Pathology & Oncology Research (2020) 26:877–883
https://doi.org/10.1007/s12253-019-00633-0

ORIGINAL ARTICLE

microRNA-138-5p as a Worse Prognosis Biomarker in Pediatric, Adolescent, and Young Adult Osteosarcoma

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Received: 5 February 2018 / Accepted: 5 March 2019 / Published online:12 March 2019

Abstract

Osteosarcoma (OS) is the most common primary malignant bone tumor with two peaks of incidence, in early adolescence and the elderly. Patients affected with this malignancy often present metastatic disease at diagnosis, and despite multimodality therapy, survival has not improved substantially over the past 3 decades. Recently, miR-138-5p, proposed as a crucial intracellular mediator of invasion, has been recognized to target the Rho-associated coiled-coil containing protein kinase 2 (ROCK2). Dysregulation of ROCK1 and ROCK2 was also described in OS, being associated to higher metastasis incidence and worse prognosis. Nonetheless, the specific roles of miR-138-5p in pediatric and young adult OS and its ability to modulate these kinases remain to be established. Thus, in the present study, the expression levels miR-138-5p were evaluated in a consecutive cohort of exclusively pediatric and young adult primary OS samples. In contrast to previous reports that included adult tissues, our results showed upregulation of miR-138-5p associated with reduced event-free survival and relapsed cases. In parallel, *ROCK1* mRNA levels were significantly reduced in tumor samples and negatively correlated with miR-138-5p. Similar correlations were observed after studying the profiles of ROCK1 and ROCK2 by immunohistochemistry. Our data present miR-138-5p as a consistent prognostic factor in pediatric and young adult OS, reinforcing its participation in the post-transcriptional regulation of ROCK kinases.

Keywords miR-138 · ROCK · microRNA · Osteosarcoma · Prognosis

Introduction

Osteosarcoma (OS) is the most common bone malignant tumor in children and adolescents [1], representing the octave malignancy of childhood [2]. Nonetheless, a second peak of incidence appears during the sixth decade of life [3]. Despite the remarkable reduction of mortality shortly after the introduction of chemotherapy, survival has not improved substantially over the past 3 decades reaching 70%, nonetheless, for metastatic cases overall survival remains under 20% [4–6].

Initial steps of metastasis include loss of adhesion, alterations of cellular shape, polarization and invasion, all of which require cytoskeleton remodeling [7]. Among the numerous proteins described to be related to cytoskeleton control are the Rho-associated coiled-coil containing protein kinases ROCK1 and ROCK2. These kinases have frequently been described as dysregulated and associated to higher metastasis incidence and worse prognosis in several tumors, including OS [8, 9].

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Although the mechanisms underneath such dysregulation may vary [10–12], both ROCK isoforms, like most proteins, are also post-transcriptionally regulated by microRNAs (miRNA). Specially, miR-138-5p, highlighted in the literature as a tumor suppressor involved in cell cycle control and metastasis [13, 14], has been recognized to target ROCK2 [15], which has been proposed to be a crucial intracellular mediator regulating OS migration [12]. However, the specific roles of miR-138-5p in non-adult OS and its ability to modulate ROCK kinases remain to be established.

Methods

Subjects and Samples

Forty-eight consecutive exclusively primary pediatric, adolescent, and young adult OS samples were obtained by surgeons from the Oncologic Orthopedic Group of the university hospital (Ribeirão Preto Medical School–University of Sao Paulo) between May, 2005 and September, 2015. The survival analysis was followed until June 2016. Surgical samples from patients that had underwent systemic chemotherapy were emphatically excluded to avoid biases on gene/protein expression. All OS patients were included in the first peak of osteosarcoma incidence, being considered pediatric (<15 years) or young adults (>15 years). The cohort consisted of 27 females and 21 males (range: 5–26 years old), being 60% younger than 15 years. Twelve non-neoplastic bone tissues were used as controls and were obtained from patients who underwent malformation bone corrective surgeries (median age of 14.5 years old; range: 7–27 years old). Local relapse or pulmonary metastasis specimens from nine patients were also evaluated. All samples were obtained with informed consent and the research approved by the ethics committee of our institution (n° 43.619.215.9.0000.5407). Tissues were collected and immediately snap frozen in liquid nitrogen and stored at −80 °C for further analysis.

RNA Isolation, Reverse Transcription and Quantitative Real-Time PCR

Total RNA of tumor and non-tumor samples was extracted using TRIzol Reagent (Invitrogen, Karlsruche, Germany) following the manufacturer's protocol. The concentration and quality of the RNA was accessed using a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies). cDNA was synthetized using the High Capacity kit (Applied Biosystems, MA, USA) according to the manufacturer's instructions. For miRNA quantification, miRNA-specific primers were used to produce the cDNA. The qRT-PCR was performed using Taqman® miRNA and gene assays [*ROCK1* (Hs01127699-m1), *ROCK2* (Hs00178154-m1), and miR-138-5p (002284)] according to the manufacturer's protocol on the 7500 Real Time PCR System (Applied Biosystems, Waltham, MA, USA). As reference genes, the small nuclear RNU6B and RNU48 were used to normalize the miRNAs levels and *GAPDH* and *GUS* used to normalize the gene levels. The MRC5 cell line were used as reference sample. Relative expression was calculated by $2^{-\Delta\Delta CT}$ analysis method [16].

Immunohistochemistry

Immunohistochemical reactions were performed with the EXPOSE Mouse and Rabbit Specific HRP / DAB Detection IHC kit (Abcam® - ab80436; Cambridge, MA, USA) according to the manufacturer's recommendations. Antigen retrieval was performed in steam cooker for 35 min in Tris-EDTA pH 9.0 buffer. The primary antibodies for ROCK1 and ROCK2 (Anti-ROCK1 Ab45171, 1: 250; Anti-ROCK2 Ab125025, 1: 200; Abcam) incubated at room temperature for two hours. Finally, the slides were incubated with diaminobenzidine solution (DAB) for a standard time for each antibody and counterstained with Harris Hematoxylin. A colon adenocarcinoma biopsy was used as a positive control for the anti-ROCK1 antibody, whereas a non-neoplastic kidney sample was used as a control for ROCK2. For the immunostaining analysis, the slides were scanned with an Olympus BX61VS Slide Scanner system (Olympus Optical do Brasil Ltda) and at least five regions representative of the tumor were analyzed using the IHC Profiler plugin, according to Varghese et al. (2014) [17]. For subsequent statistical analysis, the samples were classified into two groups: negative (no staining) or positive (Low positive, Positive or High positive).

Statistical Analysis

The association between the following variables: age (<15 years versus >15 years old); local of primary tumor (appendicular versus axial); histologic grade at diagnosis [low (I - II) versus high (III - IV)]; tumor volume detected by magnetic resonance at diagnosis (<200cm^3 versus >200cm^3); necrosis stage after chemotherapy – HUVOS level [<90% of necrotic areas (HUVOS levels 1 and 2) versus >90% necrotic areas (HUVOS levels 3 and 4)]; metastasis (presence versus absence); relapse (presence versus absence); and expression levels of microRNA and genes were determined by Mann-Whitney tests. Chi-square or Fisher's exact tests were used for associations between the mir-138-5p and ROCK1 and ROCK2 protein profiles. Survival analysis was carried out based on Log-Rank test represented on Kaplan-Meier curves using the median of the miRNA expression observed as cutoff. All tests were carried out for α=0.05. All analyses were performed using the SPSS 21.0 software (SPSS Inc., IL, USA).

Results

Upregulation of miR-138-5p Is Associated with Poor Prognosis

miR-138-5p showed higher levels in OS samples ($p = 0.0002$) when compared to controls (Fig. 1a). Kaplan Meier analysis showed that higher expression levels of miR-138-5p are associated with lower event-free survival rate, which includes local relapse, metastasis and death ($p = 0.026$) (Fig. 1b). Confirming this result, upregulation of miR-138-5p was also associated with relapse ($p = 0.046$), known to be a poor prognosis feature (Fig. 1c).

ROCK1 Is Downregulated in OS and Negatively Correlated with miR-138-5p

ROCK1 was found significantly underexpressed in tumor tissues ($p = 0.001$) (Fig. 1d). Interestingly, a negative correlation ($p = 0.02$; $\rho = -0.447$) was observed between *ROCK1* and miR-138-5p in OS samples and non-neoplastic bone tissue (Fig. 1e). Contrariwise, *ROCK2* gene expression was comparable between OS samples and controls ($p > 0.05$) (Fig. 1f) and no significant correlation with miR-138-5p expression was found ($p = 0.876$ and $\rho = 0.027$). Nonetheless, no associations with any clinical features were found for either *ROCK1* or *ROCK2*.

ROCK1 and ROCK2 Protein Expression Does Not Entail OS Prognosis

Aiming to validate *ROCK1* and *ROCK2* mRNA levels, their protein expression was further analyzed by immunohistochemistry. The protein profiles were very similar in tumor samples, which were 46.5% (20/43) positive for ROCK1 and 43.6% (17/39) positive for ROCK2 (Fig. 2a–d). Among the metastatic specimens, five from nine samples showed identical immunostaining to their corresponding primary tumors while the rest presented antagonistic immunostaining, being two positive and two negative metastases.

Different from miR-138-5p, the ROCK1 and ROCK2 protein expression was not associated with tumor relapse nor with HUVOS grade, metastasis, death or event-free-survival. Nonetheless, patients with ROCK1 positive samples showed 9.8 higher risk of developing bigger tumors, as described in Table 1. Interestingly, we observed that miR-138-5p levels were 2.2 times higher in ROCK1 negative samples ($p = 0.102$) and 3.2 times higher in samples with negative immunostaining for ROCK2 ($p = 0.03$) (Fig. 2e and f). A bigger cohort might be needed to confirm these data.

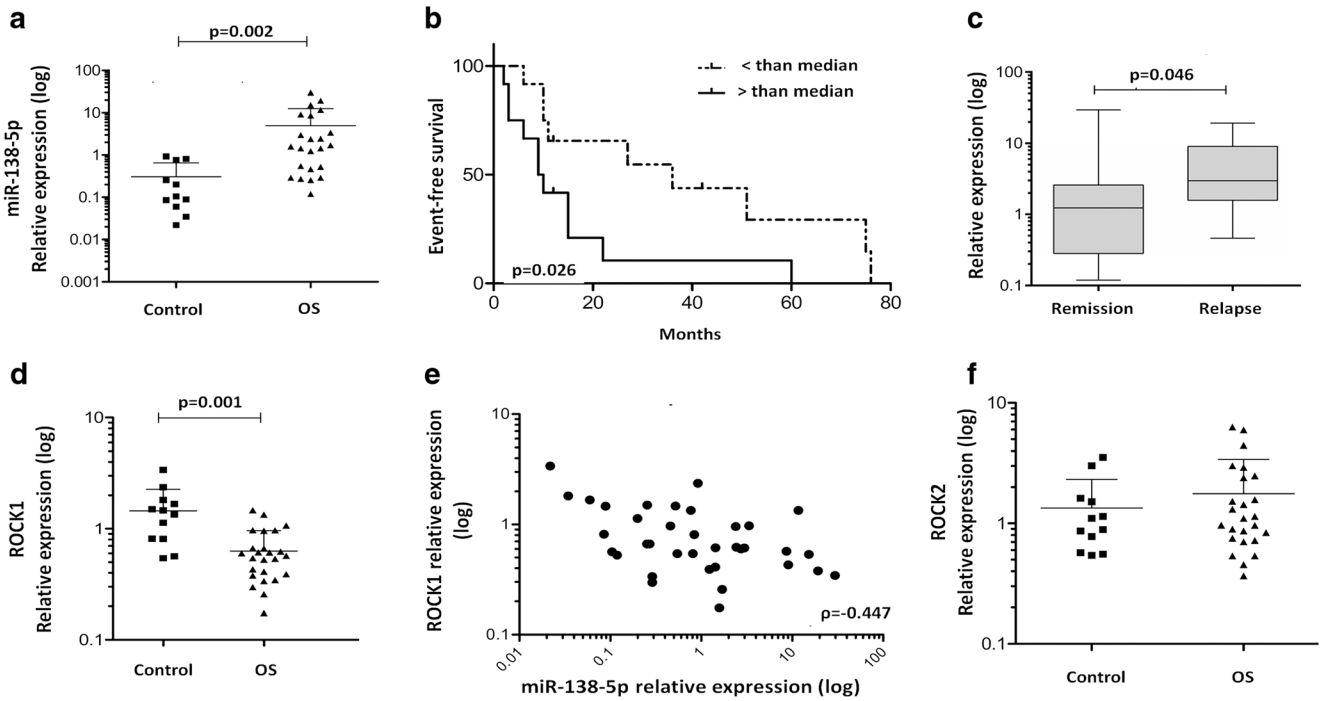

Fig. 1 miR-138-5p and *ROCK1* gene expression is dysregulated in Osteosarcoma. **a** miR-138-5p is overexpressed in OS samples; **b** Higher expression of miR-138-5p is associated with shorter event-free survival and **c** tumor relapse occurrence; **d** *ROCK1* was found downregulated in OS samples and **e** negatively correlated with miR-138-5p expression; **e** *ROCK2* mRNA levels are not altered in tumor samples. Results of relative expression are represented as log10 scale; n.s. means not significant

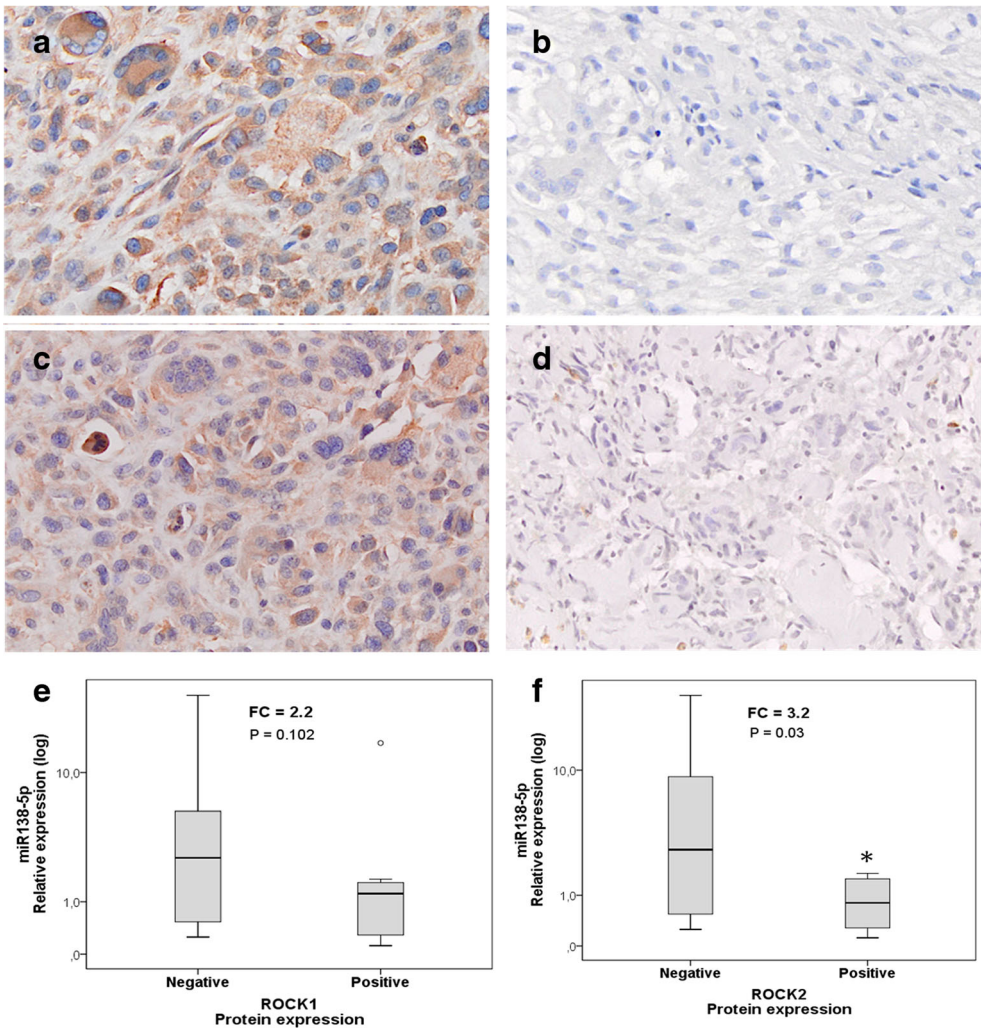

Fig. 2 **a**–**d** Representative microphotographs of immunodetection staining for ROCK1 and ROCK2 in pediatric OS samples: **a** and **c** Tumor samples positive for ROCK1 and ROCK2 respectively; **b** and **d** ROCK1 and ROCK2 negative tumor samples respectively. Original magnification 200x. **e** miR-138-5p expression levels were 2.2 times higher in ROCK1 negative samples and 3.2 times higher in samples with negative immunostaining for ROCK2 ($p = 0.03$). Results of relative expression are represented as log10 scale

Discussion

The potential clinical usefulness of miRNAs as prognostic and predictive markers for aggressive and metastatic cancers such as OS, has long been recognized. In this context, miR-138-5p has been regularly described as downregulated in cancer [18–24] and to affect tumor growth and metastasis by targeting MMP2/MMP9 [25], SOX4 and HIF1A [26], c-Met [18], TWIST2 [20] and FOXC1 [24]. Moreover, this microRNA was reported as underexpressed in OS [27]. Opposing that previous report, our data not only showed overexpression of miR-138-5p in OS samples compared with normal bone tissue, but also higher levels of it in patients with tumor relapse and shorter event-free survival, suggesting that the expression of this microRNA may be useful for distinguishing pediatric/young adult patient outcome. Moreover, a significant negative correlation between increased mir-138-5p and mRNA expression levels of *ROCK1* was observed.

The Rho-associated kinases ROCK1 and ROCK2 have been already described as involved with the regulation of metastasis-related processes in several studies. These kinases are mostly found upregulated in several tumors including gliomas, breast and gastric cancer [28–30]. Moreover, ROCK1 inhibition leads to decreased tumor invasion and proliferation in lung cancer and hepatocellular carcinomas [31, 32]. Also, in OS, *ROCK1* and *ROCK2* expression was previously found significantly higher in tumors than in non-neoplastic tissue and related with worst prognosis [8, 9]. However, our analysis of pediatric/young adult OS tissues did not confirm such results: *ROCK1* was significantly downregulated in tumor samples compared to non-tumor bone tissue, while there were no differences on *ROCK2* gene expression. Additionally, no associations were found between *ROCK1* and *ROCK2* profiles and any clinic-pathological parameters.

Besides ethnic variations [33, 34], opposing expression data might be a consequence of cohort age. Liu et al. [8], who described ROCK1 overexpression in osteosarcoma, used a commercial array of tumor samples for immunohistochemical analysis and even though the proportion between adult and pediatric samples was not informed, it contained

Table 1 Relative expression values (Median and range) of miR-138-5p and ROCK1/2 immunostaining profiles according to the clinical and pathological characteristics of patients with osteosarcoma

Characteristics	miR-138 expression ($N = 25$)		ROCK1 ($N = 43$)				ROCK2 ($N = 39$)			
	Median (range)	p-value[a]	(−) $n = 23$	(+) $n = 20$	Odds Ratio (95% CI)	p-value[b]	(−) $n = 22$	(+) $n = 17$	Odds Ratio (95% CI)	p-value[b]
Gender										
Female	1.56 (0.25–19.2)	0.682	15	9	2.3 (0.67–7.84)	0.183	13	9	1.3 (0.36–4.60)	0.701
Male	1.58 (0.12–29.5)		8	11			9	8		
Age										
<15 years	1.43 (0.12–29.5)	0.111	8	12	0.4 (0.11–1.23)	0.098	7	11	0.2 (0.07–0.97)	**0.041**
>15 years	6.63 (0.55–19.2)		15	8			15	6		
Tumor volume[#]										
< 200 cm^3	20.6 (11.7–29.5)	0.223	7	1	9.8 (1.04–92.7)	**0.041**	6	1	7.2 (0.74–70.2)	0.093
> 200 cm^3	1.44 (0.12–19.2)		10	14			10	12		
HUVOS[#]										
1–2	5.16 (0.12–29.5)	0.257	10	12	1.4 (0.34–6.16)	0.622	10	10	2.0 (0.39–10.3)	0.454
3–4	1.43 (0.29–1.43)		6	5			6	3		
Tumor grades[#]										
Low (I–II)	0.42 (0.29–0.55)	0.330	5	2	2.7 (0.45–15.5)	0.414	5	2	2.4 (0.39–14.0)	0.427
High (III–IV)	1.70 (0.12–29.5)		17	18			16	15		
Skeletal location[#]										
Axial	8.84 (2.44–15.25)	0.210	2	2	0.9 (0.11–6.72)	1.000	2	1	1.6 (0.13–19.3)	1.000
Appendicular	1.51 (0.12–29.5)		21	18			20	16		
Events:										
Metastasis[#]										
No	0.27 (0.12–29.5)	0.141	6	6	0.8 (0.20–2.93)	0.695	5	6	0.5 (0.13–2.20)	0.387
Yes	1.70 (0.55–19.2)		17	13			17	11		
Relapse[#]										
No	1.33 (0.12–29.5)	**0.046**	13	12	1.0 (0.28–3.31)	0.952	12	11	0.7 (0.19–2.72)	0.635
Yes	8.63 (0.55–19.2)		9	8			9	6		
Death[#]										
No	1.33 (0.12–29.5)	0.240	14	10	1.2 (0.36–4.35)	0.732	14	8	1.7 (0.47–6.48)	0.401
Yes	8.63 (1.58–11.7)		9	8			8	8		

Bold = statistically significant

(#) Complete clinicopathological data was not available for all patients analyzed though immunohistochemistry due to loss of follow-up;

[a] Mann-Whitney U test;

[b] Fisher's exact test 2-tailed was considered when one of the four cells of 2 × 2 table has <5 observations. Chi-square test was applied for the other cases

samples from patients up to 61 years-old. Comparatively, the study by Jiang et al. [27] included 65 osteosarcoma specimens, from which, more than 60% were adult cases. Although our sampling was smaller, we certified that all samples were within the younger peak of incidence (first and second decade of life) and without any previous treatment to avoid biases on gene expression.

Osteosarcomas in elderly patients are often considered secondary neoplasms attributed to transformation of other benign bone lesions [3]. Importantly, in recent years, there have been a constant accumulation of evidence proving that pediatric solid tumor pathophysiology differs greatly from adult counterparts [35]. Many, if not all pediatric cancers differ from adult equivalents in varied aspects that include incidence (specific types occur more frequently), primary sites, behavior, histology, genetics and other molecular features [36–40]. Such differences indicate that findings in adult tumors cannot be simply extrapolated to younger patients, and that only through separate studies such differences might predict outcomes and allow more efficient therapeutic decisions.

ROCK1 and ROCK2 immunostaining profiles have provided significant information concerning clinical aspects of different tumor types, such as oral carcinoma, vascular tumors and vulvar cancer [41–43]. However, our study showed that protein expression of both ROCK kinases was quite similar among OS pediatric samples and that their expression patterns bring no relevant information about tumor prognosis or patient outcome. In addition, we observed significant higher miR-138-5p levels

in OS samples with negative immunostaining for ROCK2. Previous studies have already described Rho-GTPases related proteins, such as RhoC, LIMK1 and ROCK2, as miR-138-5p targets, reinforcing our findings regarding miR-138-5p and ROCK2 protein profile in OS [13, 15, 44].

Taken together, our data suggests that miR-138-5p expression profile may be a reliable biomarker for pediatric OS prognosis, showing higher levels in patients with tumor relapses and lower event-free survival. Moreover, our data reinforce its participation in the post-transcriptional regulation of ROCK1/2.

Funding FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo): Grant 2014/03877–3 and LEAD and GMV fellowships, 2014/07117–3 and 2014/07118–0.

Compliance with Ethical Standards

Conflict of Interest All authors declare that they had no conflict of interest that could be perceived to impair the impartiality of the research reported.

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