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RESEARCH ARTICLE

Circulating miR-215-5p and miR-642a-5p as potential biomarker for diagnosis of osteosarcoma in Mexican population

Lucero Monterde-Cruz[1] · Eric G. Ramírez-Salazar[2,3] · Genaro Rico-Martínez[1] · Luis M. Linares-González[1] · Roberto Guzmán-González[1] · Ernesto Delgado-Cedillo[1] · Eréndira Estrada-Villaseñor[1] · Margarita Valdés-Flores[1] · Rafael Velázquez-Cruz[2] · Alberto Hidalgo-Bravo[1]

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Abstract

Osteosarcoma is the most common malignant bone neoplasia affecting individuals in the second decade of life. The survival rate has not been improved during the last 25 years, in part because of the lack of specific markers. The microRNAs have been identified as important regulators of gene expression, experimental evidence suggests these molecules as key players in cancer development and progression. To identify miRNAs differentially expressed in serum from patients with osteosarcoma compared to healthy donors in Mexican population. Fifteen osteosarcoma patients and fifteen age and sex matched healthy individuals were recruited. Two pools of total RNA extracted from serum per study group were prepared and the miRNA expression profiles were analyzed through TaqMan Low Density Arrays. Validation was carried out through RT-qPCR using individual TaqMan assays for those miRNAs differentially expressed. Fifteen miRNAs were differentially expressed in osteosarcoma patients compared to healthy controls. Overexpression of miR-215-5p and miR-642a-5p was confirmed by validation through RT-qPCR. The expression analysis of miRNAs from serum in osteosarcoma patients revealed differential expression of miR-215-5p and miR-642a-5p. Both microRNAs are potential markers for osteosarcoma diagnosis.

Keywords Osteosarcoma · Serum · microRNAs · Target genes · Biomarkers

Introduction

Osteosarcoma (OS) is a primary malignant tumor of bone that is characterized by the production of osteoid or immature bone by the malignant cells. It is the most common malignant neoplasia affecting bone and usually is localized in the appendicular skeleton. Osteosarcoma has a bimodal age distribution, having the first peak during adolescence and the second peak in older adulthood. The first peak is in the 10–14-year-old age group, coinciding with the pubertal growth spurt. This suggests a close relationship between the adolescent growth spurt and osteosarcoma. The second osteosarcoma peak is in adults older than 65 years of age; it is more likely to represent a second malignancy, frequently related to Paget's disease [1, 2]. The OS global incidence is 0.2–0.3/100,000 cases per year which increases to 0.8–1.1/100,000 cases per year in people between 15 and 19 years old. Osteosarcoma cases represent more than 10% of all solid tumors in adolescents [3–5]. The incidence in the USA is 900 new cases per year [6]. In Mexico, the OS represents 5.2% of all types of cancer in childhood and

L. Monterde-Cruz and Eric G. Ramírez-Salazar first authors.

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✉ Rafael Velázquez-Cruz
rvelazquez@inmegen.gob.mx

✉ Alberto Hidalgo-Bravo
dr_genetica@yahoo.com

[1] Department of Genetics, National Institute of Rehabilitation, Calzada Mexico-Xochimilco 289, Arenal de Guadalupe, 14389 Mexico City, Mexico

[2] Genomics of Bone Metabolism Laboratory, National Institute of Genomic Medicine (INMEGEN), Periferico Sur 4809, Arenal Tepepan, 14610 Mexico City, Mexico

[3] CONACYT-National Institute of Genomic Medicine (INMEGEN), Periferico Sur 4809, Arenal Tepepan, 14610 Mexico City, Mexico

adolescence. The incidence in Mexico is 0.6–0.8/100,000 per year, according to the National Ministry of Health [7, 8]. OS is a localized disease in 80% of cases, the most frequently affected locations are the distal femur, proximal tibia, and the proximal humerus. Clinical presentation includes a palpable fixed tumor accompanied with swelling of the affected area. Pain begins insidiously and afterwards becomes constant, which could be erroneously associated with physical trauma; joints can also be affected. Approximately 35% of patients develop metastasis when OS is not diagnosed early, the lung is the main localization for metastasis, despite the chemotherapy [9]. Clinical data and imaging studies are helpful in establishing clinical suspicion. OS typically appears as a mixed radiodense and lytic lesion arising in an eccentric manner from the metaphyseal bone [4]. The biopsy is the gold standard for confirming the diagnosis. According to the histological features, OS can be classified as high-grade (80–90% of all OS) and low grade. The current management of OS includes neoadjuvant chemotherapy and surgical resection. The first line of chemotherapy includes cisplatin, doxorubicin, methotrexate and ifosfamide [3]. After the neoadjuvant chemotherapy and surgery, the percentage of tumor necrosis is assessed. An excellent response is considered when the percentage of necrosis is above 95%. According to the percentage of necrosis, additional cycles of chemotherapy could be prescribed. One of the reasons for a late diagnosis is the lack of specific markers for OS. Current research has been focused in finding molecules with the ability to reveal the tumorigenic process at early stages. Development of high-throughput genomic technologies have made possible to increase the knowledge about the underlying molecular mechanisms of several diseases [10]. microRNAs (miRNAs) are small non-coding RNA molecules compound by 22–24 nucleotides. The function of miRNAs is to regulate gene expression in virtually all cellular processes. To exert their function on gene expression, miRNAs are able to promote mRNA degradation or block its translation by pairing to the 3′UTR of the mRNA. In addition, miRNAs are present in different body fluids (plasma, serum, urine, tears, seminal fluid), this property makes them potential and attractive biomarkers. miRNAs have been identified as key factors in the development and progression of numerous diseases, including cancer [11]. Changes in miRNAs expression cause loss of control of critical biological pathways, leading to oncogenesis, metastasis, angiogenesis and drug resistance [12]. The knowledge about miRNAs' target genes allows the identification of metabolic pathways involved in cellular transformation. The data obtained has been useful to unravel interaction networks behind cancer biology. Previous studies have investigated the expression of miRNAs in serum from OS patients aiming to identify those miRNAs able to identify affected individuals [13–15]. Although some molecules seem promising, results are not constant among populations; therefore, more patients need to be analyzed to find the more useful marker. In the present study, we analyzed the miRNAs expression pattern in serum from Mexican patients with osteosarcoma compared to healthy individuals. To the best of our knowledge, this is the first study carried out in Mexican population.

Materials and methods

Patients with primary osteosarcoma, treated at the Department of Bone Tumors in the National Institute of Rehabilitation (NIR), Mexico during the period of time from February 2015 to April 2017 were recruited. All individuals were from Mexican origin with clinical and histological diagnosis of primary osteosarcoma. Age and sex matched healthy individuals were recruited as controls. The research protocol was approved by the Institutional Ethics Committee and all the participants signed and informed consent form. When the patient was a minor, informed consent form was signed by the parents of the legally authorized representative.

RNA isolation

5 ml of peripheral blood were obtained from each participant using a syringe and a SST tube (BD Vacutainer®), the day before surgery. Serum was separated by centrifugation at 5000*g* for 10 min. All blood samples were processed within the first 30 min after collection. Serum was stored in 1.5 ml tubes at − 80 °C. miRNAs were enriched from 200 µl of serum using the miRNeasy Serum/Plasma kit (QIAGEN) according to the manufacturer's instructions. RNA was quantified using a NanoDrop spectrophotometer (ThermoFisher, Inc.). Typically, the yield was 20–50 ng/µl of small RNA.

MicroRNA profiling

We classified the samples according to age of individuals into < 18 and > 18 years old and with respect to presence of disease into osteosarcoma and control samples. We included five samples in each pool of RNA. The pools were as follows: minors with OS, adults with OS, minors control and adults control. Quantitative global profiling of serum miRNAs was performed using the TaqMan Array Human MicroRNA Panel v2.0 (Applied Biosystems), which includes Cards A and B in a 384-well format. Card A contains 384 TaqMan MicroRNA Assays enabling the simultaneous quantitation of 377 human mature miRNAs plus four endogenous controls. Card B contains assays for 290 human mature miRNAs plus seven endogenous controls. Cards A and B were prepared according to the manufacturer's instructions. Briefly, 3 µl of RNA of each pool

were reverse transcribed using the Megaplex RT primer pools A and B (Applied Biosystems). Subsequently, Megaplex RT products were pre-amplified using Megaplex PreAmp Primers (pools A and B) and TaqMan PreAmp Master Mix (Applied Biosystems). The pre-amplification cycling conditions were 95 °C for 10 min, 55 °C for 2 min and 75 °C for 2 min followed by 12 cycles of 95 °C for 15 s and 60 °C for 4 min. The pre-amplified cDNA was diluted 1:8 with distilled water and then dispensed into the 384 wells by centrifugation according to manufacturer's instructions. The real-time PCR cycling parameters were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and at 60 °C for 1 min. All cards were analyzed in a QuantStudio 7 instrument (Applied Biosystems). Only miRNAs with a raw Cq value below 35 were considered for expression analysis. The fold change was calculated with the method $2^{-\Delta\Delta CT}$. The global mean expression value of all miRNAs was used as the normalization factor [16].

RT-qPCR validation analysis

3 µl of total RNA were used as template for reverse transcription performed with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). After completion, each reverse transcription reaction was diluted to 100 µl with RNase-free water, and 5 µl were used as template to analyze the expression profile for miR-215-5p, miR-320a, miR-376c-3p, miR-642a-5p, miR-99b-5p and miR-655-3p using TaqMan microRNA assays (Assay ID. 000518, 002277, 002122, 001592, 000436 and 001612, respectively, Applied Biosystems). qPCR was performed on an Applied Biosystems QuantStudio 7 Flex system (Applied Biosystems; Foster City CA, USA). The expression levels were normalized with respect to RNU6 (Applied Biosystems, Foster City CA, USA). All the qPCR analyzes were done by triplicate in each one of the samples. The relative quantification (RQ) of the miRNAs of each sample was determined by the $2^{-\Delta\Delta Ct}$ method, where the $\Delta\Delta Ct = [Ct\ miRNA\ of\ interest - Ct\ U6] - [\Delta Ct\ global\ mean]$.

Statistical analysis

The differential expression of miRNAs between study groups was assessed using the Expression Suite Software v1.0.3 (Life Technologies) with a p value ≤ 0.05 and a cutoff value for fold-change ≥ 1.5 or ≤ 1.5. For the RT-qPCR analysis, we used Mann–Whitney test to compare fold changes of osteosarcoma patients against healthy controls using GraphPad Prism 7 software. Outliers for each miRNA were detected and eliminated using the ROUT method built in the GraphPad Prism 7 software.

Classification analysis

ROC-curves and corresponding area under the curve (AUC) estimates were performed for individual miRNAs at 95% confidence intervals for AUC-values.

Target prediction and gene set enrichment analysis

Target prediction was performed using the publicly available database miRSystem (http://mirsystem.cgm.ntu.edu.tw/) [17]. miRSystem includes seven algorithms that predict miRNA targets: DIANA-microT, miRanda, mirBridge, PicTar, PITA, rna22, and TargetScan and it also includes two experimentally validated databases: TarBase and miRecords. The targets predicted by at least 3 databases were included for the analysis. Analysis for pathway enrichment was carried out using the miRNet database (http://www.mirnet.ca/) [18]. miRNet utilizes two curated pathway databases (Kyoto Encyclopedia of Genes and Genomes [KEGG] and Reactome) to obtain the enrichment pathways. Additionally, network interactions were built. Pathways that were significantly enriched by the predicted targets (p value ≤ 0.001) were selected for further network analysis.

Results

Clinical data

We collected serum from 15 osteosarcoma patients and 15 sex and age matched healthy donors. The OS patients were nine males and six females, with a sex ratio 1.5:1. According to the place of origin, two were from the north of Mexico, seven from the center, five from the east and one from the south. The age range was from 10 to 36 years old, with a mean age of 20 years. The most constant symptom was pain (93.3%), some patients attributed the pain to physical trauma. The most common localization was the distal femur (53.3%) followed by proximal tibia (13.3%). Right side body was most affected. Enneking classification is used to cluster musculoskeletal sarcomas, to decide the surgical treatment and to stablish the prognosis. According to this classification two patients were in stage IIA, two in stage IIB and the rest in stage III, this late stage implies poor prognosis. Osteoblastic osteosarcoma was found in six patients (three of them presented other components in addition to the osteoblastic component). Clinical features are summarized in Table 1 and table A (Online Resource 1).

miRNA expression analysis

To identify the microRNAs differentially expressed in serum from osteosarcoma patients we perform TaqMan

Table 1 Clinical features of osteosarcoma patients

Variable	Osteosarcoma ($n=15$)	
	No.	%
Age (years)		
< 18	8	53.3
> 18	7	46.7
Sex		
Male	9	60
Female	6	40
Localization		
Distal femur	8	53.3
Distal femur and proximal tibia	1	6.7
Proximal tibia	2	13.3
Distal tibia	1	6.7
Proximal humerus	2	13.3
Fibula	1	6.7
Subtype OS		
Osteoblastic	3	20
Osteoblastic with another component	3	20
Other	9	60
Metastasis		
Yes	13	86.7
No	2	13.3

Low Density Arrays (TLDAs) using serum from healthy donors as control. We considered microRNAs with fold change > 1.5 or < 1.5 and p value < 0.05 as statistically significant. Volcano plots of cards A and B are shown in Fig. 1. The list of miRNAs with differential expression is displayed in Table 2. In silico analysis using miRSystem was performed to find the cell cycle and cancer-related target genes of the miRNAs with differential expression (Table B, Online Resource 1). Based on their targets, six miRNAs were selected for validation through individual TaqMan MicroRNA Assays in all participants. The selected miRNAs were miR-99b-5p, miR-215-5p-5p, miR-320a, miR-376c-3p, miR-642a-5p and miR-655-3p.

Real time RT-PCR validation

We were able to identify statistically significant overexpression for miR-215-5p and miR-642a-5p in osteosarcoma serum samples ($p=0.0051$ and 0.0229, respectively). The miR-320a had a borderline p value of 0.0592, however, we did not consider it as statistically significant. The other three miRNAs, miR-99b-5p, miR-376c-3p and miR-655-3p did not have a significant p value (0.1139, 0.7984, and > 0.9999, respectively) (Fig. 2).

Receiver operating characteristic (ROC) curve analyses of miR-642a-5p and miR-215-5p were further performed to evaluate their discriminatory value between osteosarcoma and healthy individuals. Area under ROC curve of miR-642a-5p and miR-215-5p were > 0.8 (Fig. 3a, b), indicating that both miRNAs could be potential biomarkers for classifying osteosarcoma patients. Multivariable combination of miR-642a-5p and miR-215-5p was generated, demonstrating good values for AUC when both miRNAs are evaluated as a panel (Fig. 3c).

Enrichment analysis

We constructed interplay with genes and metabolic pathways related with the two miRNAs validated through real-time PCR using miRNet (Fig. 4). We found 21 pathways with a p value < 0.001. We observed pathways that have been related to cancer such as signaling pathways regulating pluripotency, cell cycle, colorectal cancer, transcriptional misregulation in cancer, pathways in cancer, p53 signaling pathway, Wnt signaling pathway (Table 3) (http://www.mirnet.ca) [18].

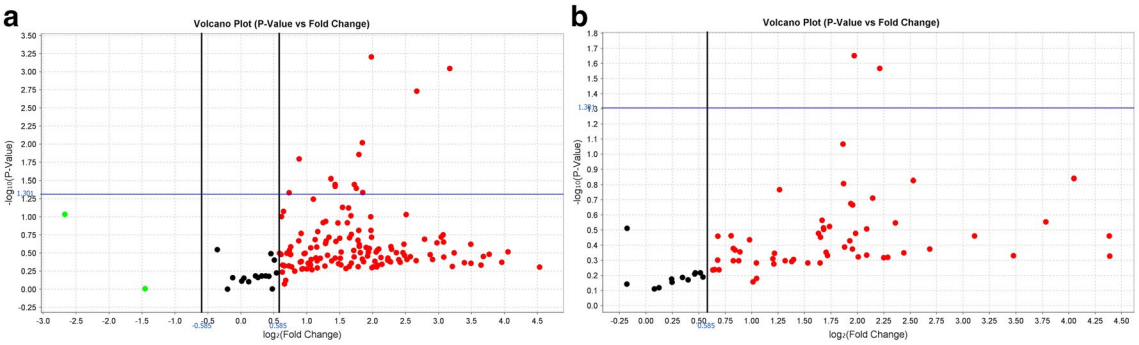

Fig. 1 Volcano plot showing the expression analysis of miRNAs from serum of osteosarcoma patients (OS) compared to healthy individuals through TaqMan Array Human MicroRNA Panel V2.0 (TLDA). TLDA includes Card A and B. Card A contains 384 probes (**a**) and card B contains 297 probes (**b**). miRNAs displaying a $\log_2$ fold change ≥ 1.5 were considered as differentially expressed

Table 2 Differentially expressed microRNAs in serum of osteosarcoma patients

miRNAs differentially expression	*p* value	Fold change
hsa-miR-99b-5p	0.03	2.5
hsa-miR-126-3p	0.036	2.6
hsa-miR-150-5p	0.016	1.8
hsa-miR-215-5p	0.04	3.3
hsa-miR-224-5p	0.002	6.3
hsa-miR-320a	0.046	3.6
hsa-miR-324-5p	0.01	3.58
hsa-miR-376c-3p	0.001	9.0
hsa-miR-454-3p	0.001	3.9
hsa-miR-576-3p	0.038	2.6
hsa-miR-584-5p	0.022	3.9
hsa-miR-628-5p	0.036	3.3
hsa-miR-642a-5p	0.014	3.4
hsa-miR-655-3p	0.047	1.6
hsa-miR-720	0.027	4.6

Discussion

Osteosarcoma is the most common malignant neoplasia affecting bone. Current treatment includes chemotherapy and surgery; however, the survival rate has not been improved in the last 25 years. One of the main reasons for this is the scarcity of non-invasive biomarkers. In recent years, miRNAs have been extensively investigated as non-invasive biomarkers in several diseases. Its presence in biological fluids makes them promising candidates. Here we present the clinical features and analysis of miRNAs expression profiles of 15 individuals diagnosed with osteosarcoma from Mexican population. 73% of them were classified as stage III of Enneking's. This high proportion of patients in the most advanced stage could be explained by a late diagnosis (mean diagnosis in this series 5.49 months).

Our expression analysis of the miRNAs was able to identify 15 differentially expressed. After validation through RT-qPCR in all subjects individually, miR-215-5p and miR-642a-5p showed a clear overexpression in patients compared to controls. However, miR-320a, miR-99b-5p, miR-376-3p and miR-655-3p did not have a significant *p* value, therefore, overexpression in osteosarcoma could not be validated in

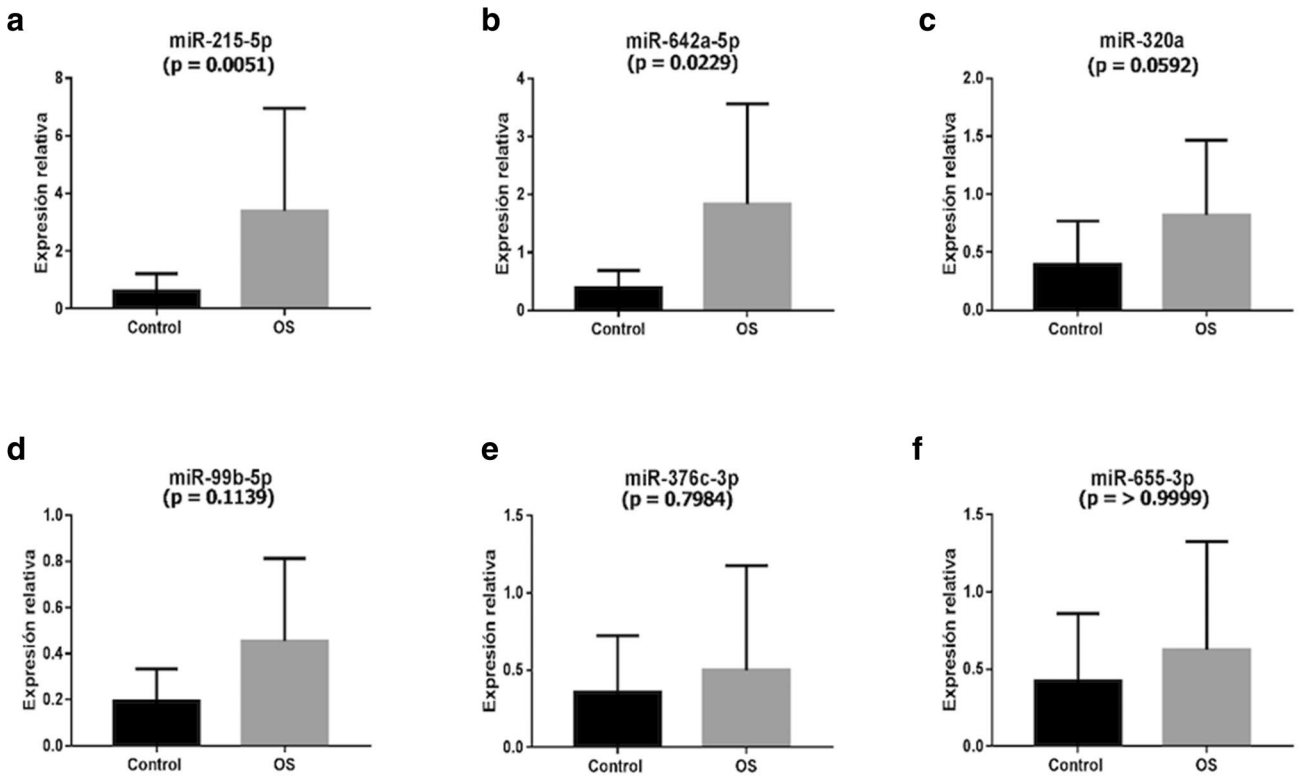

Fig. 2 Relative expression of the six validated miRNAs. Six miRNAs were validated through TaqMan MicroRNA Assays in all participants. Differential expression of **a** miR-215-5p, **b** miR-642a-5p was validated while **c** miR-320a, **d** miR-99b-5p, **e** mir-376c-3p and **f** miR-655-3p did not show statistically significant expression after validation. Graph presents mean and standard deviations

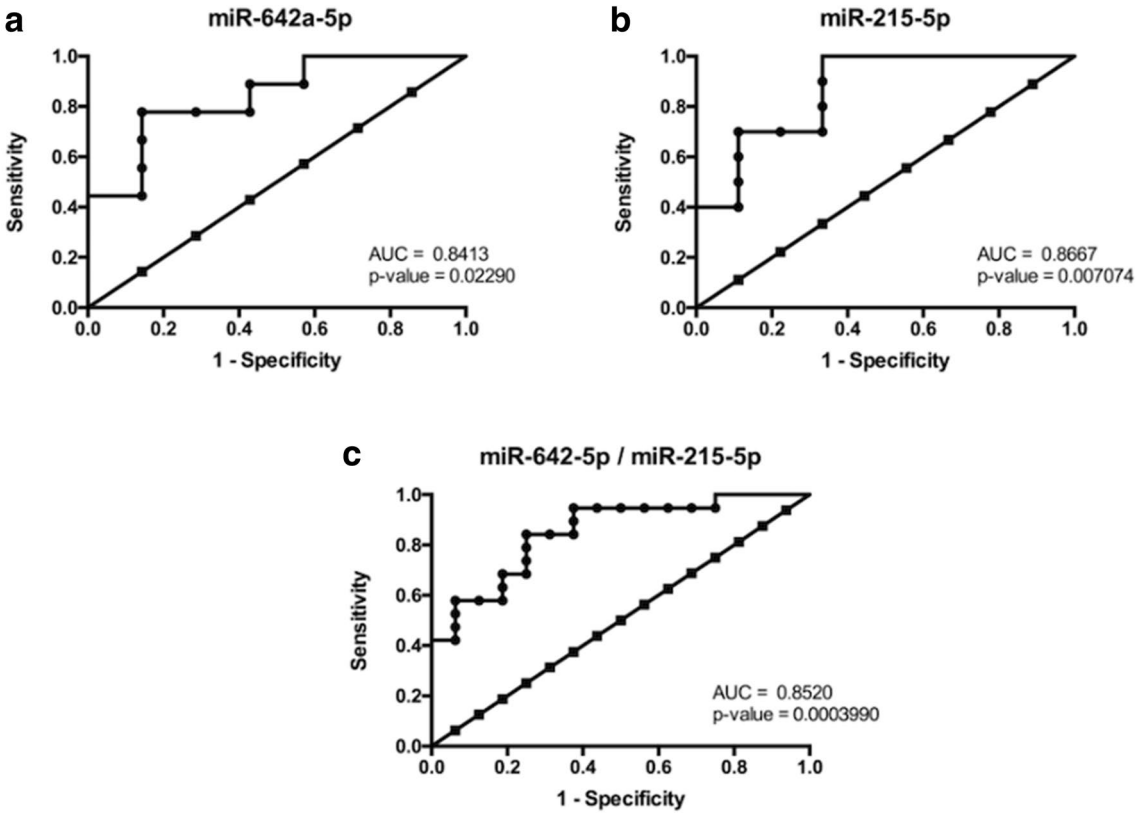

Fig. 3 Diagnostic value of miRNAs identified for osteosarcoma. Receiving Operating curve (AUC) for miR-642a-5p (**a**) and miR-215-5p (**b**) and the multivariable combination of both microRNAs (**c**) are shown. Area under the curve (AUC) and the corresponding *p* values are indicated for each ROC curve

this series of patients. In the case of miR-320a, which exhibited a borderline *p* value, it is possible that the sample size could be influencing the inability for its validation.

The two miRNAs statistically significant overexpressed have been previously reported as important participants in different cancers. miR-215-5p is upregulated in different cancers such as gastric cancer and glioma. In those tumors, miR-215-5p promotes cancer progression through targeting the 3′UTR of the *RB1* transcript [19, 20]. *RB1* is one of the genes most constantly mutated genes in osteosarcoma tissues [21]. Studies in cell lines derived from osteosarcoma and colon cancer have demonstrated that overexpression of miR-215-5p is associated with resistance to chemotherapy, especially to methotrexate. This effect seems to be related to reduction of the cell proliferation rates due to cell-cycle arrest. Previous work has evidenced that slowly proliferating cancer stem cells are more resistant to chemotherapeutic drugs [22]. The patients presented here were treated with a multidrug scheme, which always included methotrexate. Clinical assessment after chemotherapy revealed poor response in all the patients. miR-215-5p could be a very helpful resource to plan the chemotherapy in OS patients. To the best of our knowledge, regarding cancer biomarkers, serum levels of miR-215-5p have only been proposed as a potential biomarker for hepatocellular carcinoma [23]. Known targets for miR-215-5p include *RAB2A, RB1, BLCAP, CCNT2* making it an attractive candidate for further investigations.

In the same sense, we were able to identify differential expression for miR-642a-5p. This miRNA has been identified as down regulated in bladder cancer cell lines and it is related with reduced cell viability [24]. Clinical samples from gastric cancer patients displayed overexpression of miR-642a-5p supporting the oncogenic potential of this miRNA [25]. Previous work using ovarian cancer cell lines showed that overexpression of miR-642a-5p was related to Cisplatin resistance. Some of the proposed targets for miR-642a-5p are also in the MAPK and TGF-beta signaling pathways [26].

In conclusion, the mechanisms that regulate the biological behavior of tumors have not been fully elucidated yet. Expression analysis of miRNAs is a powerful approach to identify genes and metabolic pathways involved in tumor progression. The identification of novel osteosarcoma biomarkers is necessary to reduce the delay in diagnosis and provide opportune treatment to patients. Our study

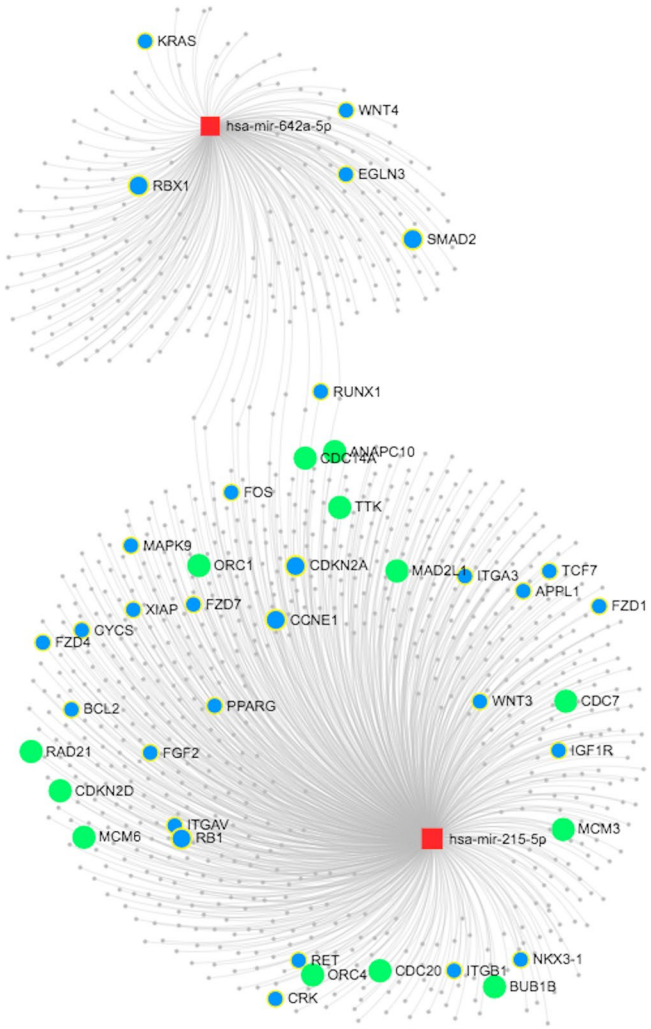

Fig. 4 Interplay with genes and metabolic pathways related with miR-215-5p and miR-642a-5p, which differential expression was validated through real-time PCR in serum of osteosarcoma patients. Signaling pathways in cell cycle (green) and pathways in cancer (blue) showed a significant enrichment

Table 3 Pathways enrichment analysis of microRNAs validated by RT-qPCR

Pathways	*p* value
Cell cycle	0.0133
Pathways in cancer	0.0935

suggests that miR-215-5p and miR-642a-5p could be in the list of potential and useful markers. It is important to mention that miR-215 has been previously related with methotrexate resistance in osteosarcoma. Further investigations in new cohorts of patients are needed to corroborate these findings. The findings observed in this group of patients contribute to understand the molecular mechanisms that are involved in malignant transformation, pathophysiology and the aggressive behavior of osteosarcoma.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were approved by the Institutional Ethics Committee. Personal data of the participants were protected.

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