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Clinical Study

MicroRNA-10a, -210, and -563 as circulating biomarkers for ossification of the posterior longitudinal ligament

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Abstract

BACKGROUND CONTEXT: The presence of ossification of posterior longitudinal ligament (OPLL) can lead to symptomatic spinal cord compression and myelopathy. The surgical approach in patients with myelopathy is influenced by the presence of OPLL. Diagnose of OPLL currently requires computed tomography which incurs a large dose of radiation. Circulating disease-specific microRNAs (miRNAs) may serve as promising diagnostic markers with no radiation and easy accessibility for OPLL patients.

PURPOSE: The purpose of this study is to evaluate the accuracy and significance of OPLL-specific microRNAs in discriminating OPLL from normal and intervertebral disc degenerated (IDD) patients by detecting the microRNAs' plasma level.

STUDY DESIGN/PATIENT SAMPLES: The level of microRNAs in OPLL patients' plasma or serum were detected and compared to that of normal and IDD patients to evaluate the accuracy and significance of diagnosing OPLL.

METHODS: Taking advantage of the high through-put microRNA sequencing data, we selectively tested the ten most differentially regulated microRNAs in patients with: (1) radiologically diagnosed OPLL (n = 68), (2) radiologically diagnosed disc herniated patients with no evidence of OPLL (n = 45), (3) non-OPLL and nonmyelopathy patients (n = 53).The feasibility of the biomarkers in identifying OPLL was assessed through analysis of sensitivity, specificity, accuracy, negative predictive value, positive predictive value, and area under the curve (AUC) values.

RESULTS: Of the ten miRNAs validated, miR-10a-3p, miR-10a-5p, miR-563, miR-210-3p, and miR-218-3p showed significance between OPLL and non-OPLL blood samples. While miR-10a-5p, miR-563, and miR-210-3p showed high accuracy and significance in identifying OPLL from other groups individually, and an index that combines these miRNAs achieved the highest accuracy and AUC among these individual miRNAs.

CONCLUSIONS: Analysis of miR-10a-5p, miR-563, and miR-210-3p may be of important value in diagnosing OPLL. These markers maybe useful in a clinical setting in the early detection of OPLL patients by blood testing. © 2018 Elsevier Inc. All rights reserved.

Keywords: Diagnostic markers; MicroRNAs; OPLL; Serum marker.

FDA device/drug status: Not applicable.

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Introduction

The most common location of posterior longitudinal ligament (OPLL) is the cervical spine where it can lead to symptomatic spinal cord compression [1]. However, the etiology of such disease is still largely obscure. Hereditary and regional factors seem to be important, for it was first described in Japanese patients. In Japan, the incidence of OPLL is estimated to be between 1.9% and 4.3% [2] while the estimated incidence is about 0.1% to 1.7% among North Americans and Europeans.

Advances in radiology, especially the use of high resolution computed tomography (CT), have led to more frequent identification of OPLL. Although CT is considered the gold standard for diagnosis, radiation exposure and time for acquisition make CT unfeasible as a large-scale screening method. Until now, there are no definitive biomarkers of OPLL that might be used to achieve a more convenient and economic way of diagnosis.

Aside from nonspecific genes that showed some correlation with OPLL, recent reports have found differentially expressed microRNAs (miRNAs) that are OPLL specific and may serve as potential biomarkers [3–5]. MiRNAs are a class of small noncoding RNAs that principally function in the spatiotemporal regulation of protein translation by binding to the 3′-untranslated region (UTR) of target mRNAs, leading to mRNA degradation or translational repression [6]. Recently, circulating miRNAs are thought to be promising novel biomarkers for cancer, development, and other diseases with high accuracy, predictability and relative stable existence in blood [7]. However, little attention has been devoted to the relationship between circulating miRNAs and OPLL.

Untill now, there are no definitive biomarkers of OPLL that might be used to achieve a more convenient and economic way of diagnosis. The purpose of the current study was to evaluate the accuracy and significance of OPLL-specific microRNAs in discriminating OPLL from normal and intervertebral disc degenerated (IDD) patients by detecting the microRNAs' plasma level.

Methods

Study approval

All experimental procedures were approved by the Second Military Medical University Chancellor's Human Research Committee in accordance with protocol 2015-0018. This study was carried out in strict accordance with the recommendations provided by the Guide.

Clinical samples

All of the clinical samples were obtained from the department of spinal surgery from February 2014 to November 2015. Informed consent was obtained from all subjects, and the subjects were all local residents from Han ethnicity. This project was approved by the institutional clinical research ethics committee. For sample collection, whole blood samples were collected from 68 diagnosed cervical OPLL inpatients, 45 diagnosed cervical intervertebral disc degeneration inpatients, and 53 outpatients with no radiological findings of spine diseases. All OPLL subjects were diagnosed by high-resolution computed tomography (CT), all IDD patients were diagnosed by both CT and magnetic resonance imaging (MRI) with no sign of ossification of the ligament, while first time outpatients with no sign of ossification or disc degeneration in plain lateral radiographs and MRI with/without CT were considered as the normal group. All blood samples were collected in the morning before medication, meal, and surgical intervention. Patients with abnormal clinical and laboratory test findings indicating systematic inflammation, diabetes mellitus, hyperparathyroidism, hypercortisolism, chronic renal disease, or other bone/mineral disorders were excluded. Clinical information like height, weight, body mass index (BMI), and presence of comorbidities such as hypertension, hyperlipidemia, and previous spinal injuries and treatment were recorded for all study participants and listed in Table 1.

Table 1
Characteristics of the population studied

		non-OPLL (n=98)		
Variables	OPLL (n=68)	non-IDD (n=53)	IDD (n=45)	p value
Age (year)	60.9 ± 6.2	55.1 ± 7.5	58.4 ± 8.3	NS
Height (cm)	163.1 ± 5.6	160.7 ± 7.8	165.4 ± 6.6	NS
Weight (kg)	68.9 ± 11.5	64.4 ± 12.8	69.5 ± 11.8	NS
BMI (kg/m^2)	25.0 ± 0.9	24.9 ± 1.1	25.4 ± 0.8	NS
Serum calcium (mg/dl)	9.3 ± 0.3	9.1 ± 0.2	8.9 ± 0.3	NS
Osteocalcin (ng/dl)	3.8 ± 1.9	3.7 ± 1.4	3.5 ± 1.6	NS
hypertension	22.1%	18.9%	17.8%	NS
hyperlipidemia	29.4%	15.1%	20.0%	NS

OPLL, ossification of posterior longitudinal ligament; IDD, intervertebral disc degenerated; NS, not significant.
Values are shown as mean ± SD or mean ± SD (range).
Statistical analysis was conducted using the unpaired Student *t* test. $p < 0.05$ was considered statistically significant.

Plasma and serum isolation

Blood samples of venous blood were collected from the patients into two tubes, one in Ethylene Diamine Tetra-acetic Acid (EDTA) coated tube for plasma isolation, and one in dry tube for serum isolation. All samples were subjected to centrifugation at 400×g for 10 minutes no longer than 4 hours after collection. Supernatant was transferred into 1.5 ml Eppendorf tubes for another centrifugation at 1,800×g for 10 minutes and the supernatant was aliquoted 200 μl each and stored at −80°C until use.

RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (PCR)

Before RNA isolation, a *C. elegans* miRNA-39 (cel-miR-39) was spiked into the serum/plasma samples with a final concentration of 0.2 nM and used as an external reference. Thereafter, 200 μl serum/plasma was subjected to RNA isolation using miRNeasy Serum/Plasma Kit (Qiagen, Venlo, Netherlands) according to the manufacture's protocol. Isolated RNA was diluted in a final volume of 10 μl in RNase-free water. The RNA integrity and concentration were determined using 2% agarose gel electrophoresis and a NanoDrop 2000c spectrophotometer (Thermo Fisher, Waltham, USA).

An equal volume of serum RNA from each participant was reversely transcribed using the PrimeScript RT Master Mix Kit (Takara, Beijing, China) according to the manufacturer's instruction. MiRNA specific primers for reverse transcription were added during cDNA synthesis. Subsequently, quantitative real-time PCR (qPCR) with SYBR Premix Ex Taq (Takara, Beijing, China) was performed on a Step One Plus thermocycler (Roche Diagnostics, Germany). The levels of miRNA in serum/plasma samples were normalized against the level of cel-miR-39 by $2^{-\Delta ct}$, where $\Delta ct = Ct_{target} - Ct_{reference}$. For absolute quantification, synthesized cel-miR-39 was used to generate the standard curve, and the copy number of each tested miRNA was calculated according to this standard curve. The primer sequences of the miRNAs used in the article are listed in Table S1 in the supplementary files.

Analysis of RNA sequencing data

For analysis of transcriptome sequencing data, we downloaded the existing GEO dataset GSE69787 from Pubmed for further analysis. The differential analysis was carried out using edgeR35, applying TMM (trimmed Mean of M-values) library normalization and a 0.05 false discovery rate (FDR) to select expressed transcripts. The scatter plot was drawn using BRB-Array Tools (https://brb.nci.nih.gov/BRB-ArrayTools).

Statistical analysis

The receiver operating characteristic (ROC) curve was plotted using SPSS version 21.0. The pROC package of R software (version 3.0.1) was used to evaluate the significance for the difference of area under the ROC curve (AUC). Youden index was used to generate the cutoff value for each miRNA. We used the Student's *t* test for analyses that compared two groups and used Kruskal–Wallis tests for analyses that compared more than two groups. We considered a p-value (two-sided) of lower than .05 to be statistically significant.

Results

General information of the patients

The clinical characteristics of patients enrolled (from February 2014 to November 2015) in this study are described in Table 1. There are 18 of 68 OPLL patients, 20 of 53 non-IDD patients and 8 of 45 IDD patients were female, each of them were diagnosed according to the radiological findings (Fig. 1a). For OPLL patients may have influence on the overall bone turnover, so we examined whether there is significant difference in serum calcium or osteocalcin level in each group. As shown in Table 1, we found no significant differences in these measurements between OPLL and IDD or non-IDD patients, indicating serum level of bone metabolism related products were not sensitive in discriminating OPLL. Considering other factors, none showed significance in OPLL patients.

Diagnostic miRNA candidate selection

In order to find OPLL diagnostic circulating RNA, we first tried to find a clue in differentially expressed miRNAs in ligament tissues. Using the high throughput miRNA sequencing data of OPLL and PLL (normal posterior longitudinal ligament) cells [5], we manually selected the 10 most differentially expressed miRNAs (five most expressed microRNAs in OPLL and five most expressed microRNAs in PLL were picked)in ligament cells (Fig. 1b).Of the 10 miRNAs we selected, miR-10a-3p, miR-10a-5p, miR-563, miR-210-3p, and miR-885-5p were highly upregulated in OPLL tissue, while miR-129-2-5p, miR-199b-5p, miR-196b-5p, miR-212-3p, and miR-218-1-3p were highly downregulated. Before subjected to further blood tests, we first validated their expression in eight OPLL and six non-OPLL ligament tissue using real-time PCR analysis and found that they are indeed differentially expressed (Fig. 1c). As other reports have shown that differentially expressed miRNAs can influence their relative level in one's blood, we hypothesized that they may have impact on the relative expression level in patients' blood.

To determine whether OPLL specific miRNAs can serve as potential biomarkers for diagnosing OPLL. We first verified miRNA existence in different blood samples. For blood samples can be prepared in several forms, here we examined the expression level of 5 OPLL highly expressed miRNAs between serum and EDTA prepared plasma, two major forms of blood samples used in clinical testing.

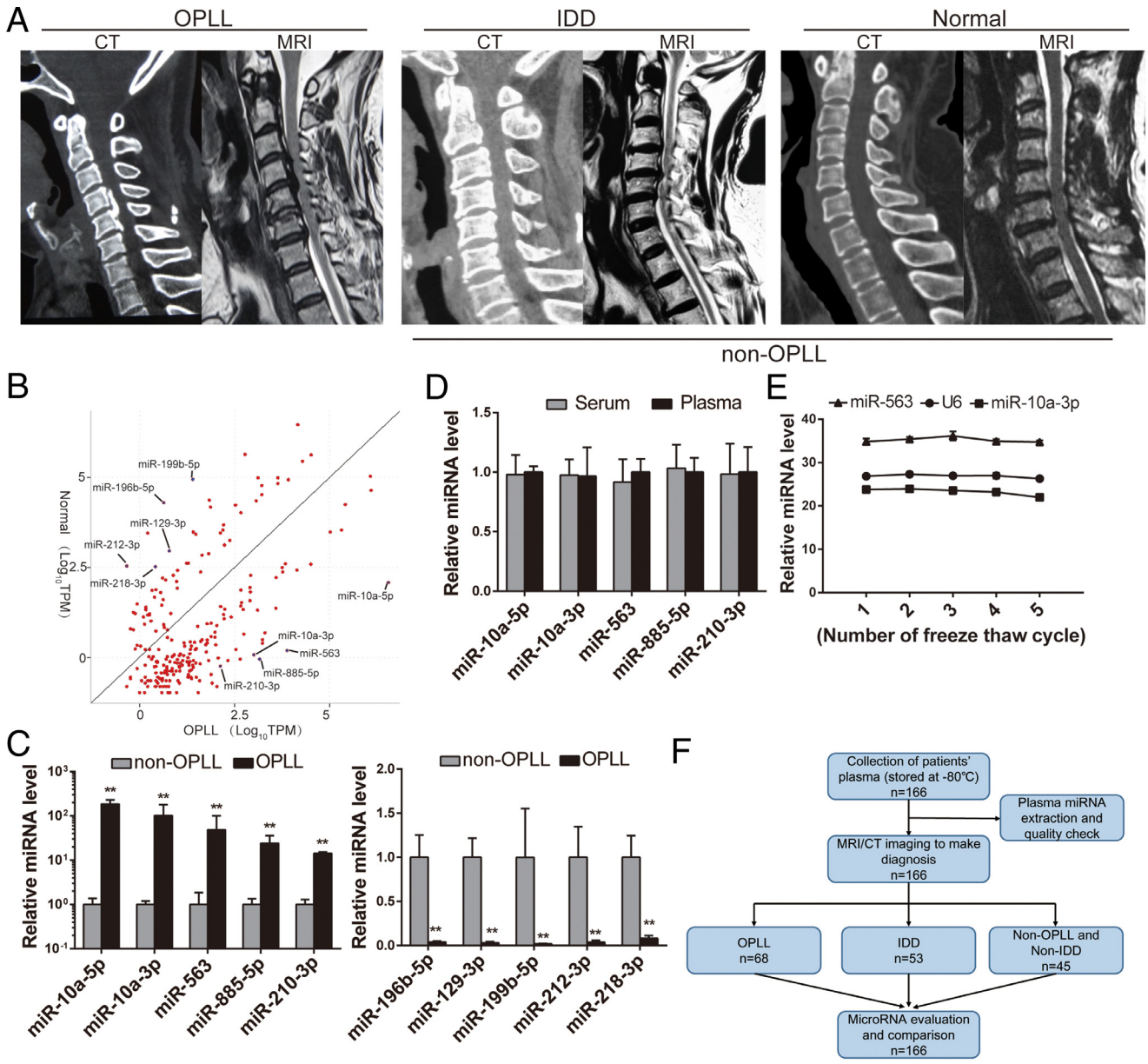

Fig. 1. (**A**) Representative radiological images of OPLL, IDD and Normal patients. (**B**) Using high through-put microRNA sequencing data, we selectively chose 5 highly upregulated and 5 highly down regulated microRNAs for further validation. (**C**) Real-time PCR validation of miRNA candidates in OPLL and normal tissue samples. (**D**) Evaluation of the abundant of candidate miRNAs in serum and plasma samples of 5 normal patients. (**E**) Analysis of miRNA level in multiple freeze-thaw cycle plasma samples to evaluate the stability of circulating miRNAs.(**F**) An STARD flow diagram depicts the study design and sample number. Data were shown as mean ± S.D. Student's *t* test is used in the comparison, and the p values were shown as *p < 0.05, **p < 0.01. (OPLL, ossification of posterior longitudinal ligament; IDD, intervertebral disc degenerated).

However, reports showed that heparin can exert inhibitory effects during cDNA synthesis [8], so we did not use plasma samples prepared using heparin in our study. By qPCR, we found that the expression of individual miRNA in serum and plasma derived from five normal patients' blood samples showed no significant difference (Fig. 1d), which indicated that circulating miRNAs are equally distributed in serum and plasma samples and that both could serve as viable forms of blood to be tested.

It has been reported that circulating miRNAs remained stable under certain harsh environment than other RNAs [9,10] so we treated plasma with multiple freeze-thaw cycles and tested and compared the expression variance of several miRNAs. Results showed that miR-563, miR-10a-3p along with U6 showed no significant expression decrease even after multiple freeze-thaws cycles (Fig. 1e), indicating that circulating miRNAs remained stable in the plasma under harsh environment.

Evaluating ossification of posterior longitudinal ligament diagnostic miRNAs in patients' blood

To evaluate the diagnostic value of these candidate miRNAs, we first examined and compared their expression levels in OPLL and non-OPLL (IDD and Normal groups) samples (Fig. 1f). As common reference genes are rarely stable in blood samples [11], we took advantage of the absolute quantification method of real-time PCR to analyze their expression. We compared their expression by acquiring the absolute copy number of each miRNA in 200 μl plasma sample that normalized to the level of cel-miR-39, a spiked-in reference gene to rule out miRNA loss during the miRNA extraction process. The expression levels of the 10 miRNA candidates measured between OPLL and non-OPLL are illustrated in Fig. 2. In all cases, the average level of miR-10a-3p, -10a-5p, -563 was significantly increased and miR-218-1-3p was significantly decreased in OPLL compared with the non-OPLL, which is consistent with their difference in the tissue samples. However, the average level of miR-210-3p was significantly decreased in OPLL compared with the non-OPLL, which showed a reverse trend compared with the expression changes in OPLL and non-OPLL tissue samples (Fig. 2).

Since IDD may cause partial calcification of the degenerated disc, and the presence of OPLL may affect the treatment of IDD, it is important to distinguish between OPLL and IDD. Thus, we evaluated the difference of these 10 miRNAs again between OPLL, IDD and normal groups. We found that although miR-10a-3p showed significance in distinguishing OPLL and non-OPLL ($p < .0001$), but it failed to identify OPLL from IDD patients ($p = .1874$, Fig. 3). For miR-218-1-3p, it showed significant difference in distinguishing OPLL and IDD ($p < .0001$), but failed in identifying Normal from OPLL patients ($p = .9368$). On the other hand, miR-10a-5p, -563 and -210-3p showed significance between OPLL and other groups ($p < .0001$), which makes them ideal for diagnosing OPLL. Although miR-196b-5p and -129-2-3p showed significance in identifying normal or IDD from OPLL patients ($p < .0001$), for they have failed in identify OPLL from IDD ($p = .5875$) and normal ($p = .3251$) patients respectively, we did not take them for further consideration (Fig. 3).

Determining the efficiency of OPLL diagnostic circulating miRNAs

Of the 10 miRNA candidates, we selected miR-10a-5p, -563 and -210-3p for further analysis as they showed significant difference in discriminating OPLL form IDD, normal and non-OPLL patients. We compared the sensitivity, specificity, accuracy, and receiver operating characteristic curve analysis (ROC) of each miRNA in diagnosing OPLL. We found that all three miRNAs had more than 60% of both sensitivity and specificity, with miR-10a-5p the highest (Table 2). In distinguishing OPLL and non-OPLL patients, the AUC of each miRNA reached over 0.80, which showed

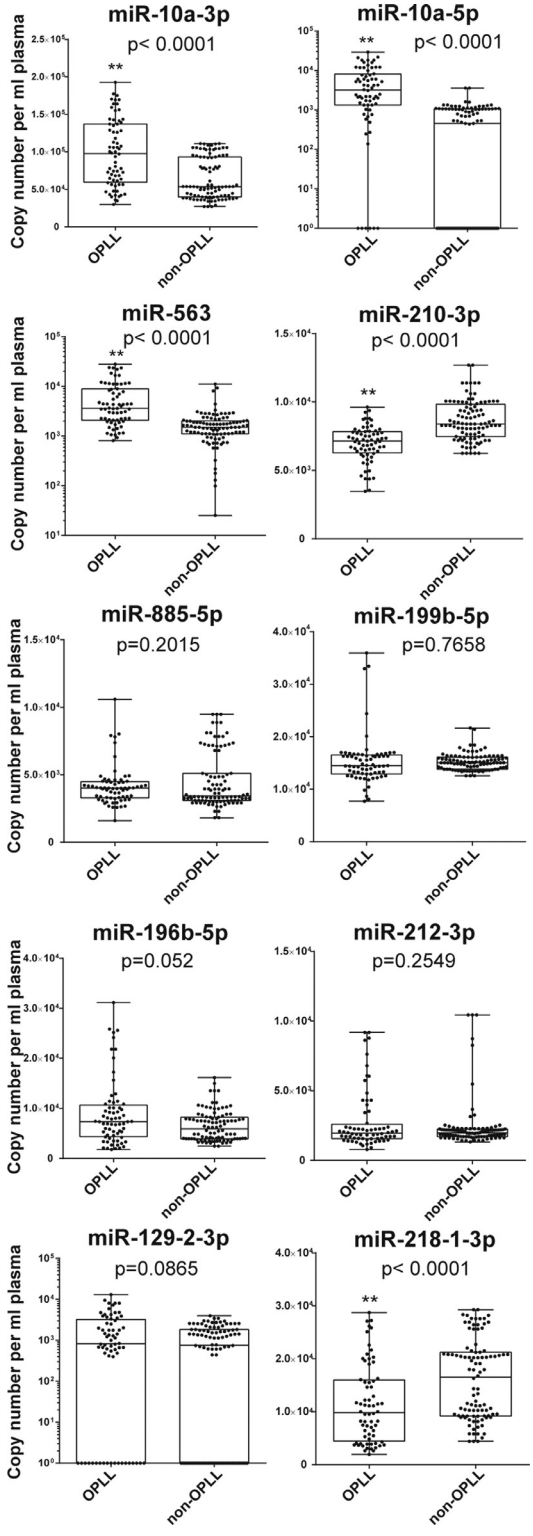

Fig. 2. Expression profiling of 10 OPLLspecific miRNAs in OPLL (n=68) and non-OPLL (n=98) patient plasma samples. OPLL refers to radiologically diagnosed OPLL patients, IDD refers to radiologically diagnosed disc degeneration patients with no sign of ossification in the posterior longitudinal ligament, and normal refers to radiologically normal patients. Data were shown as boxplot with whiskers from minimum to maximum, Student's *t* test was used to compare the significance, and the p values were labeled and shown as $*p < 0.05$, $**p < 0.01$. (OPLL, ossification of posterior longitudinal ligament; IDD, intervertebral disc degenerated).

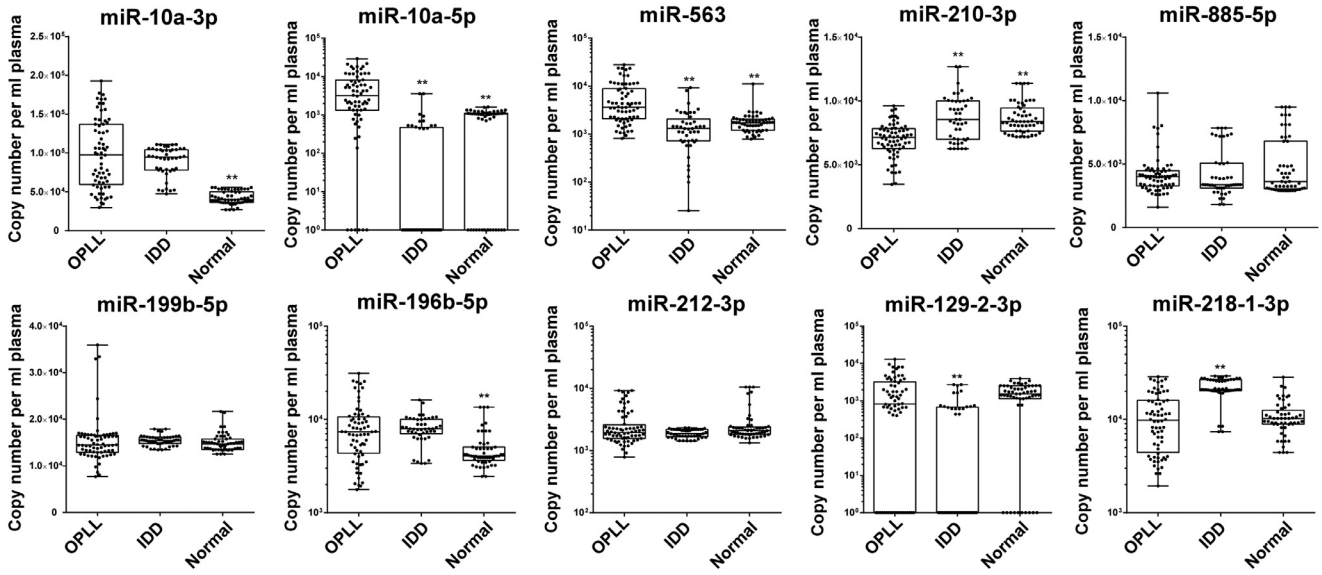

Fig. 3. Expression profiling of 10 OPLL-specific miRNAs in OPLL (n=68), IDD (n=45) and normal (n=53) patients' plasma samples. Data were shown as boxplot with whiskers from minimum to maximum, Kruskal–Wallis tests (one-way ANOVA) was used to compare the significance between groups, and multiple comparison was done to generate the p values for each group that compared to OPLL. The p values for comparison between OPLL and other groups were shown as *$p < 0.05$, **$p < 0.01$. (OPLL, ossification of posterior longitudinal ligament; IDD, intervertebral disc degenerated).

a good correlation with the disease (Fig. 4a). The combination of three miRNAs achieved an AUC of 0.945, a strong correlation, and diagnostic efficiency for OPLL. The same result of the ROC analysis is found in distinguishing OPLL from IDD patients (Fig. 4b), with miR-10a-5p again showing the highest AUC of 0.904 among the three OPLL diagnostic miRNAs. The combination of three miRNAs showed better diagnostic value than the individual miRNAs (AUC, 0.938). In analyzing the diagnostic power in discriminating OPLL and patients with no spine problems, these three miRNAs showed strong diagnostic value, with the combination achieving the highest AUC (0.950) of all (Fig. 4c). Together, these findings suggest miR-10a-5p, -563 and -210-3p a suitable circulating biomarker in diagnosing OPLL.

Discussion

In past decades, there have been major breakthroughs in detection technology of circulating biomarkers. In addition to the continued investigation about novel and unbiased serum biomarkers by different and innovative proteomics techniques, new concepts relating to circulating cells, circulating RNAs and circulating DNA have been introduced

Table 2
Performance of OPLL diagnostic circulating miRNAs

Test target	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
OPLL vs non-OPLL					
miR-10a-5p	76.5	95.9	92.9	85.5	88.0
miR-563	64.7	86.7	77.2	78.0	77.7
miR-210-3p	83.8	68.4	64.8	85.9	74.7
Combined	88.3	93.9	90.9	92.0	91.6
OPLL vs IDD					
miR-10a-5p	76.5	91.1	92.9	71.9	82.3
miR-563	64.7	75.6	80.0	58.6	69.0
miR-210-3p	83.8	62.2	77.0	71.8	75.2
Combined	91.2	91.1	93.9	87.2	91.2
OPLL vs non-IDD (Normal)					
miR-10a-5p	76.5	100.0	100.0	76.8	86.8
miR-563	64.7	96.2	95.7	68.0	78.5
miR-210-3p	83.8	73.6	80.3	78.0	79.3
Combined	88.3	96.2	96.8	86.4	91.7

OPLL, ossification of posterior longitudinal ligament; IDD, intervertebral disc degenerated.
Youden index obtained from the ROC curves were used to generate the cutoff values for each classifier to calculate the relative efficiency.

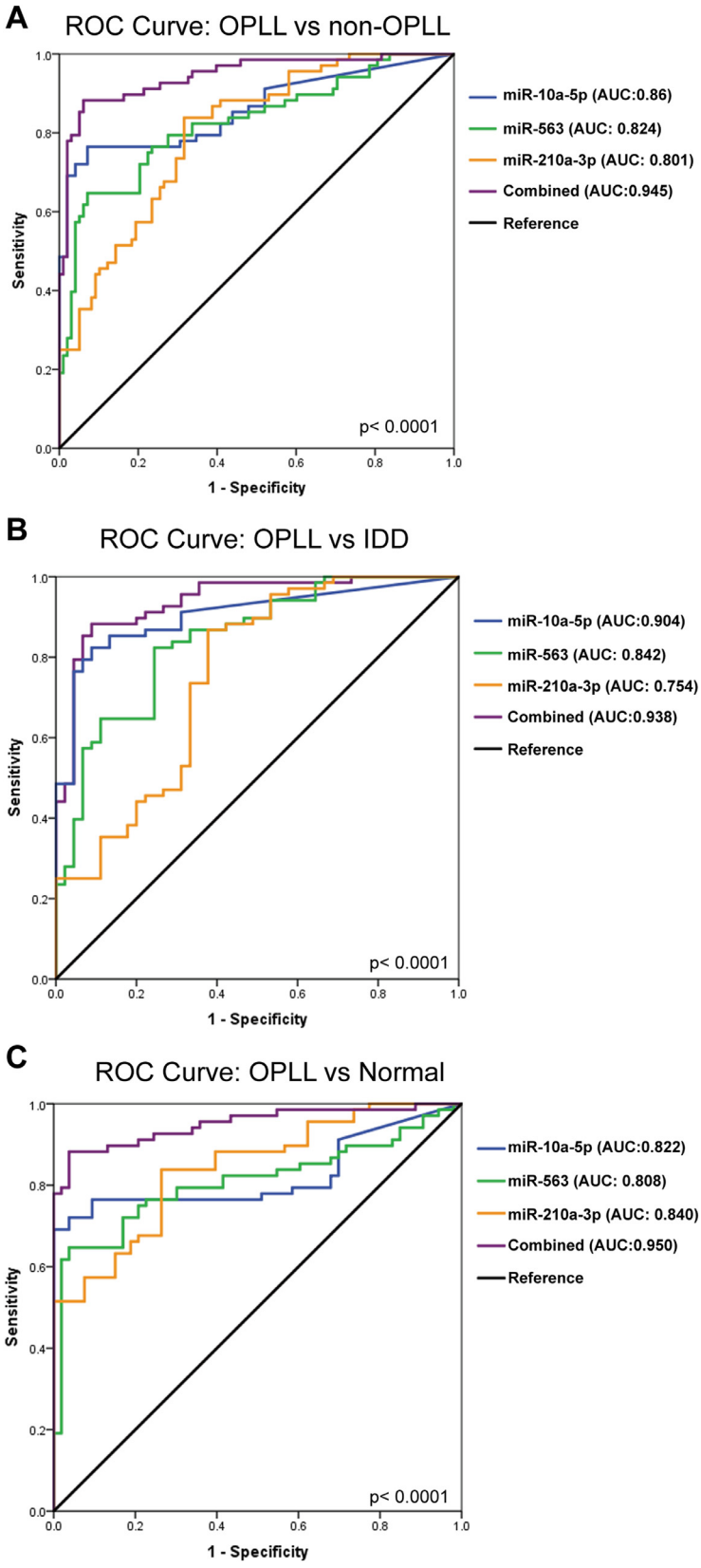

Fig. 4. Receiver operating characteristic curves obtained for miR-10a-5p, miR-210-3p and miR-563 in OPLL versus non-OPLL groups (**A**), OPLL vs IDD groups (**B**) and OPLL vs radiological normal groups (**C**). The combined values are obtained by logistic regression of three miRNAs. For p value of AUC analysis of each group were all lower than 0.0001, only one p value were shown in the figure. (AUC, area under the curve; OPLL, ossification of posterior longitudinal ligament; IDD, intervertebral disc degenerated).

[12–14]. Such novel biomarkers have been widely introduced to the field of cancer research, however, little is known in the field of spine-related diseases. Studies have been conducted to unveil the specific proteins that could be used as diagnostic tests or at least supportive biomarkers of OPLL [15,16]. However, great differences were found between each proteomic study, and few of these findings have further clinical data to demonstrate their diagnostic value [17,18].

Recently, the presence of extracellular or cell-free RNA in circulation has become a promising diagnostic and prognostic tool for all kinds of human diseases including cancers [19,20], osteogenesis imperfecta [21], heart failure [22], and other diseases. The most studied cell-free RNA is miRNA. Because of its widespread function and presence, miRNA has been found to be important to various biological and pathological processes [23]. Apart from its vital role in regulation of gene expression, studies have the found existence of stable circulating miRNAs [24]. Reports have shown that circulating miRNAs are biologically stable in the circulation partly due to its duplex structure and RISC (RNA-induced silencing complex) binding could prevent its degradation from certain type of RNAses [24]. Recent findings of circulating exosomes provided other explanations of how miRNA can be stable in the circulation [25]. To date, this is the first study of the characteristics of OPLL specific miRNAs in patient's blood, which provides novel and original data on the roles of circulating miRNAs in OPLL.

Methods for the quantification of serum/plasma RNAs have varied greatly between studies, among which quantitative real-time PCR has been widely used due to its efficiency and accessibility [11]. However, to achieve reliable and reproducible real-time PCR data, nonbiological variations resulting from technical inconsistencies should be corrected using an appropriate reference gene [26]. Although many genes have been evaluated to be suitable reference for real-time PCR analysis in blood samples, it is still hard to obtain a single universal reference gene for all diseases and ethnicities [27,28]. In this study, we analyzed the absolute levels of circulating miRNAs in the blood samples, which the copy number were generated referring to an exogenous reference miRNA, cel-miR-39. In this way, more precise data can be obtained and the copy number of these miRNAs can be further utilized in the clinical setting.

We initially showed that the expression level of miR-10a-5p, miR-10a-3p, miR-563, miR-210-3p, and miR-218-3p were significantly varied between OPLL and non-OPLL patients. However, other spinal diseases adjacent to the ligament, like intervertebral disc degeneration, may also cause its hypertrophy, a state that considered to be closely related to heterogeneous ossification of the posterior longitudinal ligament. Thus, it is important to examine whether these miRNAs can distinguish other spinal diseases like IDD. In our further exploration of these miRNAs in diagnosing OPLL specifically, we showed that miR-10a-5p, miR-563 and miR-210-3p were found to be specific to OPLL when compared with IDD or non-IDD (normal group), while miR-10a-3p and miR-218-3p showed significance only when compared with non-IDD or IDD group respectively. Although the data we presented showed promising specificity of miR-10a-5p, miR-563 and miR-210-3p in OPLL patients' blood samples, their specificity should be further investigated in other skeletal diseases like osteoarthritis.

Although we did not identify whether the changes in these circulating miRNAs are caused by cellular emission or other mechanism, it is noteworthy that of the three miRNA biomarker candidates investigated, miR-10a-5p and miR-210-3p have been reported to be related to the ossification process [29–31]. However, the underlying mechanism is largely unknown, which is worth investigating in the future study.

In analyzing the diagnostic power of each miRNA, we found that the accuracy of each individual miRNA did not reach beyond 90%, but the combined value achieved more than 91% accuracy in all sets. The ROC analysis also showed better diagnostic power of the combined value. These results indicate that the combining of relative miRNAs may well improve the specificity and may work as promising diagnostic markers for OPLL. However, we also found a considerable spread of miRNA values and these variations may be critical to selecting an underlying diagnostic marker. Since the sample numbers are relatively small (under 100 patients in each group), a larger number of patients are needed to further validate these markers and also to set up a normal value for non-OPLL patients. Nevertheless, our findings provide new markers that may have important clinical values in diagnosing of OPLL.

Author contributions statement

C.X., H.Z., H.W., Y.L., and W.Y. conceived and designed the experiments. C.X., H.Z., W.Z., and Y.C. performed the experiments. Y.C., W.Z., and H.Z. performed the patient sample collection. C.X., Y.L., W.Z., and X.S. analyzed the data. C.X. and H.W. drafted the manuscript. H.Z., Y.L., C.X., and W.Y. reviewed and edited the manuscript. All authors read and approved the final manuscript

Availability of data and materials

High throughput data of the microRNA sequencing profiles of OPLL and PLL were from the Gene Expression Omnibus database (GEO dataset) under the accession number of GSE69787. Shanghai NovelBio Bio-Pharm Technology Co., Ltd provided help with high throughput sequencing analyzing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.spinee.2018.10.008.

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