International Journal of Clinical Oncology (2019) 24:976–982
https://doi.org/10.1007/s10147-019-01433-x

ORIGINAL ARTICLE

A four serum-miRNA panel serves as a potential diagnostic biomarker of osteosarcoma

Congwei Huang[1] · Qinjue Wang[1] · Shengshan Ma[2] · Yang Sun[1] · Ashley Shane Vadamootoo[1] · Chengzhe Jin[1]

Received: 9 September 2018 / Accepted: 22 March 2019 / Published online: 20 May 2019

Abstract

Background Osteosarcoma (OS) is the most common malignant bone tumor in young adults and adolescents with approximately 3 million new cases annually. Due to the lack of sensitive and specific diagnostic biomarkers, although OS patients are curable after surgical resection, many patients suffer from metastasis or recurrence. This study aimed to investigate whether circulating microRNAs (miRNAs) could serve as biomarkers for the diagnosis of OS.

Materials and methods Healthy individuals and OS patients enrolled in this study came from Nanjing First Hospital. First, candidate miRNAs were selected by integrated analysis of two GEO datasets and a publicly available miRNA dataset. The expression of these miRNAs in tissues and serum samples were subsequently examined through qRT-PCR. The diagnostic utility of these differential miRNAs was examined by using receiver operating characteristic (ROC) curve analysis. Finally, the potential signaling pathways associated with candidate miRNAs were searched through online tools.

Results Four miRNAs (miR-487a, miR-493-5p, miR-501-3p and miR-502-5p) were selected to further investigate their diagnostic potential for OS. We discovered miR-487a, miR-493-5p, miR-501-3p and miR-502-5p were upregulated in OS tissues and serums. Besides, miR-487a, miR-493-5p, miR-501-3p and miR-502-5p in peripheral blood of OS patients were tumor-derived. The area under the ROC curve (AUC) was 0.83 (95% CI 0.71–0.97) for miR-487a, 0.79 (95% CI 0.66–0.93) for miR-493-5p, 0.82 (95% CI 0.68–0.95) for miR-501-3p, 0.83 (95% CI 0.72–0.95) for miR-502-5p, and 0.89 (95% CI 0.78–1.0) for miRNAs combination.

Conclusion Circulating miR-487a, miR-493-5p, miR-501-3p and miR-502-5p were novel potential diagnostic biomarkers of OS.

Keywords miRNAs · Osteosarcoma · GEO database · Biomarker

Introduction

Osteosarcoma (OS) is a common primary malignant bone tumor in children and young adults, which results in approximately 3,000,000 new cases annually [1, 2]. Due to the development of multiple therapeutic strategies including tumor excision, adjuvant chemotherapy and radiotherapy, the 5-year survival rate of OS patients has improved over the past decades to approximately 60% [3, 4]. However, almost 35% of patients develop metastasis when OS is not diagnosed at early stage [5]. In addition, the efficacy of therapeutic strategies currently used for metastasis and relapse of OS is limited [4]. Therefore, novel non-invasive biomarkers with high sensitivity and specificity for early detection of OS are urgently needed.

MiRNAs, short single-stranded non-coding RNAs (approximately 22 nucleotides), can functionally carry out biological effects through direct binding to 3′ untranslated regions (UTR) of target mRNAs by inducing mRNAs degradation and/or translational repression [6]. Because circulating miRNAs could exist in peripheral blood steadily [7], there are emerging studies reporting circulating miRNAs act as biomarkers of various diseases including OS [8–12]. Nonaka et al. demonstrated that circulating miR-199a-3p serves as a novel serum biomarker for colorectal cancer [13]. Maciejak et al. determined circulating miR-30a-5p as

✉ Chengzhe Jin
1985604104@qq.com

[1] Department of Orthopedic, Nanjing First Hospital, Nanjing Medical University, No.68, Changle Road, Nanjing, Jiangsu, China

[2] Department of Orthopedic, Lianyungang Hospital Affiliated to Xuzhou Medical University, Xuzhou, Jiangsu, China

a prognostic biomarker of left ventricular dysfunction after acute myocardial infarction [14]. Besides, Yao et al. reported serum miR-101 could function as potential biomarker for diagnosis and prognosis of OS [15].

In this study, we discovered four tumor-derived miRNAs (miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p) were upregulated in the serum of OS patients. Additionally, the four-circulating-miRNA panel could discriminate OS patients from healthy controls acting as potential novel diagnostic biomarkers of OS.

Materials and methods

Study population

OS patients and healthy individuals were recruited from Nanjing First Hospital affiliated to Nanjing Medical University between October 2014 and July 2017. Age and gender matched healthy individuals were recruited as controls. All OS patients have been confirmed through histopathological analysis of surgical resected tissues. Thereafter, tissues were stored in liquid nitrogen until RNA extraction. None of the patients in this study received chemotherapy or radiation therapy before peripheral blood collected. The clinical features of OS patients are listed in Table 1. Written informed consent was obtained from all participants, and this study was approved by the Research and Ethical Committee of Nanjing First Hospital.

Table 1 The clinical features of osteosarcoma patients

Variable	Osteosarcoma ($n=50$)	
	Number	Percent (%)
Age (year)		
≤ 16	26	52
> 16	24	48
Gender		
Male	22	44
Female	28	56
Tumor location		
Femur	21	42
Tibia	9	18
Fibula	13	26
Arm	7	14
Metastasis		
Yes	18	36
No	32	64
Subtype		
Osteoblastic	31	62
Chondroblastic	19	38

Samples processing

Serum samples were collected from venous blood of all participants. After being centrifuged at 4000 rpm for 10 min, these samples were transmitted into Eppendorf tubes and subsequently stored at – 80 °C until further analysis. Total RNA was isolated using Trizol LS reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. 1 µl cel-miR-39 at a concentration of 1 µM (GenePharma, Shanghai, China) was added into each serum sample to act as the external reference.

qRT-PCR for miRNAs quantitation

Reverse transcription and qRT-PCR for miR-487a, miR-493-5p, miR-501-3p, miR-502-5p, endogenous control U6 snRNA, and external reference miR-39 were performed using Hairpin-it™ microRNA RT-PCR Quantitation Kit (GenePharma, Shanghai, China) according to the manufacturer's instructions. The reactions were initiated with denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15s and 62 °C for 34s. The relative expression of miRNAs were calculated with $2^{-\Delta CtCt}$ method. $\Delta Ct = Ct_{miRNA} - Ct_{miR\text{-}39/U6}$, $\Delta CtCt = \Delta Ct_{patient} - Ct_{control}$.

Statistical analysis

Data were presented as mean ± SD. The differential expression of miRNAs among groups were determined using paired or unpaired *t* test. ROC curve and AUC were established for discriminating OS patients from healthy individuals. A cut-off values of the miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p expression were determined by using Youden index from ROC curves. A P value < 0.05 was considered statistically significant. The statistical analysis was performed using SPSS 22.0 (IBM, Chicago, IL, USA) and GraphPad 7.0 (GraphPad Software, USA).

Results

Selection of candidate circulating miRNAs

After analysis of GEO dataset (GSE28423) which conducted a comprehensive comparative analysis of miRNA expression profiles between OS cell lines and healthy human bones, we discovered 127 significantly upregulated miRNAs. (Fig. 1a, b). Andrew et al. recently published an independent, publicly available miRNA dataset [16]. Using their dataset, we found that miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were significantly associated with the prognosis of

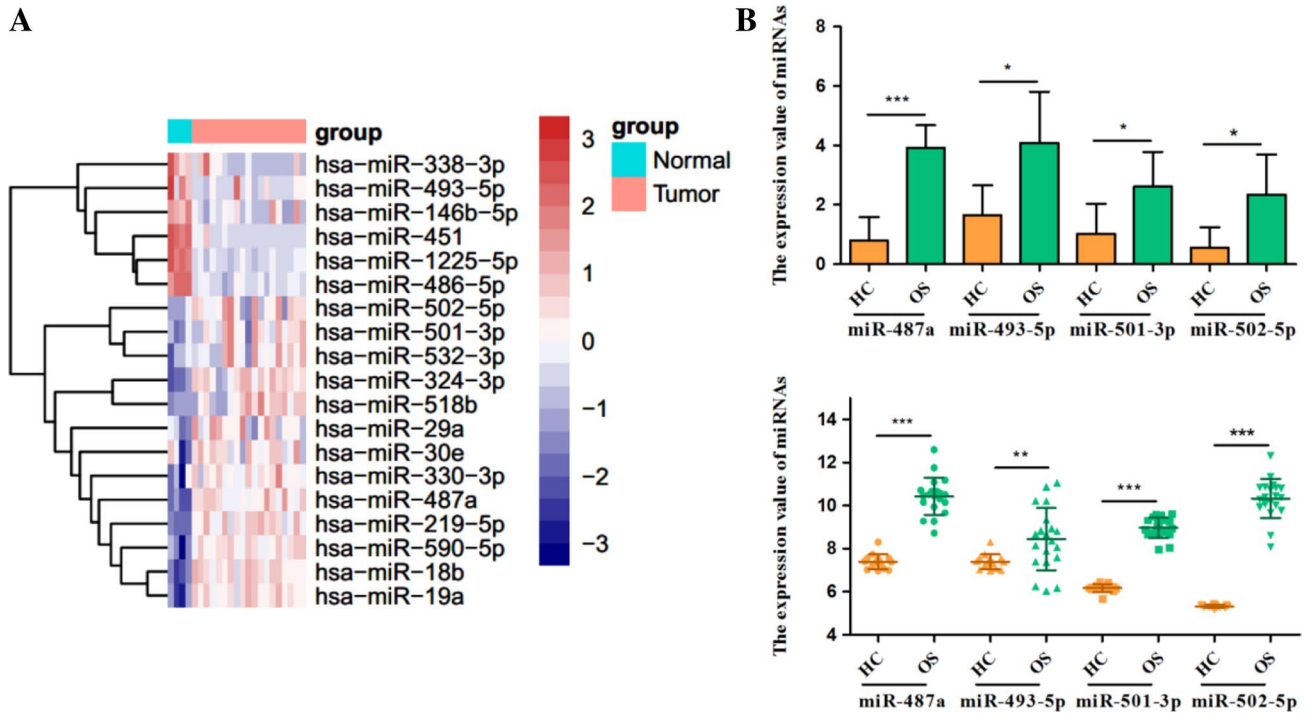

Fig. 1 Selection of candidate circulating miRNAs. **a** Part miRNAs that significantly dysregulated in OS cell lines. **b** miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were significantly upregulated in OS cell lines. **c** miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were significantly upregulated in the serum of OS patients. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

OS patients. Moreover, another GEO dataset (GSE65071) exhibited that miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were also significantly elevated in the serum of OS patients compared to that of healthy controls (Fig. 1c). Therefore, we selected circulating miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p which were potentially secreted by OS as candidates to investigate their diagnostic role in discriminating OS patients from healthy individuals.

Identification of the source and the potential diagnostic value of serum candidate miRNAs

In order to identify upregulated serum miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p that were tumor-derived, 12 matched OS tissues and adjacent normal tissues were analyzed. The expression of these four miRNAs were remarkably higher in OS tissues compared to ANTs (Fig. 2a). In addition, 15 paired pre- and post-operation serum samples were tested. When compared to that in pre-operation serum samples, the expression of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were significantly downregulated after surgical resection of malignancies (Fig. 2b). To explore the potential diagnostic value of serum miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p, ROC curve analysis was performed by using data in GSE65071. As shown in Fig. 2c, The AUC was 1.0 (95% CI 1.0–1.0) for miR-487a, 0.78 (95% CI 0.62–0.95) for miR-493-5p, 1.0 (95% CI 1.0–1.0) for miR-501-3p, 1.0 (95% CI 1.0–1.0) for miR-502-5p, which indicated these four miRNAs were promising diagnostic biomarkers of OS.

Validation of circulating miRNAs as OS diagnostic biomarkers

To further validate the diagnostic utility of serum miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p, a larger independent cohort composed of 50 OS patients and 30 healthy controls was conducted. As shown in Fig. 3a, the relative expression of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were significantly higher in the serum of OS patients compared to that of healthy controls. ROC curve revealed that the AUC was 0.83 (95% CI 0.71–0.97) for miR-487a, 0.79 (95% CI 0.66–0.93) for miR-493-5p, 0.82 (95% CI 0.68–0.95) for miR-501-3p, 0.83 (95%CI: 0.72 to 0.95) for miR-502-5p, and 0.89 (95% CI 0.78–1.0) for 4-miRNAs combination to differentiate OS patients from healthy controls (Fig. 3b). Besides, high circulating miR-487a, miR-493-5p, and miR-501-3p expressions in OS were significantly associated with metastasis. High circulating miR-502-5p expressions in OS were significantly associated with subtype of OS (Table 2).

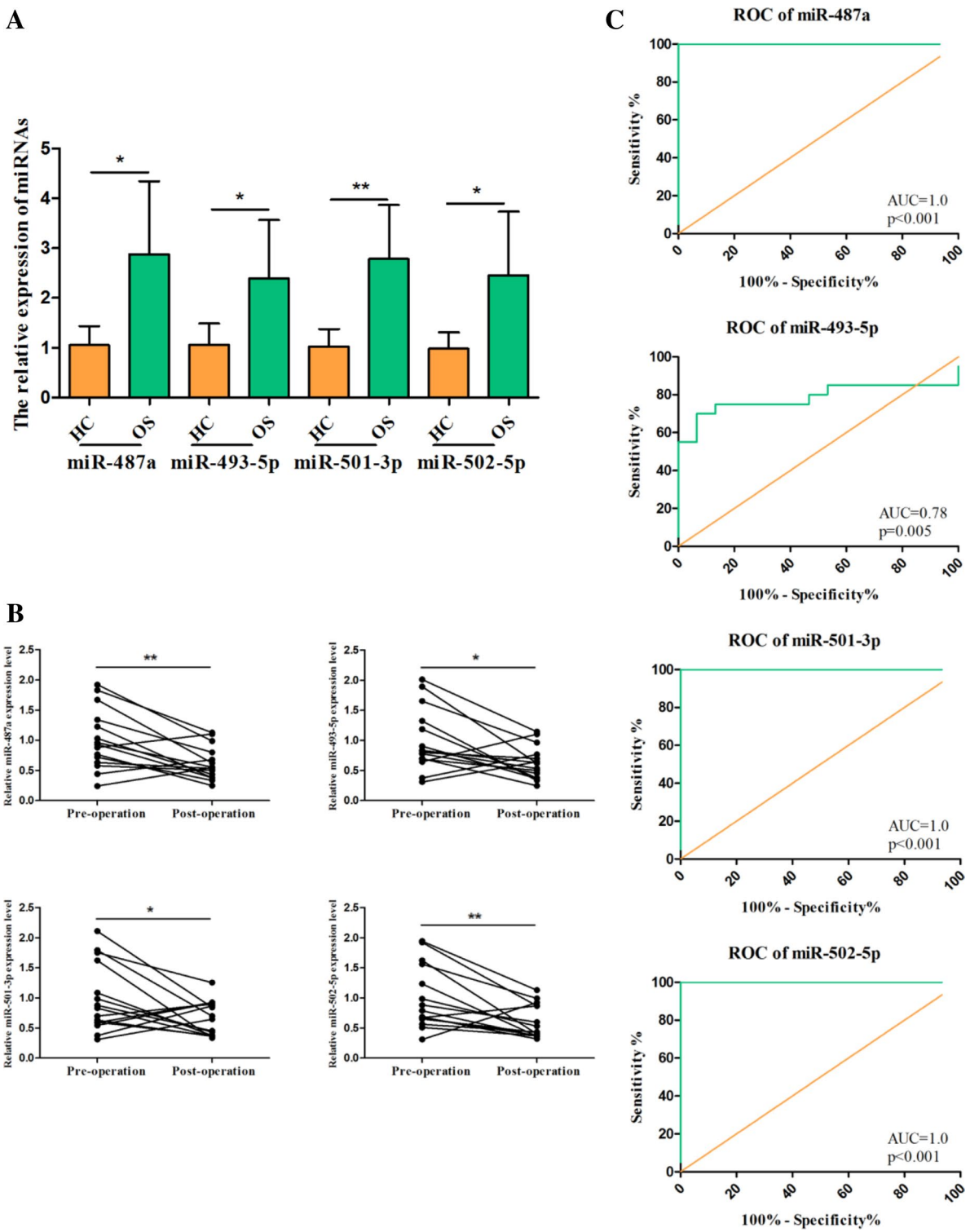

Fig. 2 Candidate miRNAs were tumor-derived and served as potential diagnostic biomarkers of OS. **a** miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were remarkably upregulated in OS tissues. **b** The expression of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were significantly downregulated after the resection of tumor. **c** The AUC of candidate miRNAs by being analyzed by ROC curves. $^{*}P < 0.05$, $^{**}P < 0.01$

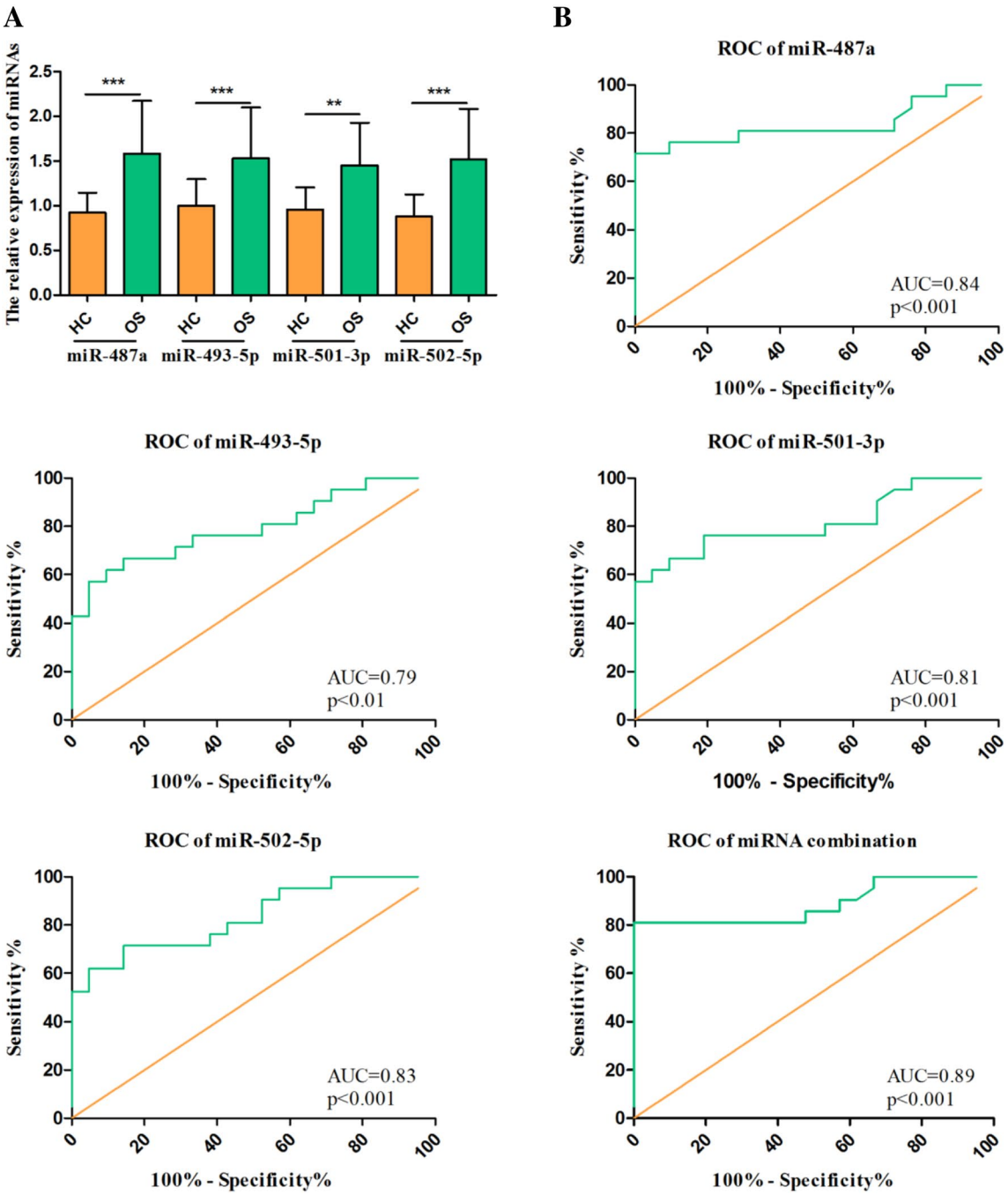

Fig. 3 Upregulated miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p acted as novel promising diagnostic biomarkers of OS. **a** miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were remarkably upregulated in the serum of OS patients. **b** The AUC of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p by being analyzed by ROC curves. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

Enrichment analysis

Considering the pathological role of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p has not been investigated in OS. We constructed a signaling pathway enrichment analysis of the targets of these four miRNAs. The potential target genes of miRNAs were predicted using TargetScan (https://www.targetscan.org/) and miRDB (https://www.mirdb.org/). Enrichment analysis was conducted by using online tools MetaScape (https://metascape.org/gp/index.html). There are several signaling pathways related with miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p, such as Wnt signaling pathway (Table 3).

Table 2 Correlations between serum miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p expression levels and clinico pathological features in osteosarcoma patients

Variables	miR-487a		miR-493-5p		miR-501-3p		miR-502-5p	
	Mean ± SD	*P*value	Mean ± SD	*P*value	Mean ± SD	*P* value	Mean ± SD	*P*value
Age (year)								
≤ 16	1.52–0.14		1.49–0.16		1.42–0.13		1.53–0.14	
> 16	1.59–0.17	0.61	1.55–0.21	0.64	1.47–0.11	0.53	1.51–0.17	0.86
Gender								
Male	1.54–0.13		1.51–0.14		1.48–0.10		1.52–0.12	
Female	1.51–0.15	0.74	1.55–0.11	0.81	1.43–0.13	0.62	1.54–0.08	0.75
Tumor location								
Femur	1.47–0.19		1.53–0.12		1.44–0.11		1.56–0.14	
Tibia	1.57–0.17		1.49–0.18		1.49–0.16		1.51–0.17	
Fibula	1.62–0.23		1.48–0.16		1.41–0.15		1.55–0.13	
Arm	1.44–0.13	0.31	1.64–0.21	0.28	1.48–0.09	0.67	1.48–0.19	0.42
Metastasis								
Yes	1.76–0.16		1.68–0.14		1.57–0.11		1.59–0.11	
No	1.36–0.13	0.028	1.31–0.12	0.033	1.21–0.13	0.021	1.37–0.09	0.062
Subtype								
Osteoblastic	1.55–0.12		1.51–0.19		1.41–0.17		1.31–0.10	
Chondroblastic	1.61–0.16	0.57	1.54–0.15	0.63	1.49–0.14	0.71	1.59–0.14	0.047

Table 3 Signaling pathway enrichment analysis of the targets of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p

miRNA	Gene ontology description	*P* value (log10)
miR-487a	Circadian regulation of gene expression	– 4.8
	Response to transforming growth factor beta	– 5.8
miR-493-5p	Cell surface receptor signaling pathway involved in cell–cell signaling	– 12.1
	Wnt signaling pathways	– 9.6
miR-501-3p	Regulation of G2/M transition of mitotic cell cycle	– 5.3
miR-502-5p	Negative regulation of mRNA catabolic process catabolic	-5.0

Discussion

In this study, we carried out an integrated analysis of two GEO datasets, and discovered miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were significantly upregulated in osteosarcoma. Furthermore, we were the first to validate that circulating miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were tumor-derived and served as novel potential biomarkers for diagnosis of OS because of their reliable ability to differentiate OS patients from healthy individuals. Finally, we pointed out that miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were associated with several signaling pathways, such as Wnt and TGF-β signaling pathways, promoting the development and progression of OS.

Circulating miRNAs acting as potential biomarkers of cancers has been widely reported. Sun et al. declared that increased plasma miRNA-30a as a biomarker for non-small cell lung cancer [17]. Zhu et al. demonstrated that circulating miR-125b as a diagnostic and prognostic biomarker for epithelial ovarian cancer [18]. Li et al. revealed that miR-199a-3p was a potential circulating diagnostic biomarker for early gastric cancer [19]. Zhou et al. reported serum miR-199a-5p as a non-invasive biomarker for detecting and monitoring osteosarcoma [20]. However, the diagnostic role of circulating miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p has not been explored in cancers. Our study is the first to demonstrate that serum miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p act as promising biomarkers for the detection of OS.

Previous studies have comprehensively investigated the pathologic role of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p in various cancers. Xu et al. reported that miR-502 mediates esophageal cancer cell TE1 proliferation by promoting AKT phosphorylation [21]. Sanches et al. discovered that miR-501 is upregulated in cervical cancer and promotes cell proliferation, migration and invasion by targeting CYLD [22]. Zhao et al. revealed that miR-493-5p suppresses hepatocellular carcinoma cell proliferation through

targeting GP73 [23]. Chang et al. demonstrated that miRNA-487a promotes proliferation and metastasis in hepatocellular carcinoma [24]. Nonetheless, the pathological effect of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p has not been studies in OS. In our study, we discovered that miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p were significantly upregulated in OS compared to that in healthy controls. Additionally, we found miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p may function on the progression of OS through several signaling pathways, such as Wnt and TGF-β signaling pathways.

Although circulating miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p appear to be promising biomarkers for diagnosing OS, there are several limitations in our study. First, the sample size included was relatively small, and we believe that prospective studies with larger sample numbers are required to clarify the utility of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p as novel diagnostic biomarkers of OS. Second, the pathologic effect of miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p ought to be further proved by conducting some experiments such as CCK8 assay, clone formation assay, migration assay, invasion assay. Third, the prognostic value of serum miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p was not explored due to the lack of follow-up observation to OS patients.

In summary, we demonstrated that serum miR-487a, miR-493-5p, miR-501-3p, and miR-502-5p are novel promising biomarkers for the diagnosis of OS with non-invasion.

Acknowledgements Funded by Jiangsu Provincial Special Program of Medical Science (BE2016618) and Postgraduate Research & Practice Innovation Program of Jiangsu Province (JX22013459).

Compliance with ethical standards

Conflict of interest There was no conflict of interest in this work.

Ethical approval Written informed consent was obtained from all participants, and this study was approved by the Research and Ethical Committee of Nanjing First Hospital.

References

1. Ma O, Cai WW, Zender L et al (2009) MMP13, Birc2 (cIAP1), and Birc3 (cIAP2), amplified on chromosome 9, collaborate with p53 deficiency in mouse osteosarcoma progression. Cancer Res 69:2559–2567
2. Kansara M, Teng MW, Smyth MJ et al (2014) Translational biology of osteosarcoma. Nat Rev Cancer 14:722–735
3. Yang J, Zhang W (2013) New molecular insights into osteosarcoma targeted therapy. Curr Opin Oncol 25:398–406
4. Bielack SS, Kempf-Bielack B, Delling G et al (2002) Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20:776–790
5. Gianferante DM, Mirabello L, Savage SA (2017) Germline and somatic genetics of osteosarcoma - connecting aetiology, biology and therapy. Nat Rev Endocrinol 13:480–491
6. Fabian MR, Sonenberg N, Filipowicz W (2010) Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 79:351–379
7. Mitchell PS, Parkin RK, Kroh EM et al (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA 105:10513–10518
8. Bekris LM, Leverenz JB (2015) The biomarker and therapeutic potential of miRNA in Alzheimer's disease. Neurodegener Dis Manag 5:61–74
9. Li SP, Su HX, Zhao D et al (2016) Plasma miRNA-506 as a Prognostic Biomarker for Esophageal Squamous Cell Carcinoma. Med Sci Monit 22:2195–2201
10. Tanase C, Albulescu R, Codrici E et al (2015) Circulating biomarker panels for targeted therapy in brain tumors. Future Oncol 11:511–524
11. Guo L, Luo C, Fan J et al (2015) Serum miRNA profiling identifies miR-150/30a as potential biomarker for workers with damaged nerve fibers from carbon disulfide. Ind Health 53:38–47
12. Lian F, Cui Y, Zhou C et al (2015) Identification of a plasma four-microRNA panel as potential noninvasive biomarker for osteosarcoma. PLoS ONE 10:e0121499
13. Nonaka R, Nishimura J, Kagawa Y et al (2014) Circulating miR-199a-3p as a novel serum biomarker for colorectal cancer. Oncol Rep 32:2354–2358
14. Maciejak A, Kostarska-Srokosz E, Gierlak W et al (2018) Circulating miR-30a-5p as a prognostic biomarker of left ventricular dysfunction after acute myocardial infarction. Sci Rep 8:9883
15. Yao ZS, Li C, Liang D et al (2018) Diagnostic and prognostic implications of serum miR-101 in osteosarcoma. Cancer Biomark 22:127–133
16. Kelly AD, Haibe-Kains B, Janeway KA et al (2013) MicroRNA paraffin-based studies in osteosarcoma reveal reproducible independent prognostic profiles at 14q32. Genome Med 5:2
17. Sun L, Chen Y, Su Q et al (2016) Increased plasma miRNA-30a as a biomarker for non-small cell lung cancer. Med Sci Monit 22:647–655
18. Zhu T, Gao W, Chen X et al (2017) A pilot study of circulating MicroRNA-125b as a diagnostic and prognostic biomarker for epithelial ovarian cancer. Int J Gynecol Cancer 27:3–10
19. Li C, Li JF, Cai Q et al (2013) MiRNA-199a-3p: A potential circulating diagnostic biomarker for early gastric cancer. J Surg Oncol 108:89–92
20. Zhou G, Lu M, Chen J et al (2015) Identification of miR-199a-5p in serum as noninvasive biomarkers for detecting and monitoring osteosarcoma. Tumour Biol 36:8845–8852
21. Xu J, Pan X, Hu Z (2018) MiR-502 mediates esophageal cancer cell TE1 proliferation by promoting AKT phosphorylation. Biochem Biophys Res Commun 501:119–123
22. Sanches JGP, Xu Y, Yabasin IB et al (2018) miR-501 is upregulated in cervical cancer and promotes cell proliferation, migration and invasion by targeting CYLD. Chem Biol Interact 285:85–95
23. Zhao J, Xu T, Wang F et al (2017) miR-493-5p suppresses hepatocellular carcinoma cell proliferation through targeting GP73. Biomed Pharmacother 90:744–751
24. Chang RM, Xiao S, Lei X et al (2017) miRNA-487a promotes proliferation and metastasis in hepatocellular carcinoma. Clin Cancer Res 23:2593–2604