential biomarker with which to differentiate a good from poor pathologic response. Therefore, circulating miR-375 might act as a candidate biomarker for chemosensitivity prediction of human malignancies.

We are aware of some limitations in our work. First, it was a single-center retrospective study. Second, the molecular mechanisms by which miR-375 regulates osteosarcoma chemosensitivity require further clarification. Third, we only detected serum miR-375 levels in this study. Several other miRs, such as miR-21 and miR-17, are also expressed abnormally in the serum of patients with osteosarcoma.[30,31] Genome-wide microarray analysis might be an ideal way to identify circulating miRs with diagnostic ability, and a blood-based biomarker panel including more miRs and other molecular markers would help to improve the sensitivity and specificity.

In summary, our study revealed a decreased serum miR-375 level in patients with osteosarcoma and its potential value as a noninvasive biomarker for osteosarcoma diagnosis, prognosis, and chemosensitivity prediction. Further prospective studies with higher numbers of patients are required to validate these results.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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References

1. Mirabello L, Troisi RJ and Savage SA. International osteosarcoma incidence patterns in children and adolescents, middle

ages and elderly persons. *Int J Cancer* 2009; 125: 229–234.

2. Laschi M, Bernardini G, Geminiani M, et al. Establishment of Four New Human Primary Cell Cultures from Chemo-Naive Italian Osteosarcoma Patients. *J Cell Physiol* 2015; 230: 2718–2727.

3. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004; 116: 281–297.

4. Wang S, Wu W and Claret FX. Mutual regulation of microRNAs and DNA methylation in human cancers. *Epigenetics* 2017; 12: 1–11.

5. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008; 105: 10513–10518.

6. Zhao Y, Song Y, Yao L, et al. Circulating microRNAs: promising biomarkers involved in several cancers and other diseases. *DNA Cell Biol* 2017; 36: 77–94.

7. Zhang H, Mao F, Shen T, et al. Plasma miR-145, miR-20a, miR-21 and miR-223 as novel biomarkers for screening early-stage non-small cell lung cancer. *Oncol Lett* 2017; 13: 669–676.

8. Yadav P, Mirza M, Nandi K, et al. Serum microRNA-21 expression as a prognostic and therapeutic biomarker for breast cancer patients. *Tumour Biol* 2016; 37: 15275–15282.

9. Menendez P, Padilla D, Villarejo P, et al. Prognostic implications of serum microRNA-21 in colorectal cancer. *J Surg Oncol* 2013; 108: 369–373.

10. Wang ZX, Bian HB, Wang JR, et al. Prognostic significance of serum miRNA-21 expression in human non-small cell lung cancer. *J Surg Oncol* 2011; 104: 847–851.

11. Yu H, Duan B, Jiang L, et al. Serum miR-200c and clinical outcome of patients with advanced esophageal squamous cancer receiving platinum-based chemotherapy. *Am J Transl Res* 2013; 6: 71–77.

12. Fu H, Fu L, Xie C, et al. miR-375 inhibits cancer stem cell phenotype and tamoxifen resistance by degrading HOXB3 in human ER-positive breast cancer. *Oncol Rep* 2017; 37: 1093–1099.

13. Yi J, Jin L, Chen J, et al. MiR-375 suppresses invasion and metastasis by direct targeting of SHOX2 in esophageal squamous cell carcinoma. *Acta Biochim Biophys Sin (Shanghai)* 2017; 49: 159–169.

14. Chen WJ, Gan TQ, Qin H, et al. Implication of downregulation and prospective pathway signaling of microRNA-375 in lung squamous cell carcinoma. *Pathol Res Pract* 2017; 213: 364–372.

15. Wei R, Yang Q, Han B, et al. microRNA-375 inhibits colorectal cancer cells proliferation by downregulating JAK2/STAT3 and MAP3K8/ERK signaling pathways. *Oncotarget* 2017; 8: 16633–16641.

16. Selth LA, Das R, Townley SL, et al. A ZEB1-miR-375-YAP1 pathway regulates epithelial plasticity in prostate cancer. *Oncogene* 2017; 36: 24–34.

17. Zhang WH, Gui JH, Wang CZ, et al. The identification of miR-375 as a potential biomarker in distal gastric adenocarcinoma. *Oncol Res* 2012; 20: 139–147.

18. Zhou N, Wu J, Wang X, et al. Low-level expression of microRNA-375 predicts poor prognosis in hepatocellular carcinoma. *Tumour Biol* 2016; 37: 2145–2152.

19. Shi ZC, Chu XR, Wu YG, et al. MicroRNA-375 functions as a tumor suppressor in osteosarcoma by targeting PIK3CA. *Tumour Biol* 2015; 36: 8579–8584.

20. Hu W and Xiao Z. Formononetin induces apoptosis of human osteosarcoma cell line U2OS by regulating the expression of Bcl-2, Bax and MiR-375 in vitro and in vivo. *Cell Physiol Biochem* 2015; 37: 933–939.

21. Kachakova D, Mitkova A, Popov E, et al. Combinations of serum prostate-specific antigen and plasma expression levels of let-7c, miR-30c, miR-141, and miR-375 as potential better diagnostic biomarkers for prostate cancer. *DNA Cell Biol* 2015; 34: 189–200.

22. Wu C, Li M, Hu C and Duan H. Clinical significance of serum miR-223, miR-25 and miR-375 in patients with esophageal squamous cell carcinoma. *Mol Biol Rep* 2014; 41: 1257–1266.

23. Komatsu S, Ichikawa D, Takeshita H, et al. Prognostic impact of circulating miR-21 and miR-375 in plasma of patients with

esophageal squamous cell carcinoma. *Expert Opin Biol Ther* 2012; 12 Suppl 1: S53–S59.

24. Yu H, Jiang L, Sun C, et al. Decreased circulating miR-375: a potential biomarker for patients with non-small-cell lung cancer. *Gene* 2014; 534: 60–65.

25. Rosen G, Caparros B, Huvos AG, et al. Preoperative chemotherapy for osteogenic sarcoma: selection of postoperative adjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy. *Cancer* 1982; 49: 1221–1230.

26. Xu L, Li M, Wang M, et al. The expression of microRNA-375 in plasma and tissue is matched in human colorectal cancer. *BMC Cancer* 2014; 14: 714.

27. Zhou N, Qu Y, Xu C, et al. Upregulation of microRNA-375 increases the cisplatin-sensitivity of human gastric cancer cells by regulating ERBB2. *Exp Ther Med* 2016; 11: 625–630.

28. Yang T, Zhao P, Rong Z, et al. Anti-tumor Efficiency of Lipid-coated Cisplatin Nanoparticles Co-loaded with MicroRNA-375. *Theranostics* 2016; 6: 142–154.

29. Lassalle S, Zangari J, Popa A, et al. MicroRNA-375/SEC23A as biomarkers of the in vitro efficacy of vandetanib. *Oncotarget* 2016; 7: 30461–30478.

30. Yuan J, Chen L, Chen X, et al. Identification of serum microRNA-21 as a biomarker for chemosensitivity and prognosis in human osteosarcoma. *J Int Med Res* 2012; 40: 2090–2097.

31. Li S, Gao Y, Wang Y, et al. Serum microRNA-17 functions as a prognostic biomarker in osteosarcoma. *Oncol Lett* 2016; 12: 4905–4910.