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Identification of circulating miR-663a as a potential biomarker for diagnosing osteosarcoma

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ABSTRACT

Purpose: Osteosarcoma (OS) is the most common malignant bone tumor in children and young adults. The overall survival rate of OS patients has not been improved during the last 25 years, in part due to the lack of sensitive and specific biomarkers. This study aimed to investigate miRNAs existing in the peripheral blood as diagnostic biomarkers of OS.

Patients and methods: OS patients and healthy controls (HCs) in this study were enrolled from Nanjing First Hospital. Candidate miRNA was selected by comprehensive analysis of GEO database. The expressions of candidate miRNA in tissues and plasma samples were subsequently examined through qRT-PCR. The diagnostic utility of candidate miRNA was examined by using receiver operating characteristic (ROC) curve analysis.

Results: After analysis of GEO database and clinical plasma samples, miR-663a was selected as a candidate miRNA to further investigate its value for diagnosing OS due to the highest differential fold change. We discovered that miR-663a expressions were remarkably elevated in both tissues and plasmas of OS patients. In addition, upregulated miR-663a in peripheral blood of OS patients was proved to be tumor-derived. The area under the ROC curve (AUC) was 0.86 (95%CI: 0.78 to 0.93) for miR-663a with 67.35% sensitivity and 89.8% specificity.

Conclusion: Plasma miR-663a was identified as a novel potential biomarker for the diagnosis of OS.

1. Introduction

Osteosarcoma (OS), the most common primary bone tumor in children and young adults, accounts for approximately 60% of pediatric bone tumor [1,2]. Despite the progression of surgical techniques and combinational neoadjuvant chemotherapy, the prognosis of patients with OS remains unsatisfactory mainly because of current poor strategies for early diagnosis [3,4]. Although alkaline phosphatase (ALP), a known serum-based tumor marker, has been used in detection of OS, its expression levels is generally high in children and also affected by organ damage, which limits its sensitivity and specificity [5]. Therefore, it is urgently needed to identify highly sensitive, specific as well as minimally invasive biomarkers for diagnosing OS.

MicroRNAs (miRNAs), a class of endogenous small non-coding RNAs, are 22–24 nucleotides in length [6]. MiRNAs play a crucial role in post-transcriptional regulation through binding to the 3′-untranslated region (3′-UTR) of their targets and thereby suppressing translation or inducing degradation of targets [7] [8]. It has been widely reported that miRNAs are significantly associated with the development and progression of OS [9–11]. Rehei et al. exhibited that miR-214, an oncogene, could significantly promote OS cell invasion and migration via targeting TRAF3 [12]. Zhuang et al. discovered that miR-524 promotes cell proliferation by down-regulating PTEN expression in OS [13]. Wang and colleagues demonstrated that miR-141-3p is a key negative regulator of the EGFR pathway in osteosarcoma and may be a new theoretical basis for the treatment of osteosarcoma [14]. Besides, mounting studies were conducted to identify circulating miRNAs as diagnostic biomarkers of cancers, such as OS [15–17]. For example, Cong et al. reported that serum miR-124 might be a promising biomarker with diagnostic and prognostic value for osteosarcoma [18]. However, currently, many circulating miRNAs identified as potential diagnostic biomarkers of OS were not selected through high throughput screening, thus other miRNAs which may have higher diagnostic value were ignored.

In this study, we analyzed the comprehensive plasma miRNA expression profiling completed using the Exiqon miRNome platform on 20

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Table 1
miRNAs significantly dysregulated with |logFC| > 2 in the plasma of osteosarcoma patients based on GEO dataset.

miRNA_ID	logFC	P.Value	miRNA_ID	logFC	P.Value
hsa-miR-663a	5.91579	1.20E-23	hsa-let-7f-2-3p	3.61325	5.39E-28
hsa-miR-31-5p	5.74147	5.97E-24	hsa-miR-214-3p	3.59456	5.11E-11
hsa-miR-203a	5.51882	1.12E-19	hsa-miR-616-5p	3.58664	3.73E-21
hsa-miR-671-5p	5.47697	1.65E-25	hsa-miR-454-5p	3.58002	1.66E-22
hsa-miR-499a-5p	5.4298	3.33E-25	hsa-miR-331-5p	3.57256	1.11E-20
hsa-miR-346	5.23055	3.52E-23	hsa-miR-450a-5p	3.48245	1.59E-19
hsa-miR-520h	5.19294	1.23E-22	hsa-miR-100-5p	3.43226	8.76E-13
hsa-miR-95	5.16319	3.11E-25	hsa-miR-205-5p	3.43211	1.39E-07
hsa-miR-375	5.10739	2.85E-16	hsa-miR-135a-5p	3.41677	5.24E-22
hsa-miR-502-5p	5.00856	3.01E-23	hsa-miR-330-5p	3.37117	6.73E-21
hsa-miR-342-5p	5.00662	2.24E-26	hsa-miR-29b-2-5p	3.33412	1.22E-17
hsa-miR-624-5p	4.99814	2.24E-30	hsa-miR-24-1-5p	3.32412	2.49E-17
hsa-miR-23b-5p	4.89943	1.22E-24	hsa-miR-629-3p	3.28145	9.59E-25
hsa-miR-206	4.77057	1.94E-17	hsa-miR-193b-3p	3.28137	2.64E-08
hsa-miR-490-3p	4.65964	8.73E-17	hsa-miR-150-5p	3.20236	6.80E-14
hsa-miR-483-3p	4.58839	1.75E-14	hsa-miR-1260a	3.20208	1.77E-18
hsa-miR-124-3p	4.58786	4.68E-16	hsa-let-7f-1-3p	3.18294	9.49E-26
hsa-miR-183-5p	4.46902	2.06E-26	hsa-miR-182-5p	3.15282	5.40E-18
hsa-miR-483-5p	4.40232	1.75E-15	hsa-miR-579	3.14197	1.41E-20
hsa-miR-200a-3p	4.35855	6.16E-19	hsa-miR-219-5p	3.11987	2.74E-18
hsa-miR-582-5p	4.2824	4.26E-20	hsa-miR-760	3.07782	1.88E-16
hsa-miR-877-3p	4.26754	4.67E-27	hsa-miR-487a	3.04344	7.58E-16
hsa-miR-885-5p	4.23953	1.85E-13	hsa-miR-204-5p	3.02722	2.45E-13
hsa-miR-10b-5p	4.17333	3.18E-14	hsa-miR-500a-5p	3.01651	3.24E-20
hsa-miR-651	4.1633	1.32E-24	hsa-miR-101-5p	2.96036	6.26E-23
hsa-miR-744-3p	4.1532	6.75E-24	hsa-miR-429	2.93878	4.63E-13
hsa-miR-452-5p	4.09595	8.93E-18	hsa-miR-191-3p	2.93589	2.60E-22
hsa-miR-181d	4.06436	6.86E-24	hsa-miR-145-3p	2.88693	1.19E-15
hsa-miR-455-3p	4.01374	4.76E-14	hsa-miR-99b-3p	2.88491	2.25E-19
hsa-miR-188-5p	3.94879	4.41E-17	hsa-miR-365a-3p	2.88394	1.41E-09
hsa-miR-628-5p	3.90509	7.80E-24	hsa-miR-576-3p	2.88063	4.35E-18
hsa-miR-127-5p	3.90211	2.99E-19	hsa-miR-369-3p	2.87104	9.78E-16
hsa-miR-20b-3p	3.80828	8.61E-25	hsa-miR-671-3p	2.84923	3.41E-20
hsa-miR-132-5p	3.78385	2.26E-22	hsa-miR-548k	2.81349	6.65E-19
hsa-miR-9-3p	3.78152	5.70E-21	hsa-miR-501-3p	2.8097	4.03E-22
hsa-miR-144-5p	3.74641	1.06E-16	hsa-miR-1	2.79888	1.65E-13
hsa-miR-193a-5p	3.73733	6.56E-13	hsa-miR-627	2.78338	3.67E-18
hsa-let-7a-3p	3.68358	7.99E-25	hsa-miR-496	2.778	4.28E-11
hsa-miR-449a	3.6548	4.60E-25	hsa-miR-576-5p	2.7621	3.30E-19
hsa-miR-1185-5p	3.64933	1.17E-19	hsa-let-7b-3p	2.75639	1.84E-13
hsa-miR-505-5p	3.63189	3.22E-28	hsa-miR-32-3p	2.75392	4.29E-19
hsa-miR-96-5p	3.62888	1.90E-18	hsa-miR-369-5p	2.73244	1.36E-14
hsa-miR-494	3.61923	1.12E-23	hsa-miR-941	2.72481	6.09E-17
hsa-miR-146b-3p	3.61687	5.72E-21	hsa-miR-379-3p	2.71114	1.32E-14
hsa-miR-33a-3p	3.61601	4.16E-25	hsa-miR-940	2.68369	2.86E-13
miRNA_ID	logFC	P.Value	miRNA_ID	logFC	P.Value
hsa-miR-345-5p	2.68165	1.43E-20	hsa-miR-1537	2.21816	1.60E-17
hsa-miR-589-5p	2.66903	4.44E-18	hsa-miR-16-1-3p	2.21359	5.03E-10
hsa-miR-629-5p	2.66167	1.21E-16	hsa-miR-2110	2.20068	3.96E-15
hsa-let-7e-3p	2.64814	1.70E-15	hsa-miR-329	2.19479	7.30E-09
hsa-miR-378a-5p	2.61742	5.66E-17	hsa-miR-141-3p	2.19279	7.74E-14
hsa-miR-486-3p	2.60808	1.45E-16	hsa-miR-758-3p	2.18467	2.07E-12
hsa-miR-190a	2.58464	1.90E-14	hsa-miR-181a-3p	2.17904	1.77E-09
hsa-miR-1271-5p	2.57782	1.82E-21	hsa-miR-34a-5p	2.17394	2.69E-08
hsa-miR-199b-5p	2.5432	2.09E-17	hsa-miR-942	2.16345	4.26E-13
hsa-miR-221-5p	2.52929	4.44E-16	hsa-miR-99a-5p	2.14719	5.29E-11
hsa-miR-9-5p	2.52035	7.82E-14	hsa-miR-185-3p	2.13951	2.13E-14
hsa-miR-130b-5p	2.50233	3.25E-18	hsa-miR-15a-3p	2.11159	1.92E-16
hsa-miR-19a-5p	2.49497	4.79E-17	hsa-miR-548c-5p	2.10986	1.31E-13
hsa-miR-200b-3p	2.46715	1.20E-17	hsa-miR-545-3p	2.10343	1.04E-15
hsa-miR-154-3p	2.44795	1.26E-13	hsa-miR-196b-5p	2.02341	5.56E-10
hsa-miR-29a-5p	2.43645	4.62E-15	hsa-miR-411-5p	2.01668	3.84E-07
hsa-miR-148b-5p	2.42442	4.14E-16	hsa-miR-484	−2.01031	3.56E-09
hsa-miR-30d-3p	2.41445	9.01E-19	hsa-miR-26a-5p	−2.16031	7.00E-16
hsa-miR-139-3p	2.41348	1.60E-14	hsa-miR-423-3p	−2.18814	3.26E-12
hsa-miR-224-3p	2.40205	5.37E-13	hsa-miR-652-3p	−2.23262	4.31E-14
hsa-miR-30a-3p	2.397	1.61E-12	hsa-miR-151a-3p	−2.29382	8.05E-17
hsa-miR-340-5p	2.39262	8.81E-16	hsa-miR-151a-5p	−2.29914	6.70E-14
hsa-miR-133b	2.38566	6.65E-07	hsa-miR-199a-3p	−2.30114	2.99E-14
hsa-miR-889	2.34551	2.34E-12	hsa-miR-27a-3p	−2.44109	2.11E-17
hsa-let-7g-3p	2.27409	1.46E-14	hsa-miR-18b-5p	−2.47142	3.00E-16
hsa-miR-187-3p	2.24657	3.23E-10	hsa-miR-18a-5p	−2.58998	1.58E-15
hsa-miR-877-5p	2.24454	1.42E-15	hsa-miR-191-5p	−2.60448	7.71E-18
hsa-miR-502-3p	2.24365	6.84E-16	hsa-miR-223-3p	−2.72998	4.43E-19
hsa-miR-1249	2.23088	4.73E-11	hsa-miR-199a-5p	−3.01081	8.83E-14

OS samples and 15 healthy controls. We focused on the selected miR-663a as a potential circulating biomarker for the detection of OS.

2. Materials and methods

2.1. Study design

First, we analyzed a GEO dataset which conducted a high-throughput analysis of circulating miRNAs in OS patients and healthy people and found 5 upregulated miRNAs with the highest fold change. Then we verified the expressions of these five miRNAs by using qRT-PCR and selected miR-663a as a candidate miRNA for further study. Next, we explored whether elevated circulating miR-663a in OS patients was tumor-derived. Subsequently, we verified the expression and diagnostic value of plasma miR-663a. Finally, a bioinformatics analysis of miR-663a was carried out to explore its potential biological functions.

2.2. Study population

All participants were enrolled from Nanjing First Hospital affiliated to Nanjing Medical University between October 2014 and June 2017. OS patients have been confirmed through histopathological analysis of surgically excised tissues. The normal plasma samples were collected from healthy people who underwent routine physical examinations. Corresponding to the clinical characteristics of OS patients, age and gender matched healthy people were selected into control group. Written informed consent was obtained from all participants, and this study was approved by the Research and Ethical Committee of Nanjing First Hospital.

2.3. Samples processing

Excised tissues were stored in liquid nitrogen immediately until RNA extraction. Plasma 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 ℃ until further analysis. Total RNA was isolated using Trizol LS

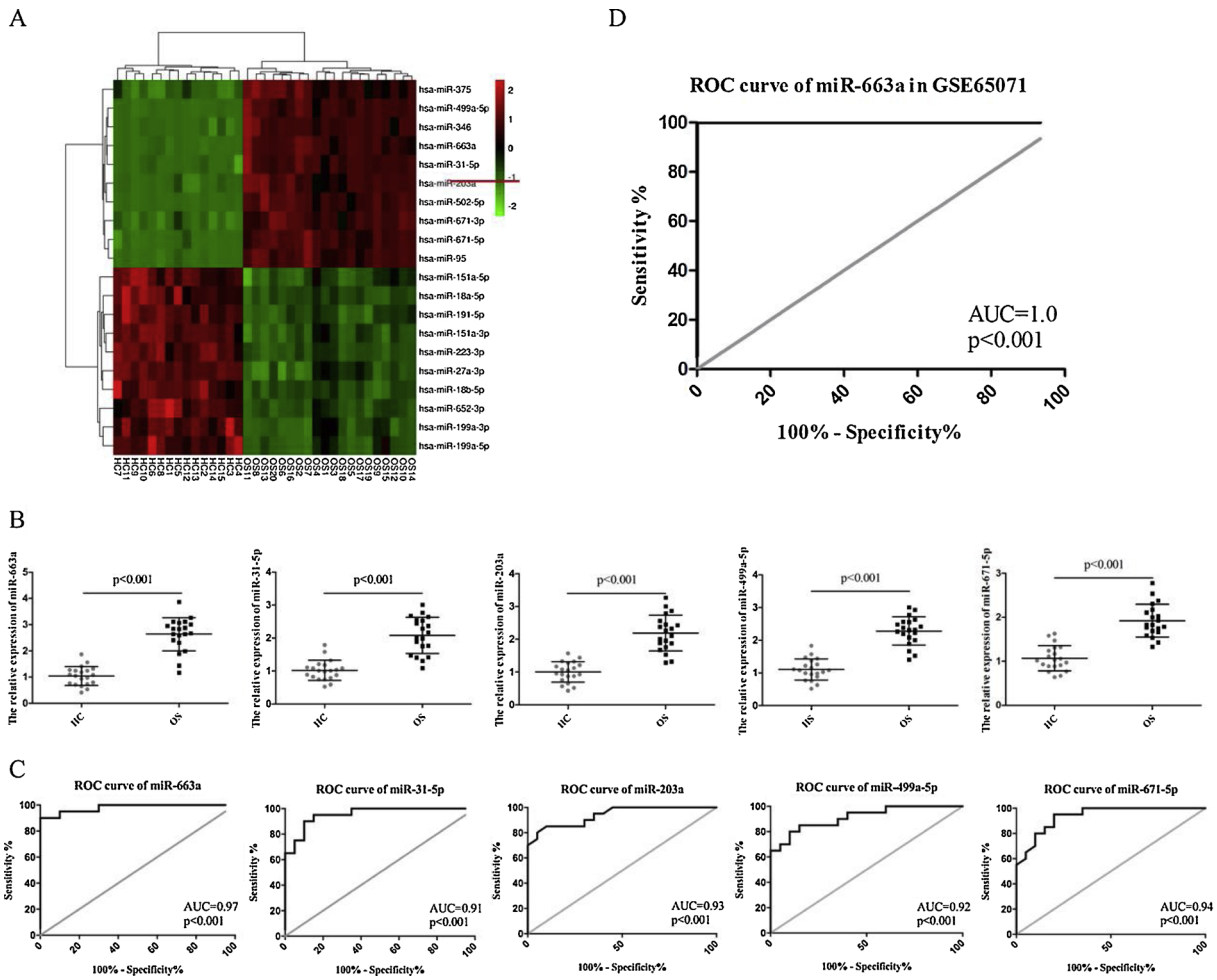

Fig. 1. Selection of candidate circulating miRNAs based on GEO database and clinical plasma samples. **A** Partial miRNAs that were significantly dysregulated in GSE65071. **B** The relative expressions of top five dysregulated plasma miRNAs in OS patients. **C** The AUC of top five dysregulated plasma miRNAs in clinical plasma samples. D The AUC of circulating miR-663a in GSE65071.

reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. 1 ul cel-miR-39-3p at a concentration of 1 uM (GenePharma, Shanghai, China) was added into each plasma sample to act as the external reference.

2.4. Cell culture

Human OS MG-63 and U-2OS cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and then cultured in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 mg/ml streptomycin (Gibco, Vienna, Austria) at 37 °C with humidified 5% CO2. Cells were plated onto 10 cm plates (5×10^5 or 1×10^6 cells/well) and the medium was collected after 24 or 48 h.

2.5. qRT-PCR for miRNA quantitation

Reverse transcription and qRT-PCR for miR-663a, endogenous control U6 snRNA, and external reference cel-miR-39-3p were performed using Hairpin-itTM microRNA RT-PCR Quantitation Kit (GenePharma, Shanghai, China) according to the manufacture's instructions. The reactions were initiated with denaturation at 95℃ for 3 min, followed by 40 cycles of 95℃ for 15 s and 62℃ for 34 s. The relative expressions of miR-663a were calculated with $2^{-\triangle Ct\ Ct}$ method. $\triangle Ct = Ct_{miRNA}\text{-}Ct_{miR\text{-}39/U6}$, $\triangle CtCt = \triangle Ct_{patient} - Ct_{control}$.

2.6. Bioinformatics analysis of miR-663a

The potential targets of miR-663a were explored through integrated analysis of TargetScan (http://www.targetscan.org/vert_72/) and miRDB (http://mirdb.org). The GO which contains three items: molecular functions (MF), biological processes (BP) as well as cellular components (CC) and KEGG pathway enrichment analysis were conducted by using online tool STRING (https://string-db.org/). If there were more than ten terms enriched in these categories, the top ten terms based on false discovery rate (FDR) were chosen.

2.7. Statistical analysis

Data were presented as mean ± SD. The statistical analysis was performed using SPSS 22.0 (IBM, Chicago, IL, USA) and GraphPad 7.0 (GraphPad Software, USA). The differential expressions of miRNAs among groups were determined using paired or unpaired t-test. ROC curve and AUC were established for discriminating OS patients from HCs. The cut-off value of the miR-663a expression was determined by using Youden index from ROC curves. A p-value < 0.05 was considered statistically significant.

3. Results

Analysis of plasma miRNA expression profiling revealed miR-663a as the most promising candidate diagnostic biomarker of OS

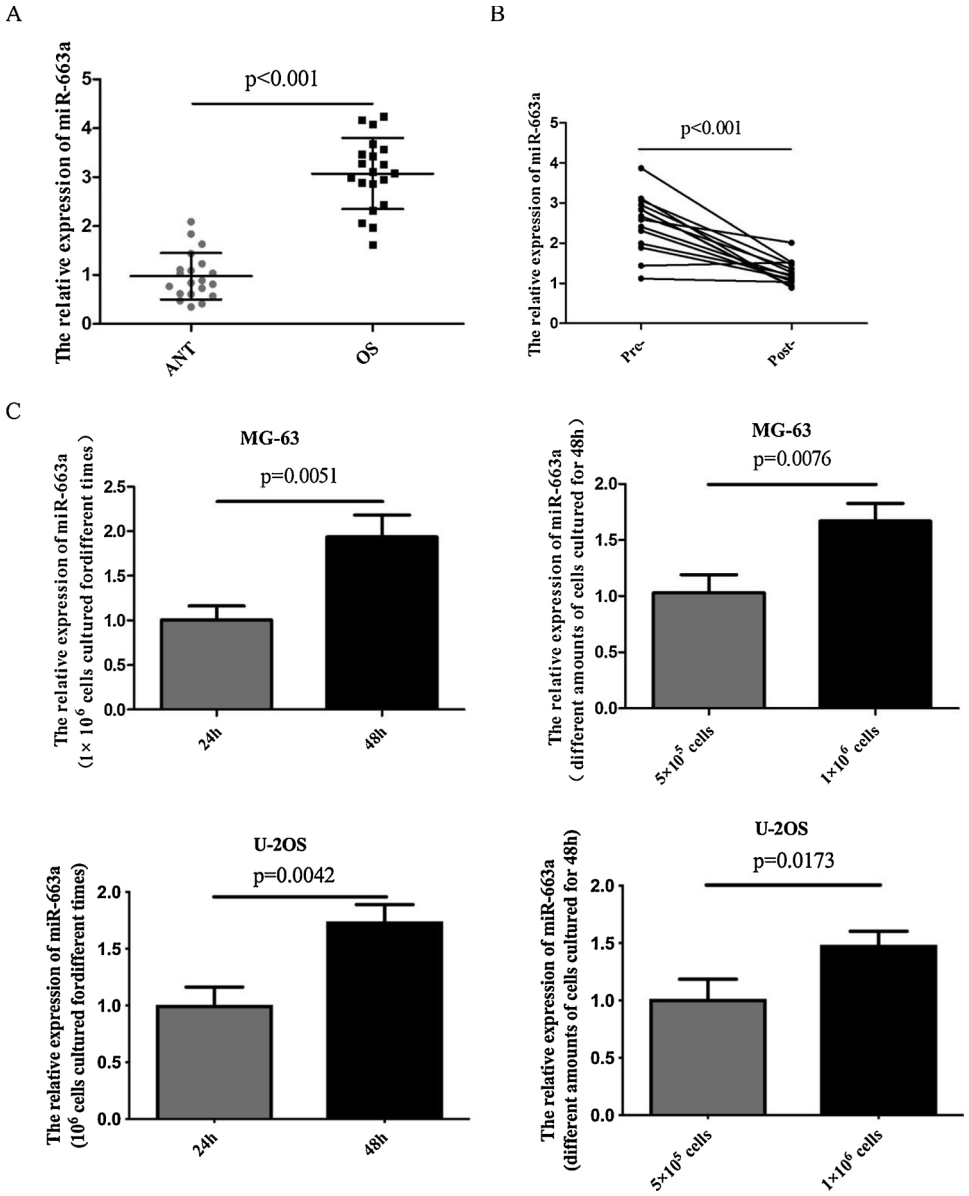

Fig. 2. Identification of the source of upregulated plasma miR-663a. **A** The relative expression levels of miR-663a in OS tissues. **B** The relative expression levels of miR-663a were significantly downregulated after surgical resection of malignancies. C miR-663a expressions in the culture media increased both with time and with increasing numbers of OS cells.

After analysis of GEO dataset (GSE65071) which conducted a comprehensive comparative analysis of plasma miRNA expression profiles between 20 OS samples and 15 HCs, we discovered 264 significantly dysregulated miRNAs with $|\log FC| > 2$ (Table 1). Among these miRNAs, miR-663a, miR-31-5p, miR-203a, miR-671-5p, miR-499a-5p were the top five with highest differential fold-change (Fig. 1A). To validate the expression levels of these miRNAs, qRT-PCR was performed by using clinical plasma samples collected from 20 OS patients and 20 HCs. As shown in Fig. 1B, plasma miR-663a expressions showed highest upregulated fold change which was consistent with the result of GSE65071. Receiver operating characteristic (ROC) analyses showed that plasma miR-663a levels could discriminate OS patients from healthy controls with an highest area under the curve (AUC) value 0.97 in clinical samples (Fig. 1C). Besides, the AUC of miR-663a to discriminate OS patients from healthy controls was 1.0 in GSE65071 (Fig. 1D). Therefore, we selected plasma miR-663a as candidate miRNA for diagnosing OS.

3.1. Identifying the source of upregulated plasma miR-663a

Emerging studies have demonstrated that cancer cells could release specific miRNAs into peripheral blood [19,20]. Subsequently, we investigated whether circulating miR-663a were tumor-derived. The expressions of miR-663a in 20 matched OS tissues and adjacent normal tissues (ANTs) were analyzed. The expressions of miR-663a were remarkably higher in OS tissues compared to ANTs (Fig. 2A). Besides, 15 paired pre- and post-operation plasma samples were tested. When compared to that in pre-operation plasma samples, the expression levels of miR-663a were significantly downregulated after surgical resection of malignancies (Fig. 2B). Studies in vitro illustrated that miR-663a expressions in the culture media increased both with time and with increasing numbers of cells (Fig. 2C).

3.2. Validating circulating miR-663a as OS diagnostic biomarker

The characteristics of participants (n = 100) in the validation phase

Table 2
: The clinical characteristics of participants.

Variable	Healthy control		Osteosarcoma	
	Number	Percent(%)	Number	Percent(%)
Age (year)				
≤14	30	60	31	62
> 14	20	40	19	38
Gender				
Male	32	64	36	72
Female	18	36	14	28
Tumor location				
Femur			29	58
Tibia			8	16
Fibula			9	18
Arm			4	8
Metastasis				
Yes			11	22
No			39	78
Subtype				
Osteoblastic			38	76
Chondroblastic			12	24

are summarized in Table 2. There was no statistically significant difference in age and gender between healthy subjects and OS patients. As shown in Fig. 3A, The relative expressions of miR-663a were significantly higher in the plasma of OS patients compared to that of HCs. ROC curve revealed that the AUC was 0.86 (95%CI: 0.78 to 0.93) for miR-663a to differentiate OS patients from HCs with 67.35% sensitivity and89.8% specificity (cut-off value = 1.845) (Fig. 3B). Moreover, high plasma miR-663a expressions in OS were significantly associated with metastasis (p = 0.0062) (Table 3).

3.3. Functional and pathway enrichment analysis of targets of miR-663a

The GO term enrichment analysis revealed that in the BP category, miR-663a targets were significantly enriched in regulation of cell communication, regulation of signaling, and cell differentiation. CC analysis showed that most targets were located in intracellular part. Furthermore, according to the results of MF analysis, targets were mainly associated with protein binding. In addition, KEGG pathway analysis showed that targets of miR-663a participated in endocytosis process of cells (Table 4).

4. Discussion

In this study, we carried out a comprehensive comparative analysis of miRNA expression profiles based on GEO database and clinical samples, and discovered plasma miR-663a was the most dysregulated miRNAs in OS patients. Then we validated that circulating miR-663a was tumor-derived and served as a novel promising biomarker for diagnosing OS because of their reliable ability to differentiate OS patients from HCs. In addition, we discovered that plasma miR-663a expression levels were significantly associated with metastasis status of OS patients.

Circulating miRNAs acting as diagnostic biomarkers of OS has been widely investigated. Monterde et al. reported that circulating miR-215-5p and miR-642a-5p as potential biomarker for diagnosis of osteosarcoma in Mexican population [15]. Liu et al. concluded that serum miR-375 expression was downregulated in patients with osteosarcoma and might serve as a biomarker for its diagnosis [21]. Besides, Li and colleagues discovered that miR-542-3p levels in peripheral blood could serve as a non-invasive biomarker for tumor monitoring and prognostic prediction in osteosarcoma patients [5]. Here, we were the first to demonstrate that plasma miR-663a expression levels were significantly elevated in OS patients and acted as a promising biomarker for the detection of OS. However, the sample size included was relatively small, and more prospective studies with larger sample numbers are needed to clarify the value of plasma miR-663a as a novel diagnostic biomarker of OS.

Several studies have investigated the pathologic role of miR-663a in cancers [22,23]. Huang et al. found that miR-663a was significantly down-regulated in hepatocellular carcinoma (HCC) tissues compared with adjacent non-tumor tissues. Gain and loss of function assays revealed that miR-663a distinctly inhibited cell proliferation, migration and invasion through targeting high mobility group A2 (HMGA2) [24]. In contrast, Ma et al. proved miR-663a was a tumor-promoting factor mediating gallbladder cancer (GBC) development through EMP3/MAPK/ERK axis which may be a novel therapeutic target for GBC treatment [25]. In our study, we discovered that miR-663a was significantly upregulated in OS tissues compared to that in ANTs. Besides, we revealed that targets of miR-663a were mainly associated with signaling communication and endocytosis process of cells through bioinformatics analysis. Considering exosomes have been reported to tart part in communication between cells, we speculated there are may be some relationships among miR-663a and exosomes [26]. Therefore, the pathologic effect of miR-663a should be further explored by conducting gain and loss of function assays both in vitro and in vivo.

In summary, we demonstrated that tumor-derived plasma miR-663a was a novel promising biomarker for diagnosing OS with non-invasion.

Disclosure of interests

None.

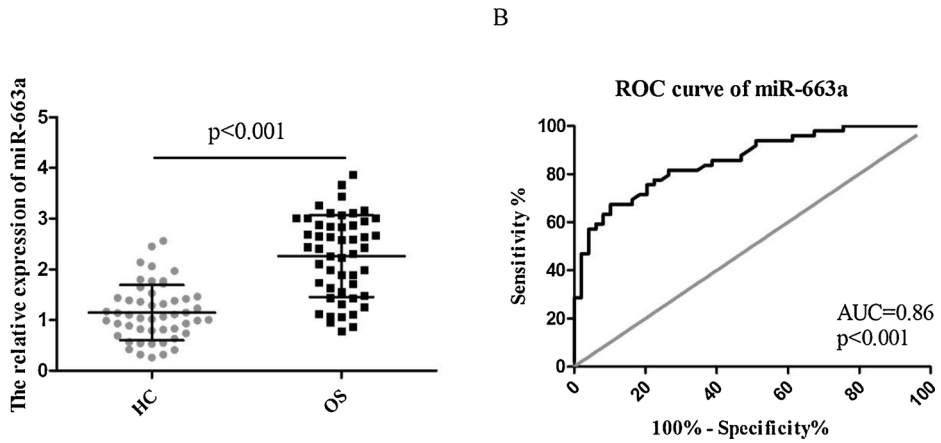

Fig. 3. Validation of circulating miR-663a as OS diagnostic biomarkers. **A** The relative expression levels of miR-663a in the plasma of OS patients. **B** The AUC of plasma miR-663a to differentiate OS patients from HCs.

Table 3
: Correlations between plasma miR-663a relative expression levels and clinicopathological features in osteosarcoma patients.

Variable	Osteosarcoma (n = 50)	
	Mean ± SD	P-value
Age (year)		
≤14	2.29 ± 0.15	
> 14	2.14 ± 0.18	0.48
Gender		
Male	2.19 ± 0.21	
Female	2.32 ± 0.14	0.27
Tumor location		
Femur	2.16 ± 0.27	
Tibia	2.37 ± 0.17	
Fibula	2.12 ± 0.22	
Arm	2.27 ± 0.19	0.31
Metastasis		
Yes	2.59 ± 0.11	
No	2.06 ± 0.15	0.0062
Subtype		
Osteoblastic	2.4 ± 0.13	
Chondroblastic	2.26 ± 0.14	0.46

Table 4
The functional enrichment and pathway enrichment analysis of targets of miR-663a.

GO-term	description	counts	FDR
BP-GO:0001501	skeletal system development	17	0.0047
BP-GO:0010646	regulation of cell communication	58	0.0047
BP-GO:0023051	regulation of signaling	59	0.0047
BP-GO:0002520	immune system development	19	0.0047
BP-GO:0051094	positive regulation of developmental process	30	0.0058
BP-GO:0002076	osteoblast development	4	0.0058
BP-GO:0010941	regulation of cell death	33	0.0083
BP-GO:0030097	hemopoiesis	17	0.0083
BP-GO:0030099	myeloid cell differentiation	10	0.0083
BP-GO:0030154	cell differentiation	56	0.0083
CC-GO:0044297	cell body	19	0.0005
CC-GO:0043025	neuronal cell body	17	0.00071
CC-GO:0097458	neuron part	32	0.0014
CC-GO:0045202	synapse	22	0.0031
CC-GO:0036477	Somatodendritic compartment	19	0.0088
CC-GO:0043005	neuron projection	25	0.0088
CC-GO:0030054	cell junction	22	0.0148
CC-GO:0030425	dendrite	15	0.0148
CC-GO:0097060	synaptic membrane	21	0.0148
CC-GO:0044424	intracellular part	154	0.0187
MF-GO:0005515	protein binding	96	0.00038
MF-GO:0019904	protein domain specific binding	18	0.039
MF-GO:0030306	ADP-ribosylation factor binding	3	0.039
KEGG-hsa04144	Endocytosis	10	0.0243

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