MicroRNA screening identifies circulating microRNAs as potential biomarkers for osteosarcoma

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Abstract. MicroRNAs (miRNAs) are a family of small non-protein coding RNAs, which regulate the expression of a wide variety of genes at the post-transcriptional level to control numerous biological and pathological processes. Various circulating miRNAs have been identified as potential diagnostic and prognostic biomarkers in multiple types of cancer and disease. The aim of the present study was to identify potential miRNA biomarkers for the early diagnosis and relapse prediction of osteosarcoma (OS). miRNA profiling was performed on serum from patients with osteosarcoma and healthy controls. All putative miRNAs were verified by reverse transcription-quantitative polymerase chain reaction analysis of 20 pre-therapeutic OS patients and 20 healthy individuals. The expression of miR-106a-5p, miR16-5p, miR-20a-5p, miR-425-5p, miR451a, miR-25-3p and miR139-5p was demonstrated to be downregulated in the serum of OS patients when compared with that of the healthy controls. Receiver-operating characteristic curve analyses indicated that these 7 miRNAs may be used as diagnostic biomarkers with the ability to discriminate between the healthy cohort and patients with OS. These results provide novel insights into the use of miRNAs in early blood screening for OS.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor amongst children and adolescents, and is localized to the metaphysis of the long bones (1). Through a combination of chemotherapy and advanced surgery, the 5-year survival rate for patients with OS has significantly improved, to 70% (2). However, a considerable proportion of OS patients possess a significant risk of local relapse or distant metastasis, even subsequent to neoadjuvant chemotherapy and beneficial surgery (3). Local and metastatic relapses have been consistently demonstrated to markedly reduce survival (4,5). Although numerous studies have focused on improving the treatment of OS in order to enhance patient prognosis, early detection of cancer remains highly recommended for the improvement of survival rates (6).

MicroRNAs (miRNAs), initially discovered in *Caenorhabditis elegans* as products of the Lin-4 gene (7), are a family of small non-protein coding RNAs that regulate a wide variety of genes at the post-transcriptional level. miRNAs have a critical role in numerous biological and pathological processes, including cell proliferation, differentiation and apoptosis (8,9). It was reported that miRNAs modulate almost 60% of protein-coding genes in humans by base-pairing with the 3'-untranslated regions of their target miRNAs (10), resulting in messenger RNA (mRNA) degradation or translation inhibition. In addition to this silencing effect, certain miRNAs are able to activate gene expression (11). Attention has turned to the examination of the role of miRNAs in carcinogenesis. In cancer, miRNAs may function as oncogenes and/or tumor suppressors through various mechanisms, including deletions, amplifications or mutations in miRNA loci, as well as epigenetic changes, dysregulation of transcription factors targeting specific miRNAs or inhibition of miRNA processing (12).

Following the initial demonstration that elevated serum levels of miRNA-21 were associated with relapse-free survival of diffuse large B-cell lymphoma patients (13), several circulating miRNAs were identified as potential diagnostic and prognostic biomarkers for various types of cancer, including nasopharyngeal carcinoma (14) and glioma (15), as well as breast (6,16,17), gastric (18,19), prostate (20,21), pancreatic (22) and colorectal cancer (23). As a tumor screening marker, circulating miRNAs have several advantages, including the availability of serum samples and their stability during storage relative to that of tissue samples. Serum miRNAs are particularly stable, even following exposure to harsh conditions, for example high/low pH, boiling and multiple freeze-thaw cycles (11,20).

The aim of the present study was to identify potential circulating miRNA biomarkers for the early diagnosis and relapse prediction of osteosarcoma.

Materials and methods

Patients. Serum samples were collected from 20 consecutive OS patients at the Second Xiangya Hospital of Central

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South University (Changsha, China) between 2012 and 2013. All patients exhibited a positive diagnosis for OS based on pathological analysis of biopsies or surgical samples. All OS samples were collected prior to any therapeutic interventions. The 20 gender and age-matched controls were also recruited from the Second Xiangya Hospital, and were confirmed to be free of any other types of cancer or chronic disease. The demographics and clinicopathological characteristics of the patients are indicated in Table I. The present study was approved by the Ethics Committee of the Second Xiangya Hospital. Furthermore, informed consent was obtained from all participants included in the project prior to blood sampling.

RNA isolation. Peripheral blood samples (5 ml) were collected from all participants according to protocols approved by the Second Xiangya Hospital. Whole blood samples were stored at room temperature for 60 min then centrifuged at 1000 x g for 10 min at 4˚C. The serum samples were stored in phased liquid nitrogen or used for RNA isolation directly following collection. Total RNA was isolated from 250 μl human serum using TRIzol-LS Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 5 μl artificial microRNA (cel-miR-39; RiboBio, Guangzhou, China) was added to each sample prior to the isolation procedure to serve as an internal control.

miRNA profiling. The expression levels of 168 unique human miRNAs, previously selected from an earlier study (3) and the Exiqon company database (http://www.exiqon.com/plate-layout-files), were detected using the miRCURY LNA Universal reverse transcription (RT) microRNA polymerase chain reaction (PCR) system and ready to use Serum/Plasma Focus microRNA PCR Panel (V3.M; Exiqon, Kangchen, China). Each Exiqon miRNA quantitative PCR panel included 168 target miRNA probes, 5 internal control primer sets and 2 reference miRNA probes (miR-103-3p and miR-191-5p). The PCR amplification was performed at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 60 sec and subsequent melting curve analysis. The expression levels of miRNAs were normalized to the internal controls and evaluated using the ΔΔCt method, where ΔCt was calculated by subtracting the average Ct values of reference miRNA from the average Ct value of the miRNA of interest.

RT-qPCR. RT and qPCR kits produced specifically for accurate miRNA analysis (GeneCopoeia, Inc., Rockville, MD, USA) were used to evaluate the expression of miRNAs in serum samples. Equal quantities of RNA (1000 ng/reaction) were reverse-transcribed. The 25 μl RT reactions were performed using an All-in-One™ miRNA RT-qPCR Detection kit (catalog no. AOMD-Q050; GeneCopoeia, Inc.) and incubated for 60 min at 37˚C, 5 min at 85˚C and then maintained at 4˚C. RT products were subsequently diluted 3-fold to a total of 75 μl. For PCR analysis, 2 μl diluted RT products were used as templates in 20 μl reaction mixtures containing primers for each miRNA, according to the manufacturer's instructions. All reactions were run on the 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA) using the following conditions: 95˚C for 10 min, followed by 50 cycles at 95˚C for 15 sec, 60˚C for 20 sec and 72˚C for 15 sec. Each sample was run in duplicate for analysis. The relative miRNA quantities in serum from participants were determined using the comparative Ct method, and the miRNA levels were normalized to cel-miR-39. Fold-change of miRNA expression between groups was determined using the ΔΔCt equation, where ΔCt was calculated by subtracting the Ct values of cel-miR-39 from the average Ct value of the miRNA of interest.

Table I. Demographic and clinical features of OS patients and healthy individuals.

Variable	OS patients	Controls	P-value
Age, years	13±4.38	14.3±4.5	0.360
Gender, n			
Male	13	12	0.744
Female	7	8	
Tumor location, n			
Femur	13		
Tibia	5		
Others	2		
Enneking stage, n			
IIA	7		
IIB	11		
III	2		

P-values were calculated using independent Student's t-test or the χ^2 test. OS, osteosarcoma.

Statistical analysis. Data is presented as the mean ± standard deviation. Differences between groups were analyzed using a two-tailed Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference. Receiver operating characteristic (ROC) and the area under ROC curve (AUC) were used to estimate the diagnostic value of candidate miRNAs in OS. Cut-off values for the expression of each miRNA were defined according to the Youden index from the ROC curve. All statistical analyses were performed by using GraphPad PRISM software, version 5 (GraphPad Software, La Jolla, CA, USA).

Results

miRNA profiling of pre-therapeutic OS patients. Three samples from patients with pre-therapeutic OS and three healthy individual samples were selected for the identification of differentially expressed miRNAs. A volcano plot was constructed to present the results of miRNAs profiling, which are presented in Fig. 1. In the plot, the black line indicates a fold-change of 1 and the pink lines indicate a 1.8 fold-change in gene expression, while the blue line indicates the threshold P-value of 0.05 for the t-test. Fourteen candidate miRNAs were selected using the following inclusion criteria: i) The Ct value of each miRNA was <35; ii) the fold-difference between the two groups was >1.8; and iii) the value of P was <0.05. Due to the small size of the sample for screening, a 1.8-fold change was used as the cut-off value to avoid eliminating

Table II. Differentially expressed serum microRNAs in osteosarcoma, identified by reverse transcription-quantitative polymerase chain reaction.

MicroRNA	Fold-change[a]	Ct value[b]	P-value
miR-451a	-2.40	23.31±0.25	0.0191
miR-551b-3p	-4.94	34.79±0.49	0.0070
miR-20a-5p	-2.00	25.53±0.16	0.0055
miR-34a-5p	2.48	31.63±0.42	0.0057
miR-2110	-2.70	32.45±0.37	0.0096
miR-95-3p	2.48	33.63+0.03	0.0011
miR-16-5p	-1.96	21.61±0.05	0.0364
miR-186-5p	-1.97	30.52±0.26	0.0044
miR-320a	1.97	27.15±0.13	0.0029
miR-106a-5p	-1.91	25.36±0.26	0.0122
miR-25-3p	-1.89	25.36±0.14	0.0023
miR-223-5p	-1.91	31.18±0.14	0.0228
miR-139-5p	-1.84	29.05±0.13	0.0305
miR-425-5p	-1.90	26.38±0.37	0.0368

[a]OS/healthy control ratio; [b] in OS. miR, microRNA.

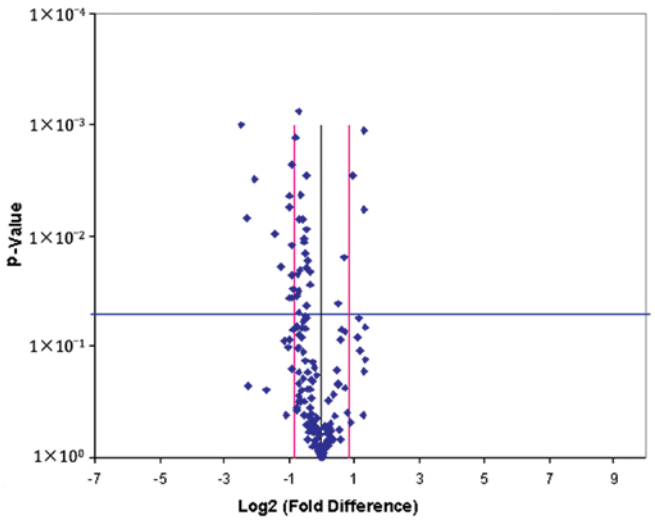

Figure 1. Volcano plot of the results of microRNA profiling. Black line, fold-change in gene expression of 1; pink lines, 1.8 fold-change in gene expression threshold; blue line, threshold for the P-value (0.05) of the t-test.

potentially significant biomarkers (3). The identified miRNAs have been reported in previous studies as being differentially expressed in OS or other tumors. Information regarding these 14 miRNAs is summarized in Table II. The initial selection of 14 miRNAs was further validated in the larger 20 patient and 20 control cohorts. The following 14 miRNAs were shown to be differentially expressed: miR-451a, miR-551b-3p, miR-20a-5p, miR-34a-5p, miR-2110, miR-95-3p, miR-16-5p, miR-186-5p, miR-320a, miR-106a-5p, miR-25-3p, miR-223-5p, miR-139-5p and miR-425-5p.

Validation of candidate miRNAs. To confirm the results of the miRNA profiling, miRNA levels in serum were examined by RT-qPCR in the 20 pre-therapeutic OS patients and 20 healthy controls. The 14 differentially expressed miRNAs were validated in the larger cohort by RT-qPCR; however, only 7 miRNAs demonstrated a statistically significant difference. The data indicating that the expression of 7 miRNAs was downregulated in the pre-therapeutic OS group were validated by RT-qPCR analysis when compared with that of the control group (Fig. 2). The data was analyzed using the ΔΔCt equation as previously described.

An ROC curve was constructed to estimate the sensitivity and specificity of these 7 miRNAs as a means of discriminating OS patients from healthy controls. The AUC for the expression of these 7 miRNAs in serum for OS diagnosis were 0.7255 [95% confidence interval (CI), 0.5435-0.9075], 0.7686 (95% CI, 0.6067-0.9306), 0.8471 (95% CI, 0.7155-0.9786) and 0.7961 (95% CI, 0.6438-0.9484) for miR-106a-5p, miR-16-5p, miR-20a-5p and miR-25-3p, respectively; while the AUC for miR-425-5p, miR-451a and miR-139-5p were 0.7765 (95% CI, 0.6163-0.9366), 0.7961 (95% CI, 0.6420-0.9501) and 0.7098 (95% CI, 0.5308-0.8888), respectively (Fig. 3). These results indicated that these 7 miRNAs may be used as diagnostic biomarkers, with the ability to resolve OS patients from the healthy cohort.

In addition, in order to confirm the prognostic value of the 7 miRNAs at early stages of OS, the serum levels of these 7 miRNAs were analyzed in patients with OS at different Enneking stages. No significant differences were noted between miRNA expression in OS patients at Enneking IIA and Enneking IIB. Due to the small sample size of Enneking III (2 samples), statistical analysis between Enneking IIA/B and Enneking III was not performed.

Discussion

OS is the most common primary malignant bone tumor amongst adolescents and young adults (2,24). However, the

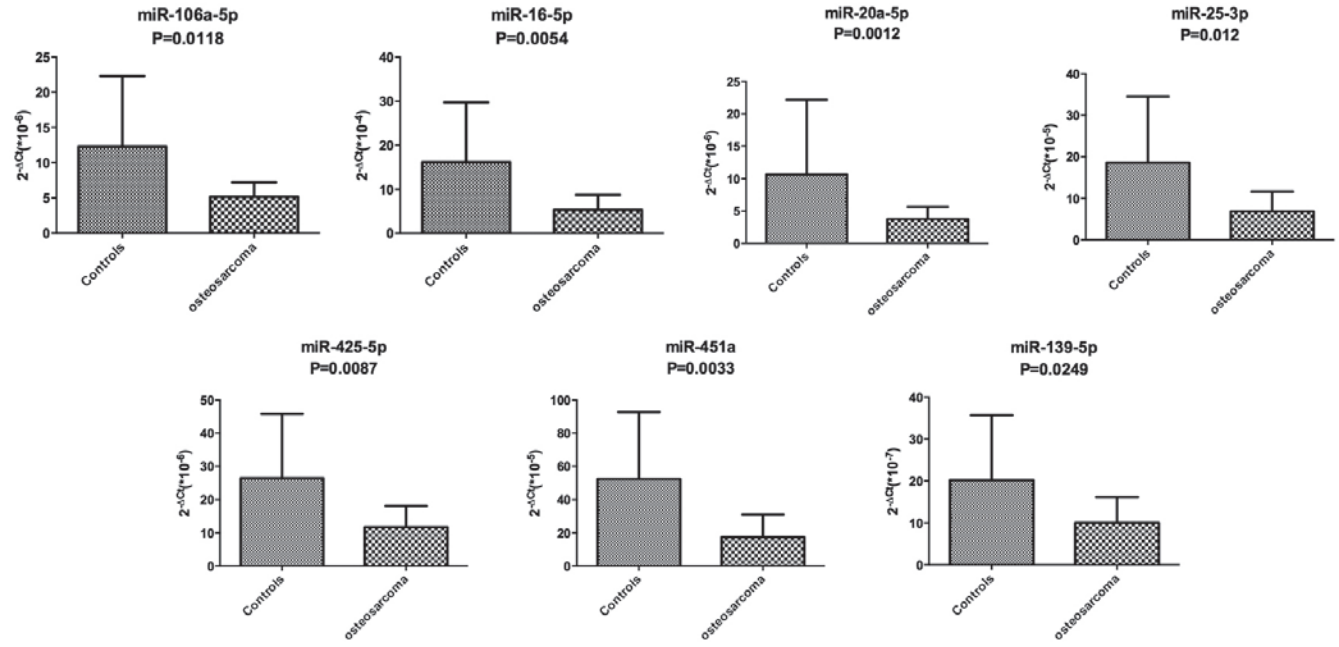

Figure 2. Expression of 7 candidate microRNAs in serum from the pre-therapeutic osteosarcoma (n=20) and healthy control (n=20) groups, determined using reverse transcription-quantitative polymerase chain reaction. miR, microRNA.

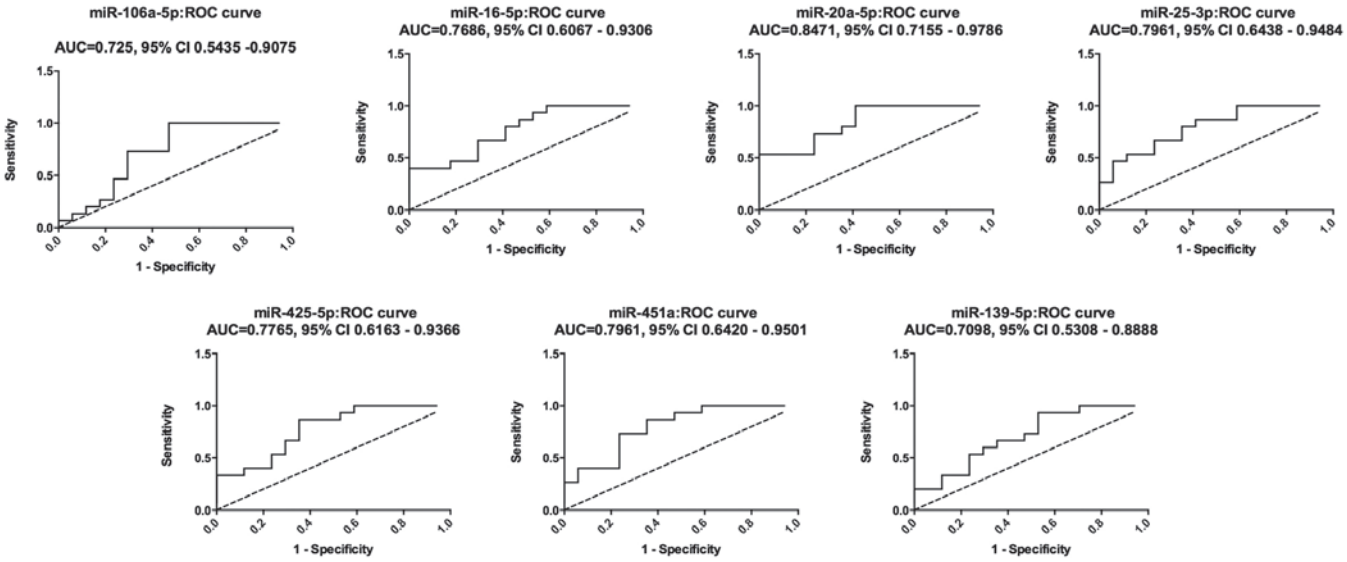

Figure 3. ROC curve analysis using expression levels of 7 serum microRNAs for discriminating osteosarcoma from healthy controls. ROC, receiver-operating characteristic; AUC, area under ROC curve; miR, microRNA; CI, confidence interval.

molecular mechanisms underlying disease development have remained elusive. Typically, diagnosis of OS is initiated by patient complaints, followed by imaging tests, including bone X-ray, magnetic resonance imaging (MRI) and computed tomography, with a final diagnosis often requiring confirmation by biopsy (2,24). This series of examinations/tests are time-consuming and expensive, particularly in developing countries. Furthermore, the majority of patients with OS are diagnosed at an advanced stage. The patients included in the present study all exhibited OS beyond Enneking stage IIA. The identification of novel screening biomarkers represents a promising technique for facilitation of the early detection of OS, which may improve treatment outcomes.

The present study demonstrated the utility of miRNA profiling as a means of diagnosing OS. Previous studies reported the expression of miR-9, miR-99, miR-195, miR-148a and miR-181a to be elevated in OS cell lines, while miR-143, miR-145, miR-335 and miR-539 were decreased (25). Another group reported elevated levels of miR-181a, miR-181b, and miR-181c in human osteosarcoma tissues, while miR-16, miR-29b and miR-142-5p were downregulated (26). Multiple target genes of miRNAs and associated downstream signaling pathways have been identified, which are correlated with the pathogenesis and progression of OS. For example, miR-221 stimulates cell survival and cisplatin resistance by targeting the phosphatase and tensin homolog (PTEN) gene in the

phosphoinositide 3-kinase/Akt pathway (27). Furthermore, increased levels of miR-128 are negatively correlated with PTEN levels, and increased proliferation of MG63 and U2OS OS cells (28). In contrast to these oncogene-like miRNA functions, miR-335 and miR-340 function as tumor suppressors by targeting the Rho-associated, coiled-coil containing protein kinase 1 gene and, thereby, inhibit OS cell migration and invasion (29,30). miR-126 inhibits the proliferation of OS cells by targeting sirt1, a histone deacetylase (31). A previous study by our group confirmed that miR-125, which is typically downregulated in OS samples, negatively regulates signal transducer and activator of transcription 3, which suppresses proliferation and migration of OS cells (32).

Based on the differential expression patterns of miRNAs in OS, numerous miRNAs have been identified as potentially valuable diagnostic biomarkers. Compared with their tissue counterparts, the potential significance of circulating miRNAs in OS diagnosis is poorly understood. In the present study, miR-106a-5p, miR16-5p, miR-20a-5p, miR-425-5p, miR451a, miR-25-3P and miR139-5p were demonstrated to be downregulated in the serum of patients with OS when compared with that of healthy controls.

These 7 miRNAs may also be involved in the pathogenesis of other cancers. miR-106a was previously shown to be downregulated in glioma, colon cancer, squamous cell carcinoma and astrocytoma, and functions as a tumor suppressor by targeting solute carrier family 2 (facilitated glucose transporter), member 3 (33), E2F transcription factor 1 (34) and Fas-activated serine/threonine kinase (35). Similarly, miR-20a is downregulated in hepatocellular carcinoma (HCC) and correlated with HCC recurrence and poor prognosis (36). In addition, miR-425 is able to reduce proliferation, impair tumorigenesis and metastasis, and increase expression of epithelial markers in aggressive breast cancer cells by targeting SATB homeobox 1, CCND2 and Fascin actin-bundling protein 1 (37). The tumor suppressive role of miR-16 was also confirmed by *in vitro* and *in vivo* functional experiments associated with OS (26). In these studies, miR-16, which is reduced in OS, was shown to inhibit cell proliferation by targeting the insulin-like growth factor 1 receptor and Raf1-mitogen activated protein kinase kinase 1/2-extracellular signal-regulated kinase 1/2 pathways (38). Furthermore, miR-451 has been shown to act as a tumor suppressor in various human malignancies, including nasopharyngeal carcinoma (39), colorectal carcinoma (40), lung cancer (41), renal cell carcinoma (42) and gastric cancer (43). Cisplatin sensitivity was increased by overexpression of miR-451 in lung cancer cells (41). Another downregulated miRNA, miR-25, may function as a tumor suppressor that inhibits cell proliferation and migration by targeting Smad 7 in colon cancer (44) and EZH2 in anaplastic thyroid carcinoma (45). Finally, studies have suggested a tumor suppressor role for miR-139-5p, which is downregulated in various types of cancer, including endometrial serous adenocarcinoma (46), breast carcinoma (47), glioblastoma (48), bladder cancer (49), basal cell carcinoma (50) and esophageal squamous cell carcinoma (51).

Consistent with the results of prior studies, the present findings confirmed that the expression of these 7 miRNAs were reduced in the serum of OS patients, which is concurrent with their function as tumor suppressor genes. However, an oncogenetic function was proposed for a number of these miRNAs, based on their ability to target tumor suppressor genes. The high expression of miR-106a was previously demonstrated to indicate a high risk of tumor penetration in advanced gastric carcinoma (52) and mediate proliferation and tumor differentiation in ovarian cancer through direct targeting of retinoblastoma-like protein 2, a member of the retinoblastoma tumor suppressor family (53). High expression levels of miR-20a are associated with lymph node metastasis in advanced gastric carcinoma (52). Recently, a report indicated that miR-20 was able to downregulate Fas expression, thus contributing to the metastatic potential of OS cells (54). Furthermore, miR-425 was detected at high levels in gastric cancer (55). Hummel *et al* (56) revealed that miR-425 was also upregulated in esophageal carcinoma cells following short- or long-term treatment with cisplatin. miR-25, which is overexpressed in ovarian cancer, gastric cancer and esophageal squamous cell carcinoma, promotes tumorigenesis and metastasis by targeting Bim, reversion-inducing cysteine-rich protein with Kazal motifs and cadherin 1, respectively (57-59).

In view of the controversial roles of miRNAs in various tumors, it is important for discrepancies between studies to be clarified. Heterogeneity of primary tumors may be an explanation for the differential expression profiles of identical miRNAs in various tumors. It was also reported that the levels of circulating miRNAs may differ from those in tissues (60,61), which may be explained by the differential secretory mechanisms and/or stability of miRNAs in blood (12,62). Thus, circulating miRNAs may not necessarily reflect changes in expression within the tumor tissue. In addition, the extraction quantification methods used may contribute to the conflicting results. In addition, to the best of our knowledge, there is no commonly accepted internal control for this type of analysis, therefore the various artificial miRNAs or endogenous housekeeping genes used for normalization may affect the final results.

Although the results presented in the present study are promising, several limitations should be acknowledged: i) The OS patient sample size was relatively small and therefore validation in larger patient cohorts is required; ii) research on the association between serum miRNA levels and patient survival remains a critical future goal; and iii) the association between tissue and serum miRNA levels requires further investigation.

In conclusion, the present study indicated that changes in the serum profile of 7 miRNAs are associated with OS. The results indicated that these 7 miRNAs may be used as potential circulating biomarkers in the future and provide novel opportunities for early blood screening for OS.

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