



# Circulating miR-125b as a biomarker of Ewing's sarcoma in Chinese children

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**ABSTRACT.** Previous studies indicated that microRNA-125b (miR-125b) has an important role in the progression of Ewing's sarcoma (ES). The purpose of the current study was to examine expression changes of miR-125b in the serum of ES patients and evaluate if the expression level of miR-125b could serve as a new biomarker for ES. This study was performed on patients who underwent surgical resection at our hospital between 2005 and 2013 after an initial diagnosis of ES. We measured serum miR-125b levels in 63 patients with ES and 126 healthy control patients using a real-time quantitative reverse transcriptase-PCR (qRT-PCR) method. Expression levels of serum miR-125b were distinctly decreased in ES patients when compared with healthy controls ( $P < 0.001$ ). ES cases that had a poor response to chemotherapy presented a significant down-regulation of miR-125b ( $P = 0.001$ ). The ROC curve showed that the serum miR-125b could serve as a valuable biomarker for differentiating ES patients from healthy controls with an AUC of 0.879 (95%CI = 0.817-0.924;  $P < 0.001$ ). At a cut-off value of 2.203 for miR-125b, the sensitivity was 72.8% and the specificity was 87.2% in

discriminating ES from the controls. Our results indicate that serum miR-125b may serve as a useful noninvasive biomarker for ES.

**Key words:** Ewing's sarcoma; miR-125; Biomarker; Serum; Diagnosis

## INTRODUCTION

Ewing's sarcoma (ES) is the second most frequent bone and soft tissue tumor, which is characterized by small, blue round cells (Schleiermacher et al., 2003; Iwamoto, 2007; Esiashvili et al., 2008). It occurs mainly in children and adolescents, with an overall incidence of about 1.3 cases per million people (Dorfman and Czerniak, 1995; Erisir et al., 2008). Over the past decades, there has been a great effort to elucidate the underlying mechanisms of ES development, and to identify novel diagnostic biomarkers and therapeutic targets for ES patients (Borinstein et al., 2013). However, the underlying molecular and cellular mechanisms for ES carcinogenesis remain largely unclear. It is reported that approximately 20% of individuals with ES have a poor prognosis, with an estimated free survival of less than 20% (Stahl et al., 2011). Those survivors of sarcomas, with distant metastasis, have lower health quality of life scores and require high cure costs (Barr and Wunder, 2009). Moreover, about 25% of patients suffering from ES have detectable metastasis at diagnosis. Therefore, the development of novel and more effective diagnostic biomarkers for this malignant disease is imperative.

MicroRNAs (miRs) are a large family of non-coding single-stranded RNAs of 18-25 nucleotides (Bartel, 2004). As a newly discovered class of cellular bioactive molecules, miRs play important roles in the regulation of gene expression both in normal and disease physiologies (Ghildiyal and Zamore, 2009). Currently, it is expected that there are more than 1500 miRs that might influence the expression of over 60% of human genes (Friedman et al., 2009; Kozomara and Griffiths-Jones, 2011). In recent years, many studies identified aberrantly expressed miRs in most human tumors and demonstrated their important roles in several tumors' pathogenesis and metastasis. In particular, a number of recent studies have detected the biology of miRs in the initiation and progression of ES. A specific miR may have a distinct role in different types of cancer, which could potentially contribute to an important advance in the diagnosis, prognosis, and prediction of therapeutic responses of human cancer (Yu et al., 2007; Fabbri, 2010; Ferracin et al., 2010). Therefore, the application of miR signatures can have a great potential in the identification of novel diagnostic molecular biomarkers for cancer.

miR-125b is a member of the most consistently dysregulated miRs. At present, the role of miR-125b in the progression of ES has been widely studied. De Vito et al. (2011), in their study with 11 normal bone tissues and 23 primary ES tumors under microarray analysis, have demonstrated that the expression of miR-125b decreased in ES tissues when compared to normal bone tissues. Iida et al. (2013) reported that the overexpression of miR-125b in parental ES cells leads to enhanced drug resistance via inhibition of the expression of apoptotic mediators, such as p53 and Bak. Moreover, McKinsey et al. (2011) indicated that the ectopic expression of miR-125b results in the negative regulation of the insulin-like growth factor (IGF) signaling pathway and suppression of cell proliferation for A673 cells. All these data indicate the potential effect of miR-125b for the diagnosis and therapy of ES.

However, these findings above are not enough to open a new era of ES management. Therefore, it is urgent that further understanding of aberrant miR expression and exploration of

novel miR biomarkers for ES can be achieved. The purpose of the current study was to examine expression changes of miR-125b in the serum of ES patients and evaluate whether or not the level of miR-125b could serve as a new biomarker.

## MATERIAL AND METHODS

### Patients and sampling

This study was approved by the Review Board of Shandong Provincial Hospital Affiliated to Shandong University Ethics Committee. Each participant signed a written informed consent. Sixty-three patients, who were diagnosed with ES and received surgery at our hospital between January 2005 and October 2013, were recruited for this study. The mean age of the patients was  $14 \pm 4.8$  years and none of them underwent preoperative treatment. Primary tumors location sites included the pelvis (N = 17), femur (N = 13), tibia (N = 10), fibula (N = 9), humerus (N = 8), spine (N = 1), rib (N = 3), and foot (N = 2). There were nine patients with metastatic disease at first diagnosis. Thirty-four patients responded to chemotherapy. After surgery, all tumor specimens were independently reviewed by two experienced pathologists. Tumor size was determined as the maximum tumor diameter based on radiographic images, including computed tomography (CT) scan and magnetic resonance imaging (MRI). One hundred and twenty six healthy individuals, who undertook routine health examinations, were included in the matched-pair control group based on age and gender with a ratio of 1:2. The characteristics of all subjects, including age, gender, tumor size, tumor site, metastasis, and response to chemotherapy, were collected from reviewing medical records or questionnaires.

Peripheral venous blood samples (5-10 mL) were obtained before all subjects received surgical resection or other therapeutic treatments. Then, these blood samples were centrifuged at 2000 g for 10 min, and stored at  $-80^{\circ}\text{C}$  until use.

### RNA extraction, reverse transcription and qPCR

Blood samples for miR detection were drawn into EDTA-K2 tubes. RNA extraction was performed using the RNeasy Plus Micro Kit (Qiagen, Netherlands), following the manufacturer instructions. The collected RNA yields were around 250 ng per 400 ml serum. Isolated RNA samples were reverse-transcribed to cDNA using the First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). Concentration and purification of miR were conducted with NanoDrop ND-1000 (Nanodrop, Wilmington, DE, USA).

qRT-PCR analysis of miR-125b expression profiles was performed using TaqMan miRNA assays (Applied Biosystems), following the manufacturer instructions. The primer sequences for miR-125b were: GCUCCCUGAGACCCUAAC (forward) and CAGTGCAGGGTCCGAGGT (reverse); and the PCR primers for *U6* were: CTCGCTTCGGCAGCACATATACT (forward) and ACGCTTCACGAATTTGCGTGTC (reverse). The expression level of miR-125b was measured using the  $\Delta\Delta\text{CT}$  method. SnRNA *U6* was used as the reference miR. Reactions were drawn onto a 96-well plate and were run in duplicate at  $50^{\circ}\text{C}$  for 20 s,  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 s, and  $60^{\circ}\text{C}$  for 1 min, using the 7900 Fast RT-PCR System (Applied Biosystems).

## Statistical analysis

All statistical analysis were carried out using the SPSS software (IBM, USA). Differences between two groups were determined using a paired *t*-test. The Wilcoxon-Mann-Whitney test was used to evaluate the relationships between the miR-125b expression level and clinicopathologic features of the subjects. The receiver operating characteristic curve (ROC) analysis was used to determine the diagnostic accuracy of serum miR-125b, with the area under the ROC (AUC) being used to determine the optimal sensitivity and specificity level. A P value of lower than 0.05 was considered to be statistically significant.

## RESULTS

### General characteristics of subjects

The demographic characteristics of all subjects are listed in Table 1. There were thirty-one and thirty-two patients whose tumor size was greater or lesser than 8 cm, respectively. Distant metastases were observed only in 9 cases. Thirty-four ES patients presented poor response to chemotherapy.

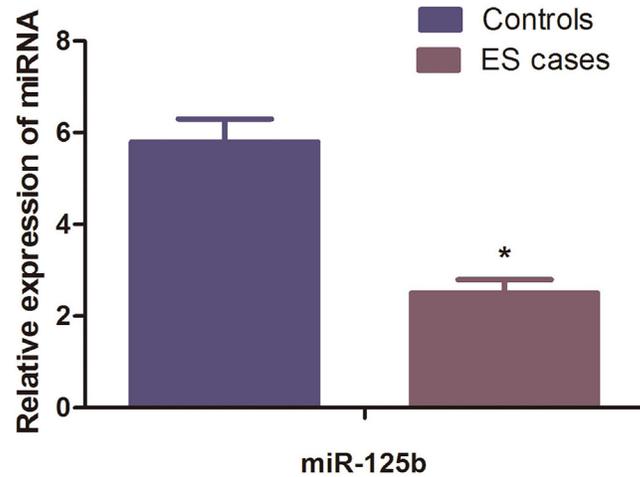
**Table 1.** Correlation between miR-125b expression and different clinicopathologic features in ES patients.

Variables	No. of cases (N = 63)	Relative expression of miR-125b*		P value
		High	Low	
Age (years)				
≥12 vs <12	39/24	14/9	25/15	0.898
Gender				
Male vs female	46/17	18/5	28/12	0.477
Tumour size (cm)				
≥8 vs <8	31/32	13/10	18/22	0.378
Tumour site				
Axial vs extremity	21/42	9/14	12/28	0.459
Tumor metastasis				
Yes vs no	9/54	4/19	5/35	0.593
RTC				
Good vs poor	29/34	17/6	12/28	0.001

RTC = response to chemotherapy. \*The median expression level of circulating miR-125b was used as the cut-off value to categorize the patients into high- and low-expression groups.

### Serum expression of miR-125b

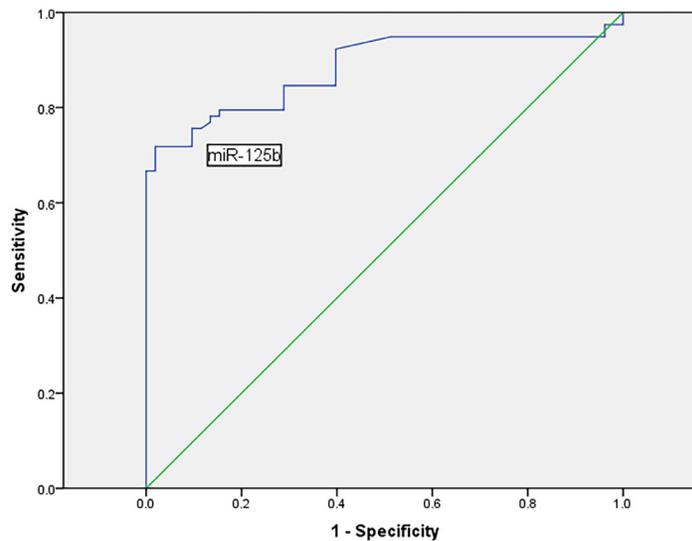
Expression of serum miR-125b was distinctly decreased in ES patients when compared with healthy controls ( $P < 0.001$ ; Figure 1). Subsequently, the correlation between the expression levels of miR-125b and the clinicopathological features of the ES cases was analyzed (Table 1). According to the expression levels of miR-125b using a cut-off value of 2.203, ES patients were divided into two groups (high and low expression groups). With respect to most of the clinicopathological features, no significant differences were found in the correlations of miR-125b expression levels with clinicopathological features, including age, gender, tumor size, tumor site, and tumor metastasis. However, ES cases who had a poor response to chemotherapy presented a significant down-regulation of miR-125b ( $P = 0.001$ ).



**Figure 1.** Relative expression level of miR-125b in healthy controls and ES patients. The expression levels were determined using a qRT-PCR assay, and the relative expression data were measured using the  $2^{-\Delta\Delta Ct}$  method. All of the assays were performed in triplicate. *U6* was used as a reference miR. \* $P < 0.001$ .

### Diagnostic accuracy of serum miR-125b for ES

The ROC curve was calculated to confirm the diagnostic accuracy of serum miR-125b for ES. The results showed that the serum miR-125b could serve as a valuable biomarker to differentiate ES patients from healthy controls with an AUC of 0.879 (95%CI = 0.817-0.924;  $P < 0.001$ ; Figure 2). At a cut-off value of 2.203 for miR-125b, the sensitivity was 72.8% and the specificity was 87.2% for discriminating ES from the controls.



**Figure 2.** Receiver operating curve (ROC) analysis of miR-125b expression level.

## DISCUSSION

The search for non-invasive biomarkers for the diagnosis of ES is currently one of the most rapidly growing areas in ES research (Marino et al., 2014). Over the past decades, researchers and clinicians have undertaken long-range efforts in early screening and multimodal regimens, including local surgery, radiotherapy, and intensive systemic chemotherapy (Bacci et al., 2006; Balamuth and Womer, 2010). However, the presence of clinically evident metastasis at diagnosis and overall survival of ES patients remain quite dismal. Although many biological and molecular markers have been investigated (Riley et al., 2003; van Maldegem et al., 2012), their practical value in clinical work still requires validation and experimental configuration.

In numerous recent studies, in the blood serum, circulating miRs were detected, which are stable, reproducible, resistant to several harsh conditions, and have already been recommended as non-invasive novel biomarkers for the diagnosis of many malignant tumors (Miller et al., 2013). Recent reports have already given more prominence to the oncogenic aspects of miR-125b, claiming that serum miR-125b could be a novel biomarker for screening human tumors, including non-small-cell lung cancer and breast cancer (Wang et al., 2012; Yuxia et al., 2012). Our previous miR array analysis in ES tissue samples has demonstrated the diagnostic potential of miR-125b (Li et al., 2014). Therefore, it is possible that miR-125b could become a biomarker for the diagnosis of ES.

Here, serum miR-125b was studied in patients with ES at initial diagnosis and compared with healthy controls. Serum miR-125b level was significantly decreased in ES patients when compared to healthy individuals. Furthermore, our findings also indicated that miR-125b alone might serve as a novel, noninvasive biomarker for ES with a high sensitivity of 72.8% and specificity of 87.2%.

Many recent studies reported that the specific profiles of serum miRs might serve as novel, noninvasive biomarkers for ES. To date, previous studies (Sato et al., 2011; Mosakhani et al., 2012; Sasaki et al., 2013; Karnuth et al., 2014; Todorova, 2014) with microarray analysis indicated significant overexpression of five miRs (miR-106b, miR-93, miR-181b, miR-101, and miR-30b), while significant underexpression of six miRs (miR-145, miR-193a-3p, miR-100, miR-22, miR-21, and miR-574-3p) in ES tissues when compared to control samples. Ban et al. (2011) showed that, as a tumor suppressor, miR-145 replacement leads to the inhibition of anchorage-independent growth of ES cells. Nakatani et al. (2012) revealed that five miRs (miR-34a, miR-23a, miR-92a, miR-490-3p, and miR-130b) might serve as independent risk predictors for the progression and survival of ES. To date, despite the dysregulation of miRs in ES tissues in overwhelming reports, no serum miR has been investigated as a noninvasive biomarker for the diagnosis of ES. Our findings revealed that the identification of miR-125b might contribute to this purpose.

Several limitations of our study should not be ignored. The sample size is relatively modest, which might cause selection bias for patients. Second, replicated investigations in other groups were not carried out. Then, other potential relevant miRs, which play important roles in ES as well, should also be investigated. Finally, the exact mechanisms of miR-125b should be identified in further studies.

In summary, we provided evidence that serum miR-125b levels were significantly decreased in ES patients, and serum ES level was significantly correlated with chemotherapy. Moreover, with the findings of its reasonable sensitivity and specificity for ES, miR-125b could become a useful biomarker for ES diagnosis. Nonetheless, further studies with larger sample size involving the exact mechanism should be carried out.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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