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ORIGINAL ARTICLE

Downregulated Adhesion-Associated microRNAs as Prognostic Predictors in Childhood Osteosarcoma

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Abstract miRNAs have been identified as key regulators of almost all cellular processes, therefore, their dysregulation is involved with several diseases, including cancer. miRNAs specifically related to the metastatic cascade are called metastamiRs and can be involved with different steps of this process, including loss of adhesion. Osteosarcoma (OS) is the most common primary malignant pediatric bone tumor that often presents metastatic disease at diagnosis; therefore, a deeper study of adhesion-associated miRNAs could shed light on its pathophysiology. Online databases were used to select four miRNAs (miR-139; miR-181b; miR-584; miR-708) predicted or validated to target proteins related to adherent junctions and focal adhesion pathways, and their expression levels and possible associations with clinical features evaluated in primary OS samples. Our results showed downregulation of miR-139-5p and miR-708-5p in OS samples compared to non-neoplastic controls. Moreover, lower expression of miR-708-5p was associated with poor overall survival and higher expression of miR-181b-5p related to worst chemotherapy response (low HUVOS level). Based on these results, we selected miR-139-5p and miR-708-5p for further functional testing. Inducing the expression of miR-139-5p diminished the clonogenic capacity of the HOS cell line, while upregulation of miR-708-5p was related to a lower cellular adhesion. In summary, this work identified new signatures of microRNA dysregulation that may serve as useful prognostic markers in this aggressive pediatric bone tumor.

Keywords microRNAs · Adhesion · Osteosarcoma · Metastasis

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Introduction

Since the discovery of miRNAs, cancer has been the most prominent of human diseases with a clear role for their dysregulation. With almost 2000 miRNAs identified in humans to date, many researchers have functionally categorized these molecules according to the cellular processes they regulate, adopting terms such as hypoximiRs, inflammamiRs and apoptomiRs [1–3]. Experimental approaches have also shown that many miRNAs act as oncogenes (oncomiRs) promoting tumor growth and progression [4]. More recently, Hurst and colleagues (2009) referred metastamiRs to those miRNAs associated with metastasis [5].

Loss of anchoring is one of the first steps of the orchestrated metastasis cascade. Several well-established metastamiRs have been linked to cellular adhesion and extracellular matrix (ECM) remodeling, including miR-29c and miR-335, which induce primary tumor cell detachment [6, 7]. Others like miR-200 or miR-205 are associated with the regulation of the epithelial to mesenchymal transition [8]. Acquisition of motility and invasive capacities are next steps for metastasis, and cytoskeleton control by miR-10b, miR-21 and miR-146 is a determinant factor [9–12].

Finally, some metastamiRs are responsible for proliferation of tumor cells in distant organs, such as miR-222 or let-7 [13–18].

Osteosarcoma is one of the most common tumors of children and early adulthood, and despite good initial prognosis, the overall survival in metastatic cases is less than 20% [19]. Several studies have demonstrated the importance of microRNAs in OS pathogenesis [20, 21], describing tumor suppressors such as miR-124, miR-133 or miR-145 [22–25] and oncomiRs, such as miR-17-92 cluster, miR-603 or miR-9 [26–29]. Also, microRNA signatures and their association with clinical features of OS patients have been described [30–33]. Among those associated with metastatic process are miR-144 and miR-217, both related to cytoskeleton control, [34, 35] and miR-195 that targets CCND1 [36]. However, few studies present a comprehensive analysis of dysregulated microRNAs in specific pathways, as Poos and colleagues, who showed the relation of microRNAs and transcription factors [37], or the present work, which shows the role of some microRNAs in cellular adhesion.

For the present study MiRWalk and KEEG Pathway Databases were used to select four miRNAs (miR-139; miR-181b; miR-584; miR-708) with 180 common targeted proteins (Fig. 1), predicted or validated to target proteins related to the adherent junctions (cell to cell attachment) and the focal adhesion (cell to ECM attachment) pathways, consequently, important for the initial steps of the metastatic process (Fig. 2).

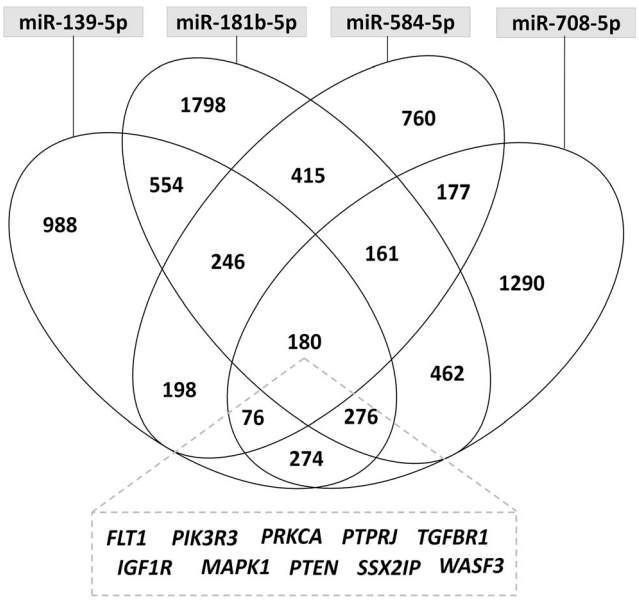

Fig. 1 Venn Diagram indicating common proteins targeted by miR-139-5p, miR-181b-5p, miR-584-5p, miR-708-5p. These proteins were indicated as predicted and validated targets through miRWalk analysis including the following databases: miRWalk, miRDB, PITA, RNA22, miRanda, RNAhybrid, PICTAR2 and Targetscan. considering a minimum seed length of seven at 3'UTR region and a *p*-value of 0.05. Common genes targeted by the four miRs simultaneously are designated in the grey box: proteins related to Adherent Junction ($p = 0.001$) and Focal Adhesion ($p = 0.053$) pathways

None of these miRNA dysregulations were described in OS tumor samples before, except for miR-181b [31]. Thereby, the expression levels of these microRNAs were evaluated in pediatric OS samples compared to non-neoplastic bone tissue, and their roles on tumor development and progression tested in in vitro models.

Material and Methods

Tissue Samples Biopsy samples of Osteosarcoma were obtained from 24 patients at the Clinics University Hospital (Ribeirao Preto School of Medicine – University of Sao Paulo), operated between 2006 and 2016. None of the patient received chemotherapy or radiotherapy prior to biopsy. The patients corresponded to 14 women and 15 men with a mean age at diagnosis of 15 years (range: 5–26 years old) - all patients were included in the first peak of osteosarcoma incidence and were considered pediatric cases. Twenty-one patients presented metastasis, and 12 had relapse (Table 1). Twelve aged-matched non-neoplastic bone tissues were collected to be used as control. These control samples were obtained from resection margin of osteochondroma ($n = 7$), condroblastoma ($n = 1$), cyst removal ($n = 2$), or bone fracture surgeries (n = 2) performed at the same institution. The mean age of these patients was 15,58 (range: 7–27 years old). This study was approved by the local Ethics Committee that follows the Helsinki convention criteria. Signed statement of informed consent was obtained from each patient (n° 43,619,215.9.0000.5407). Additional information about patient's data is summarized on Online Resourse 2.

miRNA Quantification by qRT-PCR Frozen tumor tissue samples were macrodissected by a trained pathologist. Total RNA was extracted using TRIzol Reagent (Invitrogen, Karlsruche, Germany) following the manufacturer's protocol. The quantity and quality of samples was evaluated with an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies®). Total RNA (100 ng) was retrotranscribed with miRNA specific primers using a TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). The qRT-PCR was performed using Taqman® miRNA assays (has-miR-139 ID 002289; hsa-miR-181b ID1098; hsa-miR-584 ID001624; hsa-miR-708, ID 002341) according to the manufacturer's protocol. The expression levels of studied miRNAs were measured using the ABI 7500 Real Time PCR System (PE Applied Biosystems). Relative expression was analyzed through $2^{-\Delta\Delta CT}$ method [38] with two internal controls, small nuclear RNU6B and RNU48 (ID001093, ID001006, respectively) and MRC-5 fibroblast cell line expression was used as calibrator.

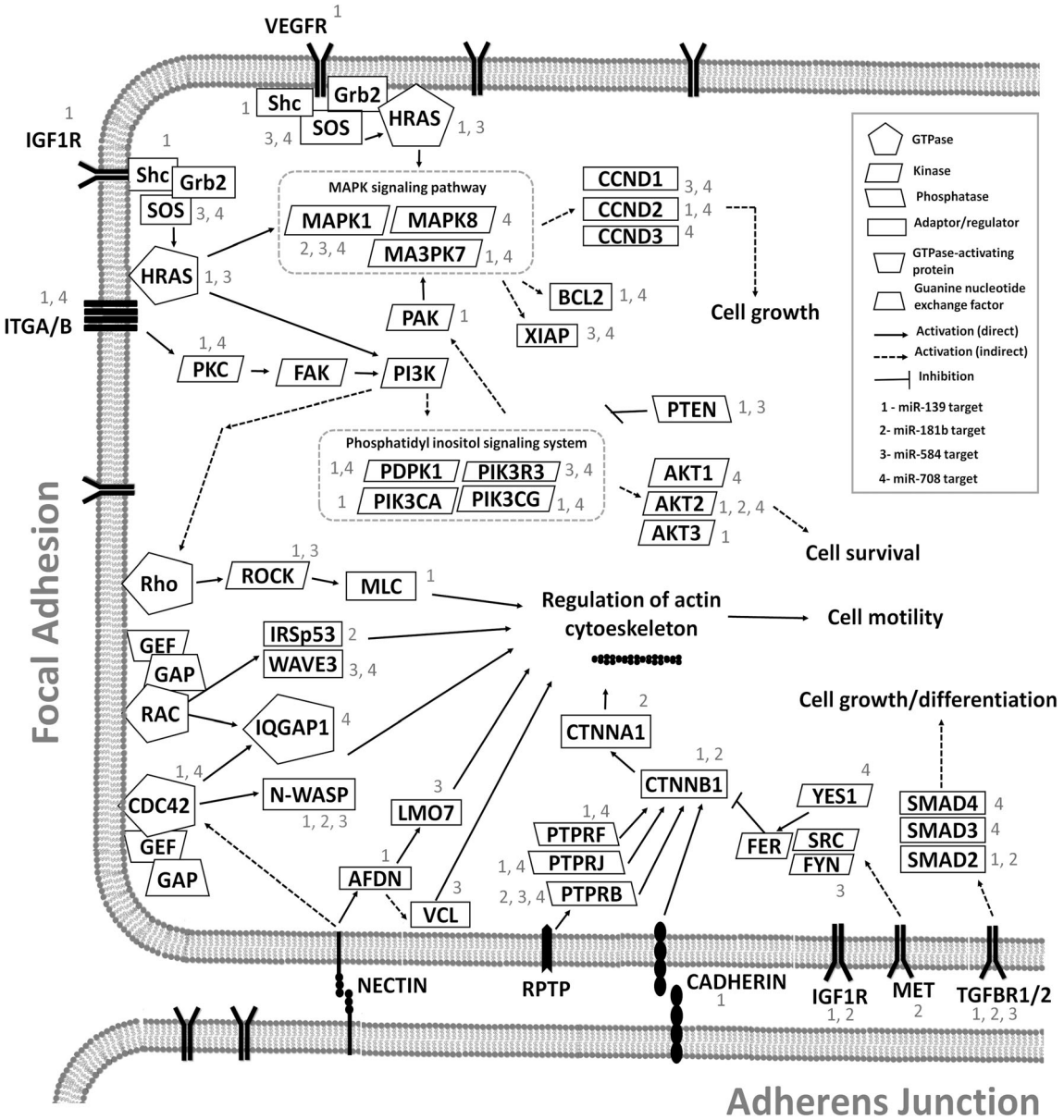

Fig. 2 Focal adhesion and adherents junction related proteins identified by KEEG Pathways analysis. The numbers above each protein indicate which microRNA could be targeting its mRNA

Cells Lines HOS and MG-63 cell lines were kindly provided by Professor Jeremy Squire (Queen's University, Canada/ Ribeirao Preto School of Medicine), while SAOS-2 was donated by Professor Keith Oswaldo Okamoto (University of Sao Paulo, Brazil). MRC-5 and HEK293T/17 cell lines were obtained from the ATCC (American Type Culture Collection, Rockville, MD). HOS, MG-63, SAOS-2 and MRC-5 were cultured with HAM-F10 medium, while HEK293T/17 was cultured in Dulbecco's Modified Eagle's Medium. Both media supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2.

Transfection of miRNA Pre-miRNA miR-139-5p (mirVana™ miRNA mimic ID: MC11749, Ambion®) and control (mirVana™ miRNA mimic negative control, Ambion®) were transiently transfected into the HOS cell line using Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to fabricant's protocol (mirVana™ miRNA mimics Protocol 2013). The miRNAs transfection efficiency was monitored by qRT-PCR at 48 h.

Viral Particle Production and Transduction Recombinant lentiviruses were produced by cotransfection of HEK293T/17 cells with pCMV-VSV-G (Addgene, Cambridge, MA, #Cat. 8454) and psPAX2 (Addgene, Cambridge MA, #Cat. 12,260). pLV-miRNA-Expression vectors were used as control and pVL-[hsa-mir-708] plasmids (containing miRNA-708-5p sequence) were used to induce this miRNA expression

Table 1 Summary of Patient Clinical data from OS samples, including age, gender, tumor grade and volume at diagnosis, local, HUVOS grade, metastasis at diagnosis, relapse, status and overall survival in months

ID	Age	Gender	Tumor grade at diagnosis	Volume at diagnosis (cm^3)	Local	HUVOS	Metastasis at diagnosis	Relapse	Status	Overall survival (months)
1	21	F	not informed	401.76	right Femur	did not receive QT	present	absent	lost follow-up	
2	12	F	grade 4	1393.92	left Femur	not informed	present	absent	event (death)	6
3	18	M	grade 3	865.92	left Femur	grade 2	present	absent	event (death)	28
4	15	M	high grade	25.547	ankle	amputation	present	absent	desease free	67
5	10	F	grade 1	not informed	left Tibia	surgical removal	present	absent	desease free	65
6	12	F	high grade	221.86	right Fibula	grade 2	present	absent	event (death)	24
7	13	M	grade 3	432.00	right Femur	grade 1	present	absent	event (death)	39
8	20	M	grade 3	not informed	left Femur	amputation	present	absent	desease free	22
9	13	M	grade 3	486.00	left Femur	grade 4	present	absent	desease free	46
10	12	F	grade 3	651.78	right Femur	grade 1	present	absent	event (death)	12
11	15	F	grade 2	455	left Knee	grade 3	absent	absent	desease free	30
12	18	F	low grade	465.11	left Femur	grade 1	present	absent	desease free	22
13	11	M	grade 3	396.17	left Femur	grade 2	present	absent	desease free	25
14	21	F	grade 1	not informed	right Femur	not informed	present	present	desease free	17
15	26	F	high grade	not informed	left Iliac	not informed	present	absent	event (death)	3
16	19	F	high grade	72.99	right Femur	grade 2	present	absent	event (death)	15
17	18	M	high grade	659.02	right Iliac	grade 2	present	present	in treatment	18
18	17	F	high grade	620.74	rifht Femur	grade 2	present	present	in treatment	16
19	12	F	high grade	562.77	femur	grade 2	absent	absent	desease free	12
20	5	M	high grade	50.05	right Femur	grade 2	absent	absent	desease free	12
21	6	M	high grade	971.52	right Femur	grade 3	absent	absent	in treatment	10
22	16	F	high grade	not informed	right Humerus	grade 2	present	absent	in treatment	10
23	16	F	high grade	234.50	left Femur	grade 3	present	absent	in treatment	10
24	12	M	high grade	not informed	right Tibia	grade 2	present	present	in treatment	8

permanently. The recombinant virus-containing media was used for transduction or stocked at −80 °C. The OS cells line HOS was exposed with recombinant virus-containing medium, centrifuged (2200 rpm, 30 min, room temperature) and incubated for 24 h. This procedure was repeated 3 times. Transduced cells were selected using puromycin (1 μg/ml). The miR-708-5p expression levels were confirmed and monitored by RT-qPCR.

Colony Formation Assay Clonogenic assays were performed according to Franken and colleagues (2006) [39]. Cells were transfected with specific miRNAs and controls and after 48 h, suspensions of 300 cells were seeded into 6-well plates. Cell cultures were incubated for at least 7 days and fixed with methanol and stained with Giemsa 3% (Sigma-Aldrich, St. Louis, MO, USA). Only colonies with more than 50 cells were counted. Assays were performed in triplicate and repeated in three sets of tests.

Invasion Assay The invasion assays were performed with Matrigel-coated transwells. Transfected cells were suspended in serum free medium in a concentration of 5×10^5 and were seeded in the upper chamber of the transwell insert (24-well insert, 8- m pore size; Becton Dickinson & Co., NJ, USA). Medium containing 10% fetal bovine serum was used in the lower chamber as chemoattractant. After 24 h of incubation, non-invading cells were removed using a swab and cells that invaded the membrane were fixed in methanol and stained with Giemsa 3%. Ten random fields of each transwell were photographed under light microscope and invaded cells were counted with Image J® software [40].

Adhesion Assay Cell Adhesion assay were performed in 96 wells plate. The wells were pre-coated with 2% gelatin (G-7765, Sigma-Aldrich) for 2 h. Cells were plated at a density of 1×10^3 cells per well. After 90 min non-adherent cells were subsequently removed along with the supernatant and the attached cells were fixed with methanol and stained with Giemsa 3%. Five random fields were photographed under inverted light microscope (10X objective) and counted with the Image J® software [40].

Statistical Analysis The association between miRNAs expression and clinical data was determined by MannWhitney tests. The analyzed variables were: age (< 15 years versus 15 years); local of primary tumor (appendicular versus axial); tumor grade at diagnosis [low (1,2) versus high (3,4)]; tumor volume detected by magnetic resonance at diagnosis (<200cm^3 versus > 200 cm^3); necrosis stage after chemotherapy – HUVOS level (less than 90% of necrotic areas (HUVOS levels 1 and 2) versus more than 90% necrotic areas (HUVOS levels 3 and 4); metastasis (presence versus absence); relapse (presence versus absence). Survival analysis (event free survival – relapse/metastasis/deceased; overall survival – deceased) was carried out based on Kaplan-Meier curves. More information about clinical associations can be found on Online Resource 3. Functional assays were statistically analyzed by Student's two-tailed t-test or One-Way Repeated Measures Analysis of Variance (ANOVA) followed by the Bonferroni Pairwise Multiple Comparison. All tests were carried out for 훼=0.05. All analyses were performed using the SPSS 21.0 software (SPSS Inc., IL, USA) and expressed as the mean ± standard deviation. All Graphs were produced using GraphPad Prim 6.

Results

Adhesion-Associated microRNAs Are Dysregulated in OS

Expression of miR-139-5p and miR-708-5p were significantly decreased in OS samples compared to non-neoplastic bone samples (respectively p values of 0.002 and 0.035). The levels of these microRNAs in the three OS cell lines were even lower (p = 0.025 and p = 0.007, compared to control). miR-181b-5p was also downregulated in cell lines when compared to tumor samples (p = 0.04). Conversely, no significant alterations were observed among sample groups for miR-584-5p. Nonetheless, its expression showed great variability within each group, including not detectable expression levels in four OS samples and the SAOS-2 cell line (Fig. 3a).

miR-181b-5p and miR-708-5p Expression Levels Correlate with OS Prognosis and Progression

By using Mann Whitney tests, we investigated possible associations between miRNAs expression in OS samples with clinical features. As seen in Fig. 3b and Table 2, higher expression of miR-181b-5p are present in tumors with lower HUVOS grade, being significantly associated to worse response to chemotherapy treatment (p = 0.016). Moreover, overall survival analysis showed that lower expression levels of miR-708-5p are correlated with a poor prognosis and lower patient survival rates (p = 0.024) (Fig. 2c, Table 2). Together, these results indicate the importance of these miRNAs in OS aggressiveness and progression.

Restoration of miR-139-5p and miR-708-5p Affects the Clonogenic and Adhesive Capacity In Vitro

Once miR-139-5p and miR-708-5p were differentially expressed in OS, both miRNAs were selected for functional investigation. Aiming determine the role of these miRNAs in OS pathogenesis, we induced an independent overexpression of these miRNAs in the HOS cell lines. miR-139-5p had a medium relative increase of 7000 times, while miR-708-5p presented a medium relative increase of 114 times.

A significant diminished clonogenic capacity of cells was observed after miR-139-5p induction (Fig 4 a), without affecting their invasive ability (data not shown). Conversely, upregulation of miR-708-5p significantly reduced the adhesiveness of HOS cells (Fig 4 b) and although not statistically significant increased their invasive capacity (data not shown). These results indicate that these miRNAs can be participating in different steps during metastatic process of OS, mainly miR-708-5p is directly involved with adhesion process.

Discussion

The major cause of mortality in OS is metastasis [41], and adhesion loss is thought to be the first step of this multistep process. Thus, the identification of adhesion-associated miRNAs with a differential pattern of expression remains central to understand OS pathogenesis and might contribute for patient stratification and treatment.

miR-139-5p is already considered a biomarker [42]. Its downregulation has been demonstrated in various tumors of different origins including colorectal, bladder and hepatocellular carcinoma [43–47]. Moreover, miR-139-5p has been directly associated with metastatic breast cancer [48–50] while its role on epithelial-mesenchymal transition, invasion and migration process was repeatedly proven [46, 51–54]. Our study shows for the first time the downregulation of this miRNA in osteosarcoma. In addition, we show that by increasing miR-139-5p in vitro, the clonogenic capacity of the HOS cell line diminishes, giving support for a probable contribution in OS pathophysiology.

MiR-181b has also shown great potential as a predictive and prognostic biomarker in different cancers [55–59], though its overexpression is commonly related to better treatment response [60–62]. This miRNA has also been described as upregulated in OS samples by others (5 times higher through microarray assays) [31] and its ectopic expression in OS cell lines increased cell viability and promoted invasion and migration [63]. Nonetheless, despite a slight increase of miR-

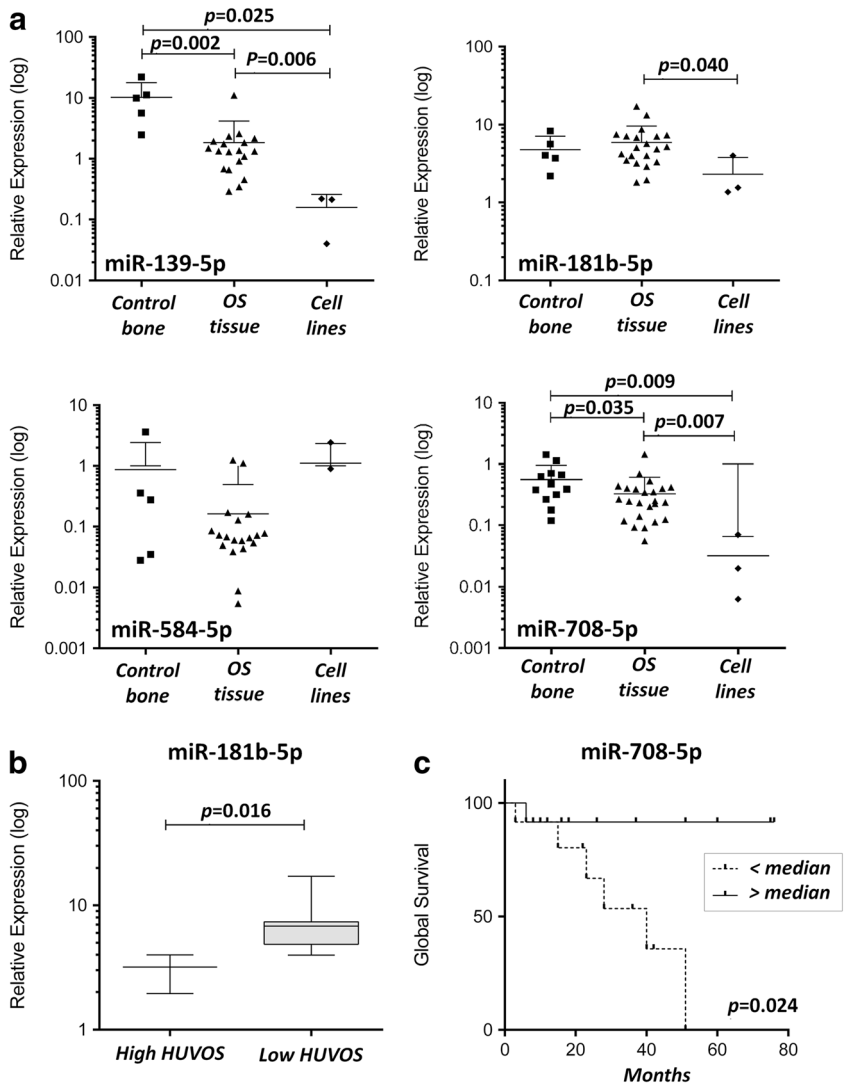

Fig. 3 a. Scatterplots of miR-139-5p, miR-181b-5p, miR-584-5p and miR-708-5p expression levels in non-neoplasic bone tissue (referred as control) OS tumor samples and OS cell lines (HOS, MG-63 and SAOS-2). Values equal to zero were excluded from the graph but included for statistical analyses. b. Higher expression of miR-181b-5p was related to a low HUVOS (low necrosis rate after QT). c. Kaplan–Meier plots of overall survival according to relative gene expression below or above the median of miR-708-5p expression. Significance level $p < 0.05$

181b-5p expression levels in our samples, we were unable to corroborate such findings. Moreover, in our cohort, increased expression of miR-181b-5p correlated with low HUVOS grade, indicating little necrotic tissue in tumor after chemotherapy. Interestingly, it was shown that complete and partial responses to S-1 treatment in colon cancer were strongly associated with patient with lower levels of miR-181b [64]. Therefore, the function of miR-181b may depend on the type of tumor and cellular context and it could still be considered an indicator of OS aggressiveness and chemoresistance.

The expression level of miR-708-5p also seems to be tumor-dependent, once it has been found either up- or downregulated [65–68]. In our study, miR-708-5p was underrepresented in OS samples and lower expression levels associated to worse overall survival. Thus, this microRNA showed to be the most intrinsically related

Table 2 Association between clinical variables and microRNAs expression levels in OS tissue samples as determined by Mann–Whitney test

	Age	Local of primary tumor	Malignancy level at diagnosis	Tumor volume	HUVOS level	Metastasis	Relapse	Death
(p value)								
miR-139-5p	0.186	0.201	0.071	0.264	0.229	0.502	0.137	0.599
miR-181b-5p	0.355	0.322	0.108	0.062	0.016*	0.688	0.722	0.709
miR-584-5p	0.692	0.909	0.244	0.152	0.147	0.231	0.075	0.306
miR-708-5p	0.469	0.834	0.685	0.779	0.614	0.588	0.543	0.014*

*Significantly statistic $p < 0.05$

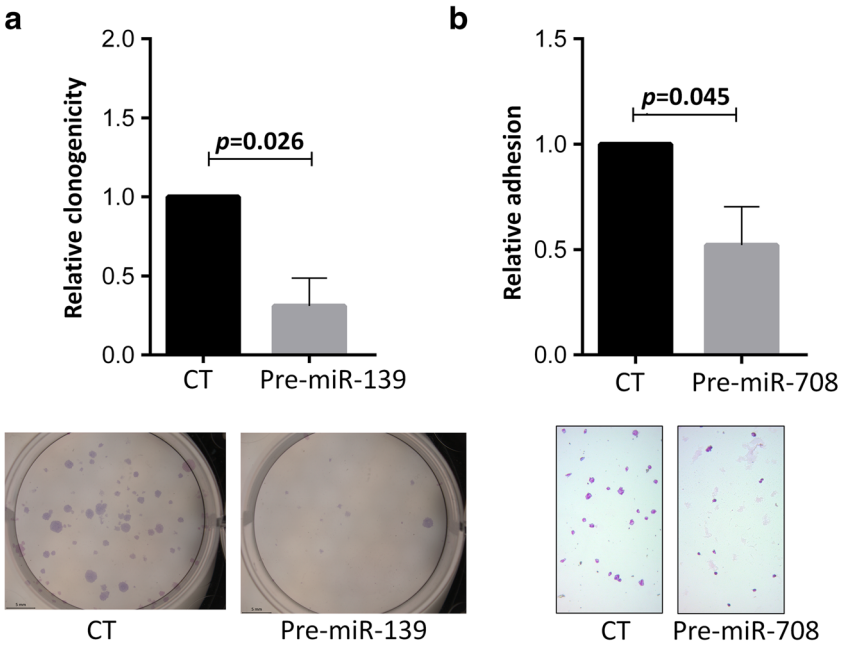

Fig. 4 Clonogenic, invasion and adhesion rates in OS cell line HOS compared to controls after miR-139-5p (a) or miR-708-5p (b) induction. Photography magnification was 40× for colonies in (**a**) and 1000× for individual cells in (**b**). Significance level $p < 0.05$

to OS pathogenesis, acting as a tumor suppressor and targeting directly adhesion process as validated in vitro. Recent investigations have also highlighted its relation with adhesion control, regulating several genes associated with this process including Rap1b in ovarian cancer [69] and FAK through aberrant Ca^{2+} regulation in breast cancer [70]. Moreover, miR-708 targets E-cadherin regulators ZEB2 and BMI1 [71].

On the other hand, few studies have explored the expression miR-584-5p in neoplastic tissue. Its role in glioma is controversial [72–74], while it was described upregulated in malignant mesothelioma and rectal cancer [75, 76]. Conversely, low levels of this miRNA have been described in renal cell carcinoma [77], lung cancer [65], and thyroid carcinoma [78]. Even though, our results did not show statistically different levels of miR-584-5p in our cohort compared to non-neoplastic bone. Most samples showed expression below the mean and not detectable expression levels in four samples and the SAOS-2 cell line suggesting that miR-584-5p might act as a tumor suppressor in OS. Of note, some works highlight miR-584-5p as a negative regulator of metastasis through its interaction with ROCK1, a serine/threonine kinase that phosphorylates downstream targets involved in cytoskeletal rearrangement and hence promotes the formation of stress fibers, focal adhesions and cellular junctions, facilitating cell movement [77, 78].

In conclusion, this study shows for the first time the downregulation of miR-139-5p and miR-708-5p in pediatric osteosarcoma and highlights the potential of miR-181b-5p and miR-708-5p as prognostic biomarkers. Moreover, through in vitro assays, our results evidence the participation of miR-139-5p in the survival and proliferation of cells and consolidates a role for miR-708-5p in cellular adhesion control.

Compliance with ethical standards

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