Cancer Genetics 230 (2019) 21–27

Cancer Genetics

ORIGINAL ARTICLE

MiR-708-5p is inversely associated with EWS/FLI1 Ewing sarcoma but does not represent a prognostic predictor

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Abstract

Background: Overall survival of Ewing sarcoma (EWS) remains poor and less than 30% of patients with metastatic or recurrent disease survive despite current treatments. Thus, there is a constant search for new biomarkers for diagnosis, prognosis and prediction of therapy. Numerous studies have reported the abnormal expression of miR-708-5p in tumors of different origins. However, its role in EWS remains unclear.

Procedure: qRT-PCR was performed in nineteen consecutive EWS samples and twelve non-tumor bone samples from age-matched controls. Functional assays were performed in SK-ES-1 cells transfected with miR-708 lentiviral-based vectors and results analyzed in terms of clonogenicity, migration, invasion and western blot.

Results: We show that miR-708-5p is downregulated in EWS tissues though no associations with any prognostic features such as HUVOS grade, event or survival were found in our cohort. Nonetheless, expression levels of this micro-RNA were inversely associated with the presence of the EWS/FLI1 translocation. When miR-708-5p was transfected into the SK-ES-1 cell line, it did not affect migration or clonogenicity, but promoted a significant increase on the invasive potential of cells endorsed with high expression of MMP2.

Conclusions: Taken together, our results suggest that despite downregulated in EWS samples, this miRNA might represent a secondary genetic alteration derived from the pleiotropic cellular effects of the abnormal EWS/FLI1 transcription factor that does not affect tumor growth but instead, is related with the promotion of tumor invasion, not being suitable for future therapeutic intervention.

Keywords miR-708, Ewing sarcoma, Invasion, Motility.

Abbreviations: EWS, Ewing sarcoma; EWSR1 gene, Ewing sarcoma breakpoint region 1; FLI1 gene, Friend leukemia virus integration site 1; ATCC, American Type Culture Collection; FBS, Fetal bovine serum.

Received September 13, 2017; received in revised form August 13, 2018; accepted November 7, 2018

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https://doi.org/10.1016/j.cancergen.2018.11.003

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Introduction

Ewing's sarcoma (EWS) is a highly aggressive and metastatic neoplasia that typically occurs in children and young adults, representing the second most common primary malignant tumor of bone [1]. EWS treatment consists of preoperative chemotherapy, followed by surgery and postoperative chemotherapy and/or radiotherapy [2,3]. However, about 25–30% of patients already manifest metastatic disease at the time of diagnosis [4], significantly decreasing 5-year survival from 60–70% to 10–40% [5].

Most cases of EWS (85%) are the result of the translocation (11;22)(q24;q12) that juxtaposes the *EWSR1* gene (Ewing sarcoma breakpoint region 1) on chromosome 22 and the *FLI1* gene (Friend leukemia virus integration site 1) on chromosome 11, resulting in the expression of the chimeric protein EWS/FLI1 [6–8]. This in-frame fusion protein has variable domains, facilitating the interaction with numerous transcription factors that leads to the alteration of the transcription of innumerous genes involved in cell cycle regulation, DNA repair, migration and apoptosis, having, therefore, oncogenic activity [9].

The EWS/FLI1 fusion has demonstrated to be prognostically relevant in EWS, independent of tumor site, stage, and size [10]. Recent studies suggest that EWS/FLI1 also modulates cellular spreading (adhesion, motility and invasion), both in vitro and in vivo [11]. Among its various targets, EWS/FLI1 also acts on the modulation of several microRNAs such as miR-145, miR-205, miR-190 and miR-35a-5p, miR-22, and let-7a [12,13].

More recently, an in vitro study showed that miR-708-5p is also negatively modulated by EWS/FLI1, and its repression upregulates the DNA repair protein EYA3 contributing to chemoresistance and cell survival of EWS cell lines [14]. Dysregulation of miR-708-5p has also been commonly demonstrated. In some carcinomas, restoration of this micro-RNA not only diminishes cell growth [15,16] but also suppresses tumor invasiveness and migration [17]. Nonetheless, its exact role in EWS tumorigenesis remains unclear.

Material and methods

EWS tissue samples

Surgical samples of EWS were obtained from 19 patients at the Clinics University Hospital (Ribeirão Preto School of Medicine – University of São Paulo), operated from 2006 to 2016 (survival analysis was followed until June 2016). None of the patients received chemotherapy or radiotherapy prior to biopsy. The patients corresponded to 7 women and 12 men with a mean age at diagnosis of 14 years (range: 4–28 years old). Seven patients presented metastasis, and ten had relapse. Each tumor sample was categorized as EWS by an expert pathologist based on the staining profiles of CD99, NSE and FLI1 complemented by FISH and/or RT-PCR. Non-tumor bone tissue were obtained from malformation bone corrective surgery. All specimens were snap frozen in liquid nitrogen and stored at −80 °C and microdissected by the pathologist before further analysis. 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° 43619215.9.0000.5407).

Cell lines

The SK-ES-1 and RD-ES cell lines (EWS/FLI1 positive) was purchased from Rio de Janeiro Cell Bank (BCRJ, Federal University of Rio de Janeiro, Brazil). MRC-5 (normal fibroblasts - used as calibrator for Real-time PCR) and HEK293T/17 (used for viral particle production) cell lines were obtained from the ATCC (American Type Culture Collection, Rockville, MD). Cells were cultivated with recommended media supplemented with 10% of fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL) and kept at 37 °C in a humidified 5% CO_2 incubator.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was extracted from tumor and non-tumor samples by Trizol Reagent (Invitrogen, Karlsruche, Germany) following the manufacturer's instructions. The quality and quantity of the RNA was evaluated through a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies). RNA was afterwards retrotranscribed using the High Capacity kit (Applied Biosystems, MA, USA) according to the manufacturer's instructions. For miRNA quantification, the Taqman® miRNA assayhsa-miR-708was used (hsa-miR-708, ID 002341). qRT-PCR reactions were performed on the 7500 Real Time PCR System (Applied Biosystems, Waltham, MA, USA). Relative expression was calculated using $2^{-\Delta\Delta CT}$ analysis method [18] with two internal controls, small nuclear RNU6B (ID001093) and RNU48 (ID001006). The MRC5 cell line was used as calibrator.

Detection of EWS-FLI1 Fusion

RT-PCR was carried out using cDNA generated from total RNA as described above. PCR reactions were performed in a Veriti 96 Well Thermal Cycler (Applied Biosystems, MA, USA) with 35 cycles under the following conditions: 2 min at 94 °C, (30 s at 94 °C, 30 s at the annealing temp, and 30″ at 72 °C), and 10 final min at 72 °C. The PCR primers to detect the *EWS/FLI1* fusions were *EWS* (5′ TCC TAC AGC CAA GCT CCA AGT C 3′) e o *FLI* (5′ ACT CCC CGT TGG TCC CCT CC 3′). Reactions were maintained at −4 °C until analysis through agarose gel electrophoresis (2%). PCR products were loaded onto the gel with 1X TBS buffer and stained with ethidium bromide. Subsequently, they were visualized through exposure to UV light and photographed in MiniBis Pro system (Uniscence, FL, USA).

Viral particle production and miRNA transduction

HEK293T/17 cells were co-transfected with pCMV-VSV-G (Addgene, Cambridge, MA, #Cat. 8,454), psPAX2 (Addgene, Cambridge MA, #Cat. 12,260) and the plasmid of interest in order to produce recombinant lentivirus. pVL-[hsa-mir-708]

plasmids (containing miRNA-708-5p sequence) were used to induce this miRNA expression permanently and pLV-miRNA-Expression vectors were used as control. The recombinant virus-containing media was stored at −80 °C. The SK-ES-1 cell line was exposed to the recombinant virus-containing medium, centrifuged at 2200 rpm for 30 min (room temperature) and incubated for 24 h. This procedure was repeated 3 times. Transduced cells were selected using puromycin (2.5 mg/mL). The miR-708-5p expression levels were confirmed and monitored by RT-qPCR.

Colony formation assay

Clonogenic assays were performed according to Franken et al. [19]. Briefly, single cell suspensions of 500 cells from control SK-ES-1 cell line or pre-miR708 SK-ES-1 cell line were seeded in 6-well plates and incubated at 37 °C for 10 days. Then, colonies were washed, fixed with methanol and stained with Giemsa 3%. Only colonies with more than 50 cells were counted by direct visual inspection with a stereo microscope at 20 × magnification. Three independent assays were performed.

Invasion assay

Cell invasion was measured by invasion of the SK-ES-1 cell line through Matrigel-coated Transwell inserts (Becton Dickinson & Co., NJ, USA). Control and transduced cells were harvested, re-suspended in serum-free medium and seeded on the top of Matrigel-coated invasion 8 µm pore size chambers (5×10^5 cell/insert). Lower chambers were filled with media containing FBS. After 24 h of incubation, noninvasive cells were removed from the membrane upper surface with swabs. The invaded cells attached to the lower side of the membrane were fixed with 100% methanol and stained with Giemsa 3%. The membranes were removed from the inserts and placed on microscope slides with Entelan (Merk, NY, USA) and photographed at 20x magnification (ten random fields of each membrane). The invaded cells were counted with the ImageJ software®. Three independent assays were performed.

Boyden migration assay

Migration was assessed using a 48-well Boyden chamber housing a polycarbonate membrane with 8 µm pores (Neuroprobe Inc., MD, USA). Each membrane was coated with 5 mg/mL of fibronectin (Sigma-Aldrich, St. Louis, MO, USA) for 24 h before the assay. The bottom chambers were filled with medium containing FBS. Control and transduced cell lines were harvested, re-suspended in serum-free medium and seeded on the upper chambers (2.5×10^4 cells/well). After 24 h of incubation, non-migrating cells were scraped from the upper surface of the membrane. Cells on the lower surface were fixed with methanol and stained with hematoxylin. The membrane was placed on microscope slides and photographed at 20x magnification (four random fields for each well). The migrating cells were counted with the ImageJ® software. Three independent assays were performed.

Western blot

Total protein samples were extracted from the EWS cell line with RIPA buffer (Thermo Scientific, CA, USA) according to manufacturer's instructions. Equal amounts of heat-denatured protein samples (50 µg per lane) were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, EUA). The antibodies included primary rabbit monoclonal anti-ROCK1antibody (Ab45171at 1:500 dilution, Abcam, Cambridge, United Kingdom), primary rabbit monoclonal anti-ROCK2 antibody (Ab125025at 1:10,000 dilution, Abcam), primary mouse monoclonal anti-RHOC antibody (Ab180785at 1:10,000 dilution, Abcam), primary mouse monoclonal anti-MMP2 antibody (MAB13405 at 1:10,000 dilution, Millipore, CA USA) and rabbit monoclonal anti-GAPDH antibody (Ab128915 at 1:10,000 dilution, Abcam). The immunoblots were developed using goat anti-rabbit secondary antibody (Ab6721 at 1:5000 dilution, Abcam) and goat anti-mouse secondary antibody (Ab6789 at 1:5000 dilution, Abcam) followed by detection with the ECL Western Blotting Substrate Kit (Abcam) and visualized in a ChemiDoc Bioimaging System (Bio-Rad, Hercules, California, EUA). Expression levels were quantified using ImageJ® software and normalized to loading controls. Assays were performed in triplicate.

Statistical analysis

Possible associations between clinical variables [age (< 14 years versus 14 years old); sex; EWS/FLI1 status; necrosis stage after chemotherapy – Huvos level (levels 1 and 2 versus levels 3 and 4); metastasis (presence versus absence); relapse (presence versus absence); disease progression (relapse/metastasis versus complete remission), death (alive versus deceased)] and expression levels the miR-708-5p were determined by Mann-Whitney tests. Survival analysis was carried out based on Log-Rank test represented on Kaplan-Meier curves using the median of the miRNA expression as cutoff. The functional assays data were statistically analyzed by Student's two-tailed *t*-test. All tests were carried out for $\alpha = 0.05$. All analyses were performed using the SPSS 21.0 software (SPSS Inc, IL, USA) and expressed as the mean ± standard deviation.

Results

miR-708-5p is downregulated in EWS and associated with EWS/FLI11 status

qRT-PCR was performed in nineteen consecutive EWS samples and twelve non-tumor bone samples from age-matched controls. Expression of miR-708-5p was significantly decreased in tumor samples compared to the control group (fold change 18.02) ($p = 0.00035$). The levels of this miRNA were even lower in the RD-ES and SK-ES cell lines (Fig. 1(A)). No associations between miR-708-5p expression levels and prognostic features, such as Huvos grade, metastasis, relapse, deceased or overall and event-free survival were found

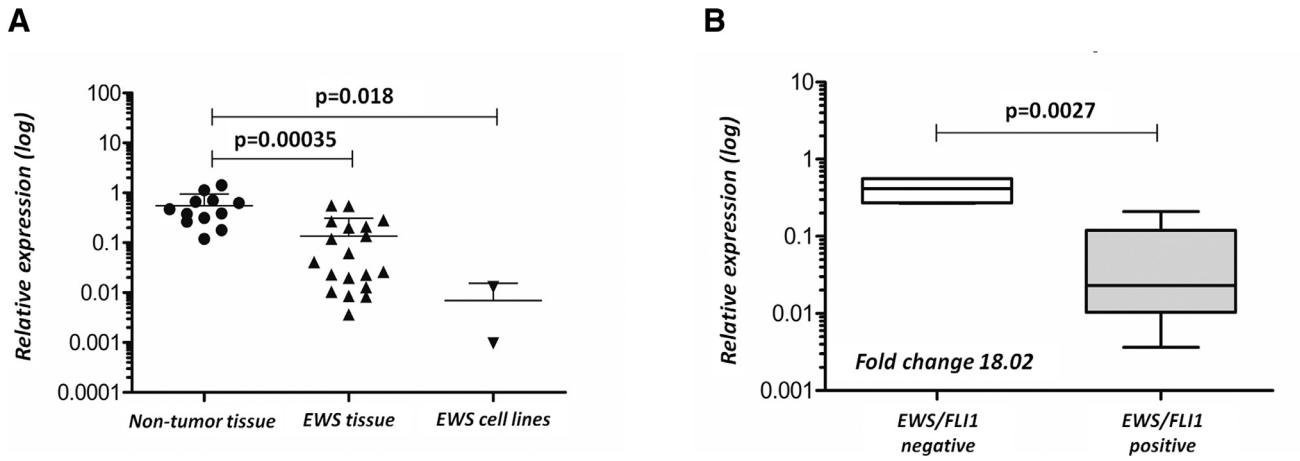

Fig. 1 *Analysis of miR-708-5p expression in Ewing Sarcoma.* **(**A) Downregulation of miR-708-5p in EWS tissue and cell lines compared to non-tumor tissue; (B) miR-708-5p is 18 times less expressed in tumors positive for EWS/FlI1 ($n=15$ for positive samples and $n=4$ for negative samples). Gene expression was determined by qRT-PCR. The horizontal lines represent the median of the data. Significant difference in gene expression was analyzed by Mann–Whitney tests ($p<0.05$).

Table 1 Association of mir-708-5p expression and clinical features in EWS (determined by Mann–Whitney tests).

Clinical features	*p* Value
Age	0.191
Sex	0.176
EWS/FLI1 status	0.003
Metastasis	0.353
Death	0.072
Relapse	0.514
HUVOS grade	1.000
Overall survival	0.201
Event-free survival	0.382

(Table 1), however this miRNA was significantly downregulated in *EWS/FLI11* positive samples compared to those negative for the translocation (Fig. 1(B)).

In vitro overexpression of miR-708-5p increases EWS invasive capacities but does not alter cell migration and colony formation

To elucidate the role of miR-708-5p in the regulation of growth and invasion, SK-ES-1 cells were permanently transfected with a pre-miR- lentivirus-based vector system. Confirmation through qRT-PCR showed a 140x stable expression compared to the mock control (data not shown). MiR-708 overexpression did not affect the colony formation and migration capacity of SK-ES-1 (Fig. 2(A) and (B)). However, forced expression of this micro-RNA strongly increased the invasive capacity stimulated by FSB attraction (Fig. 2(C)).

EWS cells transduced with miR-708-5p show higher expression of invasion-related protein

In order to better understand the mechanism under miR-708-5p invasion stimulation, we evaluated the expression of 4 invasion-related proteins: ROCK1, ROCK2, RhoC and MMP2. Our results revealed increased levels of all proteins on miR-708-5p transduced EWS cells, with significant higher levels of MMP2 expression (~4x, $p=0.029$) (Fig. 2(D) and (E)).

Discussion

Ewing sarcomas are molecularly characterized by expression of chimeric transcripts generated by the chromosomal translocation *t*(11:22). This translocation juxtaposes the *EWS* and *FLI1* genes, resulting in the fusion of EWS transcriptional activation domain with FLI1 DNA binding domain [20]. Since its discovery, several studies have pointed the importance of the resulting chimeric protein on EWS pathogenesis. EWS/FLI1 is involved on several oncogenic processes such as cell proliferation [21], transformation [22] and in vivo tumor growth [23]. More recently, Robin et al. demonstrated a role of this fusion protein on EWS chemoresistance through the repression of miR-708 that targets the EYA3, a DNA-repair protein [14]. In the present study, we demonstrate that miR-708 is indeed downregulated in EWS samples compared to non-neoplastic tissue. Moreover, lower expression of this miRNA was present on those tumors positive for EWS/FLI1 mutation.

Although the outcomes for EWS patients with localized disease have improved on the past three decades, the prognosis for those who present metastasis, who relapse or do not respond to initial therapy remains poor [4]. In addition to be pointed as a predictor of chemoresistance, miR708-5p also seems to play important roles on other oncogenic processes such as cell proliferation, migration and apoptosis. Numerous studies have reported the abnormal expression of miR-708 in several tumors of different origins including renal cell carcinoma, lung adenocarcinoma, hepatocellular carcinoma, ovarian carcinoma, bladder carcinoma, and prostate carcinoma, among others [16,17,24–28].

Mostly, in the cancer context, miR-708 has been repeatedly indicated as a tumor suppressor, since its expression is generally repressed, as is the case of EWS. In pancreatic ductal adenocarcinoma this microRNA was found downregulated

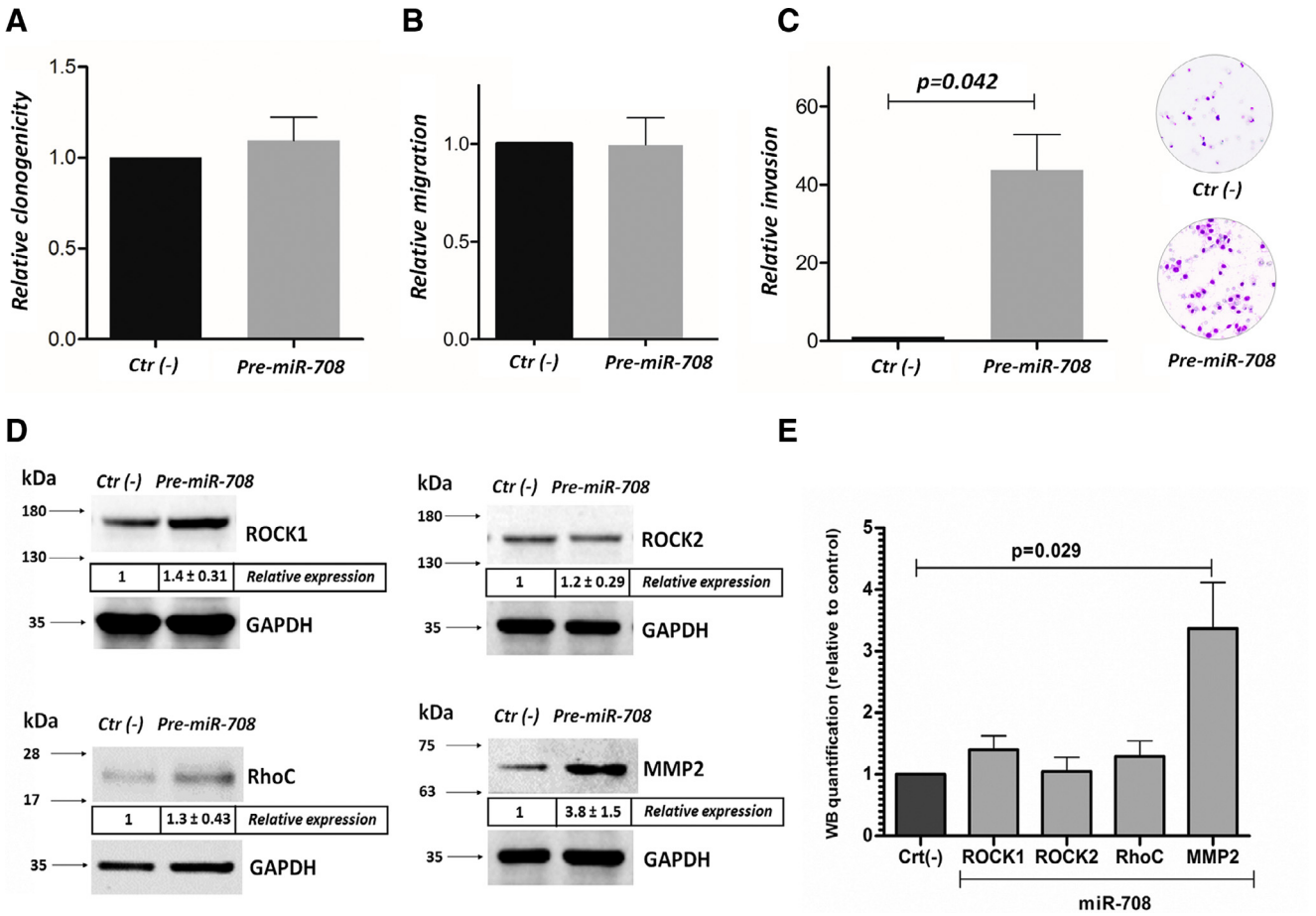

Fig. 2 *Effects of miR-708-5p on EWS cells.* (A) and (B) Restoration of miR-708-5p expression through lentiviral-based vectors did not affect the clonogenicity of SK-ES-1 cells (A) or migration (B). (C) Restoration of miR-708-5p expression stimulates SK-ES-1 cells invasion through Matrigel towards the chemoattractant (FBS). (D) and (E) Overexpression of miR-708-5p significantly increases MMP2 protein expression (4x). Levels of RhoC, ROCK1 and ROCK2 were also, increased in ~30%. Assays were performed in SK-ES-1 cell line in triplicate and the data statistically analyzed by Student's two-tailed *t*-test. All tests were carried out for ?? = 0.05 and expressed as the mean ± standard deviation.

[29] while in prostate cancer, lower miR-708 expression was also correlated with poor prognosis [26]. Similar results were described for childhood acute lymphoblastic leukemia with lower miR-708 expression associated with inferior relapse-free survival [30]. In addition, distal breast cancer metastases have shown decreased miR-708 levels compared to primary tumors [25]. Nevertheless, in lung adenocarcinoma, shorter survival was described for with higher miR-708 levels [24] suggesting that this miRNA may act in a tumor-dependent manner.

Even though we show that miR-708-5p is downregulated in EWS tissues, we were unable to detect any association with clinical features besides being inversely associated with the presence of EWS/FLI1 transcript in our cohort. Consequently, despite reports of increased mir-708-5p after EWS/FLI1 knockdown [14], its role as a tumor suppressor in EWS remains undefined. From our perspective, the low levels of this microRNA might result as a secondary genetic alteration based on the pleiotropic cellular effects of this abnormal transcription factor.

In vitro studies have demonstrated reduced growth and invasion capacities after miR-708-5p over expression [16,17,31,32]. Robin et al. [14] further demonstrated that the forced expression of miR-708 in EWS cells (A673) increases apoptotic markers (PARP cleavage and activation of caspases 3 and 7) and chemo-sensitivity to etoposide. Nonetheless, in our study, induction of miR-708-5p did not affect the colony formation or the migration capacities of SK-ES-1 cells. Rather, our results pointed miR-708-5p as an invasion stimulator, once EWS cells capacity to move through a matrix towards the chemoattractant was strongly increased by this miRNA.

According to our results, miR-708-5p and the presence of EWS/FLI1 fusion are inversely associated. Of note, Franzetti et al. [33] recently described that low EWS/FLI1 expression cell populations (supposed to have higher levels of miR-708-5p) have increased migration and invasion capacities compared to cell populations with high EWS/FLI1 expression (supposed to have low levels of miR-708-5p). Likewise, miR-708-5p has shown to accelerate migration of human mesenchymal stem cells [34] and the invasion of lung cancer cell lines [24].

In experimental cell biology, migration and invasion are separated terms although both of them are metastasis related. Migration involves directed movement of cells on

subtracts such as basal membranes or ECM fibers, therefore it occurs on 2D surfaces without obstructive fibers network. Invasion is a step ahead defined by cell movement through a 3D matrix encompassing a matrix extracellular remodeling and changes on cell shape [35]. The Rho-GTPase RhoC and its effectors, ROCK1 and ROCK2 are responsible for modulating the action of several subtracts involved on the cytoskeleton control and cell contractibility that contributes both processes [36,37]. On the other hand, the protein MMP2 is an important matrix metalloproteinase responsible for ECM degradation [38], capacity only evaluated through the invasion assay. MiR-708 has already been described regulating MMP2 expression, but in a suppressive manner leading to the inhibition of invasion [32,39]. However, in our model, MMP2 expression was found strongly increased on miR-708-5p transduced EWS cells. Also, a ∼30% increase in RhoC, ROCK1 and ROCK2 expression was seen in response to the miRNA overexpression. Although controversial, miR-708-5p may have multiple points of direct and indirect regulation as seen in other tumors [40] that may influence the invasive capacity of cells. For instance, miR-708-5p has been predicted to inhibit the expression of the tissue inhibitors of metalloproteinases, TIMP1-4. Nonetheless, more experimental testing is needed in other to decipher the mechanism through which mir-708-5p induced expression intensifies the expression of the invasion associated proteins.

Taken together, our results confirm the association between EWS/FLI1 and miR-708. However, despite downregulated in EWS samples and being previously described as a chemosensitizer, this miRNA does not affect tumor growth but instead it strongly stimulates tumor invasion not being appropriate for future therapeutic intervention.

Conflict of interest

None declared.

Financial support

FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) [Grant 2014/03877-3] and fellowships: GMR [2015/00524-5], LEAD [2014/07117-3], and GMV [2014/07118-0].

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