MOLECULAR ONCOLOGY 10 (2016) 1043–1053

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miR-200c and phospho-AKT as prognostic factors and mediators of osteosarcoma progression and lung metastasis

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ARTICLE INFO

Article history:
Received 3 February 2016
Received in revised form
23 March 2016
Accepted 15 April 2016
Available online 23 April 2016

Keywords:
Pediatric osteosarcoma
miR-200c
Mesenchymal to
epithelial transition
Phospho-AKT
Lung metastasis

ABSTRACT

Lung metastasis is the major cause of death in osteosarcoma patients. However, molecular mechanisms underlying this metastasis remain poorly understood. To identify key molecules related with pulmonary metastasis of pediatric osteosarcomas, we analyzed high-throughput miRNA expression in a cohort of 11 primary tumors and 15 lung metastases. Results were further validated with an independent cohort of 10 primary tumors and 6 metastases. In parallel, we performed immunohistochemical analysis of activated signaling pathways in 36 primary osteosarcomas. Only phospho-AKT associated with lower overall survival in primary tumors, supporting its role in osteosarcoma progression. CTNNB1 expression also associated with lower overall survival but was not strong enough to be considered an independent variable. Interestingly, miR-200c was overexpressed in lung metastases, implicating an inhibitory feed-back loop to PI3K-AKT. Moreover, transfection of miR200c-mimic in U2-OS cells reduced phospho-AKT levels but increased cellular migration and proliferation. Notably, miR-200c expression strongly correlated with miR-141 and with the osteogenic inhibitor miR-375, all implicated in epithelial to mesenchymal transition. These findings contrast epithelial tumors where reduced miR-200c expression promotes metastasis. Indeed, we noted that osteosarcoma cells in the lung also expressed the epithelial marker CDH1, revealing a change in their mesenchymal phenotype. We propose that miR-200c upregulation occurs late in osteosarcoma progression to provide cells with an epithelial phenotype that facilitates their integration in the metastatic lung niche. Thus, our findings identify phospho-AKT in the primary tumor and miR-200c later during

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http://dx.doi.org/10.1016/j.molonc.2016.04.004

tumor progression as prognostic molecules and potential therapeutic targets to prevent progression and metastasis of pediatric osteosarcomas.

1. Introduction

Osteosarcoma is the most common primary high grade bone malignancy with highest incidence in children and adolescents (Arndt et al., 2012). Current survival rates vary by age, disease stage, anatomic site and histologic response to presurgical chemotherapy, ranging from 55 to 75% for children and adolescents in most countries (Savage and Mirabello, 2011). However, survival rate is reduced to 10–30% if there is metastatic disease at diagnosis (Kager et al., 2003). After recurrence, mainly to the lungs, there are limited treatment options that are frequently unsuccessful. Thus, there is an urgent need to identify new druggable targets for precision medicine treatments.

MicroRNAs (miRNAs) are important epigenetic regulators in cellular physiology as well as in the pathogenesis of human cancers, including osteosarcomas. Several miRNAs have already been reported to regulate signaling networks in osteosarcoma cells (reviewed in Chang et al., 2015). Among them, those targeting the insulin-like growth factor-1 IGF-I signaling pathway are of particular interest since this pathway regulates pleiotropic responses that vary from cellular metabolism, proliferation, differentiation and apoptosis. Regulation of the IGF-I signaling pathway and mutations in PI3K, one of its downstream targets, have been described to be of relevance in osteosarcoma, correlating with poor prognosis (Choy et al., 2012; Su et al., 2011). In addition, the monoclonal antibody R1507 targeting IGF-IR was reported to be effective alone or in combination with rapamycin in inhibiting growth of osteosarcoma xenografts (Kolb et al., 2010). Moreover, increased expression of IGF-IR has been correlated with tumor metastasis and prognosis in patients with osteosarcoma (Wang et al., 2012). Notably, an orthotopic study in mice with phospho-specific antibodies and kinase inhibitors revealed that only IGF-IR/MEK pathway, but not IGF-IR/AKT pathway, remain active in lung metastasis, suggesting that Ras/Raf/MEK/ERK signaling may play an important role in osteosarcoma lung metastasis (Yu et al., 2011). In addition, c-Myc overexpression enhanced MG-63 and SAOS-2 osteosarcoma cells invasion through the activation of MEK-ERK pathway whereas inhibited the activity of PI3K-AKT pathway (Han et al., 2012). Further downstream in IGF-I signaling is the Nuclear Receptor Coactivator 3 (NCOA3), which was reported to be overexpressed in osteosarcomas driving its progression (Geng et al., 2014). Strikingly, activation of PI3K/AKT upregulates NCOA3 levels by inhibiting its proteasomal degradation (Ferrero et al., 2008), which may result in a feedback loop by increasing IGF-I levels that act in an auto or paracrine manner to activate both, Ras and PI3K pathways. Together, these results support IGF-I signaling as an important growth and survival pathway in osteosarcoma cells.

Different miRNAs in osteosarcoma have been reported to target IGF-I signal transducers and transcription factors responsible for cellular invasion, epithelial to mesenchymal transition (EMT), stemness and chemoresistance. The IGF-IR itself is targeted by miR-133b, frequently downregulated in osteosarcomas (Zhao et al., 2013). PTEN is regulated by miR-93 and miR-23a, resulting in increased proliferation and migration (Kawano et al., 2015; Tian et al., 2015). PTEN is also the target of miR-144 in nasopharyngeal carcinomas (Zhang et al., 2013). However, this tumor suppressor-like behavior described in epithelial carcinomas has a reversed effect in osteosarcoma cells, where its downregulation is associated with cell proliferation and invasion by downregulating its target gene, TAGLN (Zhao et al., 2014). Besides, miR-144 may also regulate migration and invasion of osteosarcoma cells by targeting the cell adhesion protein Ezrin (Cui and Wang, 2015). FOXO1 is a transcriptional factor downstream of the PI3K pathway and a positive regulator of bone formation in osteoblasts (Rached et al., 2010). Interestingly, FOXO1 has recently been reported to be the target of miR-135b and miR-374, thus promoting proliferation and invasion in osteosarcomas (He et al., 2015; Pei et al., 2015). miR-101 and miR-155 have been shown to favor and to inhibit chemosensitivity by respectively blocking and inducing autophagy (Chang et al., 2014; Chen et al., 2014). These counteracting effects are somewhat controversial since both miRNAs target the same pathway, but at different levels: miR-101 downregulates mTOR expression levels (Merkel et al., 2010), a major regulator of autophagy together with AMPK, whereas miR-155 targets the alpha regulatory subunit of PI3K (p85), an upstream activator of mTOR (Huang et al., 2012). One plausible explanation could rely on the promiscuity of miRNAs so that the final outcome depends on other targets regulated by miR-101 and miR-155. In this regard, miR23a was also shown to target molecules other than PTEN that may enhance its oncogenic potential. Overexpression of miR-23a inhibited osteosarcoma HOS cells differentiation by downregulating connexin-43 (Cx43/GJA1), a mediator of intercellular signaling critical to osteoblast development (Gindin et al., 2015). In the same cluster as miR-23a, miR-27a promotes pulmonary osteosarcoma metastasis by downregulating the transcriptional regulator CBFA2T3 (Salah et al., 2015).

Here we demonstrate that miR-200c is upregulated in lung metastasis and ectopic expression of miR-200c in U2-OS cells increased cellular proliferation and migration but reduced basal phospho-AKT levels. In addition, multivariate analysis of immunohistochemical staining for signaling pathways showed a significant association of Akt activation with poorer prognosis. Based on the inhibitory role of miR200 family on Akt signaling, these results suggest that Akt and miR-200c contribute to osteosarcoma progression through converging mechanisms.

2. Materials and methods

2.1. Patients and samples

A retrospective study was performed in a primary cohort of 36 patients with primary osteosarcomas who underwent treatment between 1990 and 2011. These cases were collected from the archives of Hospital La Fe in Valencia. Clinical characteristics for each patient are indicated in Supplementary Table 1 and summarized in Table 1. For miRNA studies, nucleic acids were collected from 11 primary osteosarcomas plus 15 pulmonary metastases. A validation cohort of 16 patients was collected from the archives of the Pathology Department of the University of Valencia and University Clinic Hospital. The clinical characteristics of patients included in this second cohort are summarized in Supplementary Table 2. All samples from each cohort were reviewed independently by two expert pathologists to confirm histology and select tumor areas for tissue microarray construction and nucleic acid isolation. Written informed consent was signed by the patients; when not possible (dead or unreachable patients), the study material was used after decoding in accordance with Spanish law and with the approval of the Institutional Review Board. All procedures were done in accordance with the Helsinki declaration.

Table 1 – Summary of clinicopathologic features of 36 patients with primary osteosarcoma included in the immunohistochemical study.

Characteristics	n (%) *Median (range)
Age	14.47 (6.71–20.99)*
Gender	
Male	18 (50%)
Female	18 (50%)
Localized disease	33 (92%)
Site of primary disease	
Femur	20 (56%)
Tibia	8 (22%)
Humerus	3 (8%)
Fibula	1 (3%)
Pelvis	1 (3%)
Other	3 (8%)
Tumor sample analyzed	
Diagnostic biopsy (pre-chemotherapy)	16 (44%)
Surgery (post-chemotherapy)	20 (56%)
Treatment	
Metotrexate	34 (94%)
Ifosfamide	24 (67%)
Doxorrubicin	36 (100%)
Cisplatin	35 (97%)
VP-16	6 (17%)
Ciclophosfamide	16 (50%)
Type of surgery	
Conservative	25 (69%)
Radical	10 (28%)
Necrosis rate	70% (0–100%)*
Relapse/Progression	23 (64%)
Survivors	17 (47%)
Follow-up time (survivors)	11.11 years (2.1–26.4)*

2.2. Tissue microarray assembling and immunohistochemical analysis

Two 1 mm diameter cores were punched from each paraffin block using a manual tissue arrayer and mounted in an array paraffin block. Internal controls were also included in the tissue array. To correlate activated signaling pathways with clinical data, we selected phospho-AKT and phospho-ERK as two major druggable pathways activated by multiple signaling molecules with high relevance in osteosarcoma, including growth factors and tyrosine kinase receptors such as IGF-I and IGF-IR. We evaluated the activation of WNT/β-catenin (CTNNB1) pathway based on the fact that dysregulation of beta-catenin signaling is common in osteosarcoma and is implicated in the pathogenesis of osteosarcoma (Haydon et al., 2002). In addition, we included the evaluation of Sonic Hedgehog (SHH)/Gli1 activation in the immunohistochemical analysis because it is a druggable pathway and has been implicated in osteosarcoma (Lo et al., 2014).

3 μm Sections from the paraffin-embedded microarray were stained with the following antibodies: Akt-pS473, Phosphorylation Site Specific (Dako M3628); β-catenin (Dako M3539); phospho-Erk1,2 (Cell Signaling 4370); Gli1 (R&D Systems AF3324). For phospho-AKT, phospho-ERK and β-catenin both, cytosolic or nuclear stainings were considered as positive. For Gli1 only nuclear stainings were considered as positive. Immunohistochemistry was performed after confirming by hematoxylin/eosin staining that the samples in the tissue microarray were representative.

For immunoreactivity evaluation we used a previously reported semi-quantitative scoring system based on the overall stain intensity and percentage of neoplastic stained cells (Hatanaka et al., 2003). Briefly, the HSCORE was based on the percentage of neoplastic cells stained (Pi) and their staining intensity (i) and was calculated in each case according to the formula: HSCORE = $\sum$(i × Pi) where i = 0, 1, 2, 3 and Pi varies from 0 to 100%. Hence, the range for the HSCORE was 0–300. Each slide was evaluated separately by 2 independent pathologists (M.H. and M.L.) and mean value of both cores was calculated per tumor sample. In case of discrepancy between pathologists, slides were re-evaluated and consensus final score agreed. The Youden index was used as a *criterion* for selecting the optimum cut-off points. Cut-off values of 40 for either phosphor-AKT or β-catenin stainings were determined by ROC curve analysis. These data are provided in Supplementary Table 1.

2.3. miRNA isolation and array hybridization

Tumor areas were carefully selected avoiding adjacent normal or necrotic tissue. Five core punches were obtained from each FFPE block. AllPrep® FFPE kit (QIAGEN) was used to isolate RNA and DNA when possible. Samples were deparaffinized according to the manufacturer's instructions and proteinase K digested 24–48 h according to visual inspection tissue disaggregation. After RNA supernatant and DNA pellet separation, RNA was incubated at 80 °C to partially reverse formalin cross-linking, column purified and DNase digested. Total RNA was eluted in 14 μl H_2O. RNA purity and concentration were evaluated spectrophotometrically and fluorimetrically by NanoDrop ND-2000 (ThermoFisher) and Qubit (Invitrogen). Spectrophotometry measurements at three different wavelengths (230, 260 and 280 nm) and A260/230 and A260/280

ratios were used to assess the presence of contaminants: peptides, phenols, aromatic compounds, or carbohydrates and proteins. Qubit RNA HS kit was applied to highly degraded RNA samples to accurate quantitation of double stranded DNA and RNA respectively. Integrity and related size of RNA were assessed by microfluidics-based platform Agilent 2100 Bioanalyzer with RNA 6000 Nano Kit. Electropherograms were visualized at the Agilent 2100 Expert software including data collection, peak detection and interpretation of profiles.

RNA tumor samples were analyzed with miRNA Affymetrix platform miRNA 3.0 to obtain the miRNA and other non-coding RNA profile. 50 ng Of total RNA were added to the labeling mix following FlashTag miRNA (Genisphere) direct labeling manufacturer procedures. The GeneChip® Scanner 3000 7G System and reagents from Affymetrix were used to hybridize, wash, stain and scan the arrays. Expression console free software from Affymetrix was used for quality control and probe set normalized values assessment.

2.4. Statistical analysis

Data were summarized using mean, median, standard deviation and range in the case of continuous variables and with relative and absolute frequencies in the case of categorical variables. Differences between survival curves were assessed using the Log rank test. Signaling pathways found to be significant were evaluated with multivariable Cox regression analysis. Differentially expressed miRNAs between primary and metastatic tumors were assessed using an Elastic Net penalized logistic regression model. Differences between primary and metastatic tumor for each miRNAs selected for validation were assessed using the Wilcoxon–Mann Whitney test. p values <0.05 were considered statistically significant. All analyses were performed using R (version 3.2.2).

2.5. Tissue culture and transfection conditions

U2-OS cells were grown in DMEM media supplemented with 10% fetal bovine serum plus antibiotics. One day before transfection cells were trypsinized, counted and 10^5 cells per well were seeded in 6 well plates. On the day of the transfection media was replaced with 1 ml of media without serum and antibiotics. miR-200c mimic (Sigma) was diluted in 150 μl Opti-MEM media (Invitrogen) and mixed with 150 μl of Opti-MEM containing 9 μl of RNAiMAX (Invitrogen). 250 μl Of the mixture containing miR-200c mimic was added drop by drop to the cells to a final concentration of 15 nM. Eight hours post-transfection media was replaced with regular fresh growing media.

Twenty four hours post-transfection cells were trypsinized and counted before using them in migration and proliferation assays. For wound healing assays 2×10^5 cells were seeded in triplicates in Cytoselect™ 24-well plates. One day later the insert was carefully removed and healing of the wound was monitored. Pictures were taken every 2 h with an inverted microscope. For proliferation assays, 10^3 or 3×10^3 cells were seeded in triplicates in 96-well plates. Colorimetric assay with XTT (Roche) was determined at the indicated days following manufacturer's instructions for use.

In parallel, miRNA was isolated with miRNeasy Mini Kit (QIAGEN) two days after the transfection and miR-200c-3p (Applied biosystems No. 002300, Cat. 442797) and RNU6B (Applied biosystems No. 001093 Cat. 4427975) as endogenous control were quantified by real-time PCR using TaqMan microRNA assays (Applied biosystems).

3. Results

3.1. Identification of prognostic signaling pathways in primary osteosarcomas

To identify druggable pathways that correlate with poor prognosis in child and adolescent osteosarcomas, we conducted a multivariate immunohistochemical analysis in a cohort of 36 primary osteosarcoma cases (see Table 1 for clinical variables). Immunostaining analysis for the activation of the PI3K-AKT pathway was determined with pAKT (Ser473) antibodies and revealed a broad variability in both, the intensity and the distribution pattern (Figure 1A). Evaluation of the immunostainings by H-score revealed a significant correlation between activated AKT and poorer prognosis in primary osteosarcomas before chemotherapy (Figure 1B), supporting the role of this pathway in osteosarcoma progression. In contrast, no significant correlation was found when the analysis was done with 20 tumor samples obtained after chemotherapy (Figure 1C), suggesting that tumor response to chemotherapy may result in temporal or permanent loss of AKT activation and therefore, tissues samples obtained post-chemotherapy may not be appropriate for phospho-AKT analysis to predict outcome. Hence, these data support a role for AKT activation in osteosarcoma progression and suggest that immunohistochemical analysis of pre-chemotherapy biopsies is a powerful predictive tool to carefully-select pediatric patients for treatment with inhibitors of this pathway as a strategy to improve outcome.

We also evaluated the role of WNT signaling pathway in osteosarcoma prognosis by analyzing the cytosolic/nuclear expression of CTNNB1 (β-catenin). Cytosolic staining was scored as positive since increased cellular cytosolic β-catenin levels have been associated with increased β-catenin target protein expression (Sellin et al., 2001); most non-membrane expression of β-catenin is cytosolic in breast cancer and is associated with poor outcome (Lopez-Knowles et al., 2010). Only 28% of the osteosarcomas expressed β-catenin (Supplementary Figure 1A), somewhat lower than pAKT positive cases (44%). Interestingly, positive immunostaining for β-catenin only correlated with lower overall survival in osteosarcoma primary tumors after chemotherapy (Supplementary Figure 1B) but not in pre-chemotherapy tumors alone (Supplementary Figure 1C). However, β-catenin positivity was not strong enough to be considered an independent variable (Supplementary Table 3), perhaps reflecting the need for additional cases in the cohort.

3.2. Differential expression of miRNAs between primary osteosarcomas and lung metastases in the primary cohort

Increasing evidence supports the role of miRNAs in cancer etiology and progression. To identify targets that may regulate osteosarcoma progression, we conducted a high-throughput screening of miRNAs by comparing primary tumors vs. resected lung metastases. In this first cohort, 11 primary

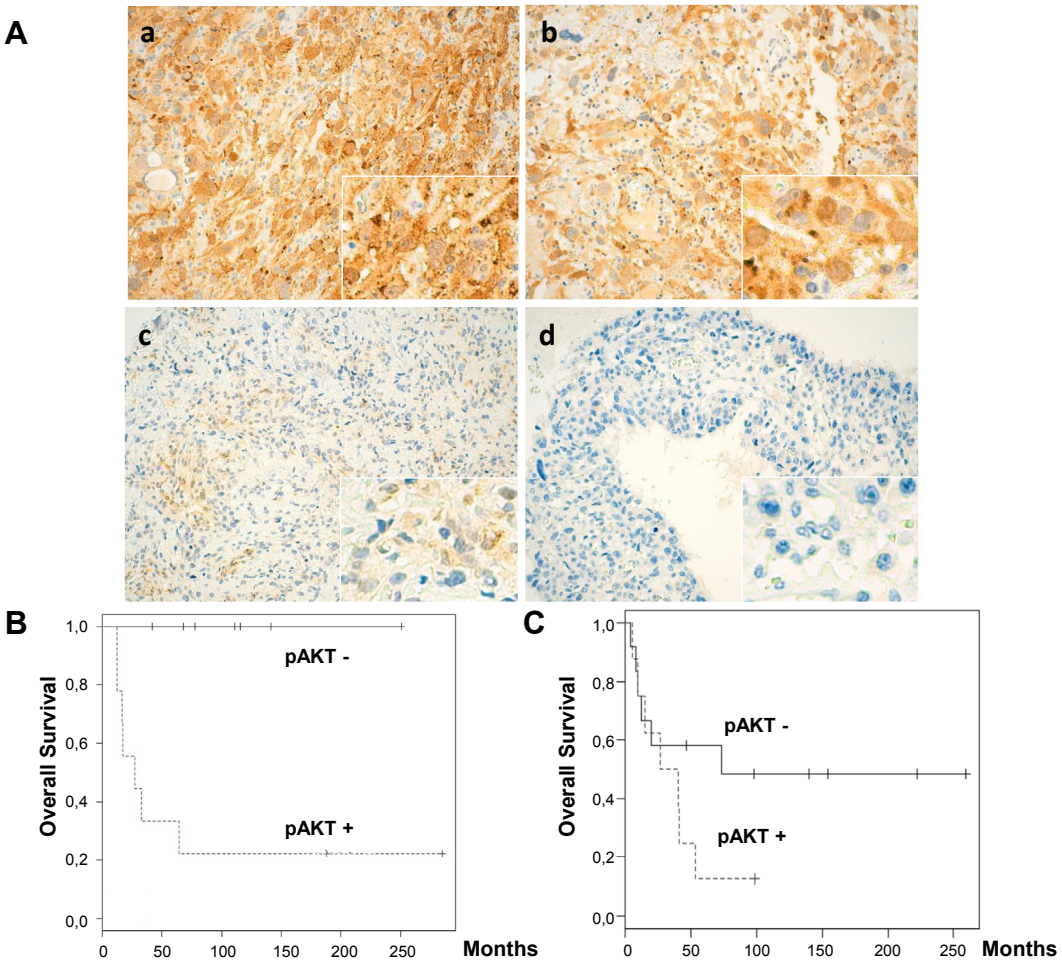

Figure 1 – Akt activation correlates with lower overall survival in primary osteosarcomas. (A) Representative immunostaining with phosphor-AKT (Ser473) antibodies showing variability from 95% of stained osteosarcoma cells with 3+ intensity (a) to 0% of stained cells (d). Inlets in the bottom right corner are magnifications to show the details of the stainings. (B) Kaplan–Meier survival curves in 16 primary osteosarcomas before chemotherapy; (Log Rank test, p = 0.003). (C) Kaplan–Meier survival curves in 20 primary osteosarcoma cases obtained after chemotherapy; (Log Rank test, p = 0.173).

tumors were compared to 15 lung metastases, all from La Fe hospital. Punches from paraffin-embedded tissues were selected carefully by pathologists to avoid contamination by non-tumorigenic tissue. Using Elastic Net to perform a penalized logistic regression analysis of the microarray data, we generated a heatmap model of 26 miRNAs that were differentially expressed between primary and metastatic tumors (Figure 2A). Of these, only means for mirR200c, miR4786-3p and ENSG00000212490_x displayed at least a two-fold difference and were thus selected for further validation.

3.3. *Differential expression of miRNA between primary osteosarcomas and lung metastases in the validation cohort*

A second cohort of 10 primary tumors and 6 metastases from a different hospital (University Hospital, Valencia) were used to validate the results of the miRNAs in osteosarcoma metastases. In this second analysis, only expression of miR-200c was significantly changed between primary and metastatic osteosarcomas (Figure 2B). Collectively, these results strongly support a role for miR200c in the molecular processes of lung metastasis. Interestingly, expression analysis revealed a strong correlation between miR200c and either miR-375 or miR141 (Figure 3); these particular miRNAs are involved in EMT as well as in a negative regulation of PI3K/AKT pathway. Expression of miR-30a also displayed a correlation with miR200c, but to a lower extent than miR-375 and miR-141 ($R = 0.73$, $p = 0.008$, data not shown), suggesting a role in immunosuppressive functions as well as in mesenchymal to epithelial transition (MET) and in inhibition of osteogenic differentiation. We did not detect an inverse correlation between the expression of any miRNAs and pAKT, β-catenin, or other clinical parameters except lung metastases. This lack of correlation between miRNAs and activated AKT may reflect the need for increasing the n numbers of osteosarcoma cases or alternatively, these may simply be unrelated variables. Thus, although we cannot yet conclude whether miR-200c and pAKT are regulated by independent pathways, our results clearly demonstrate that both molecules have significant value in predicting osteosarcoma progression.

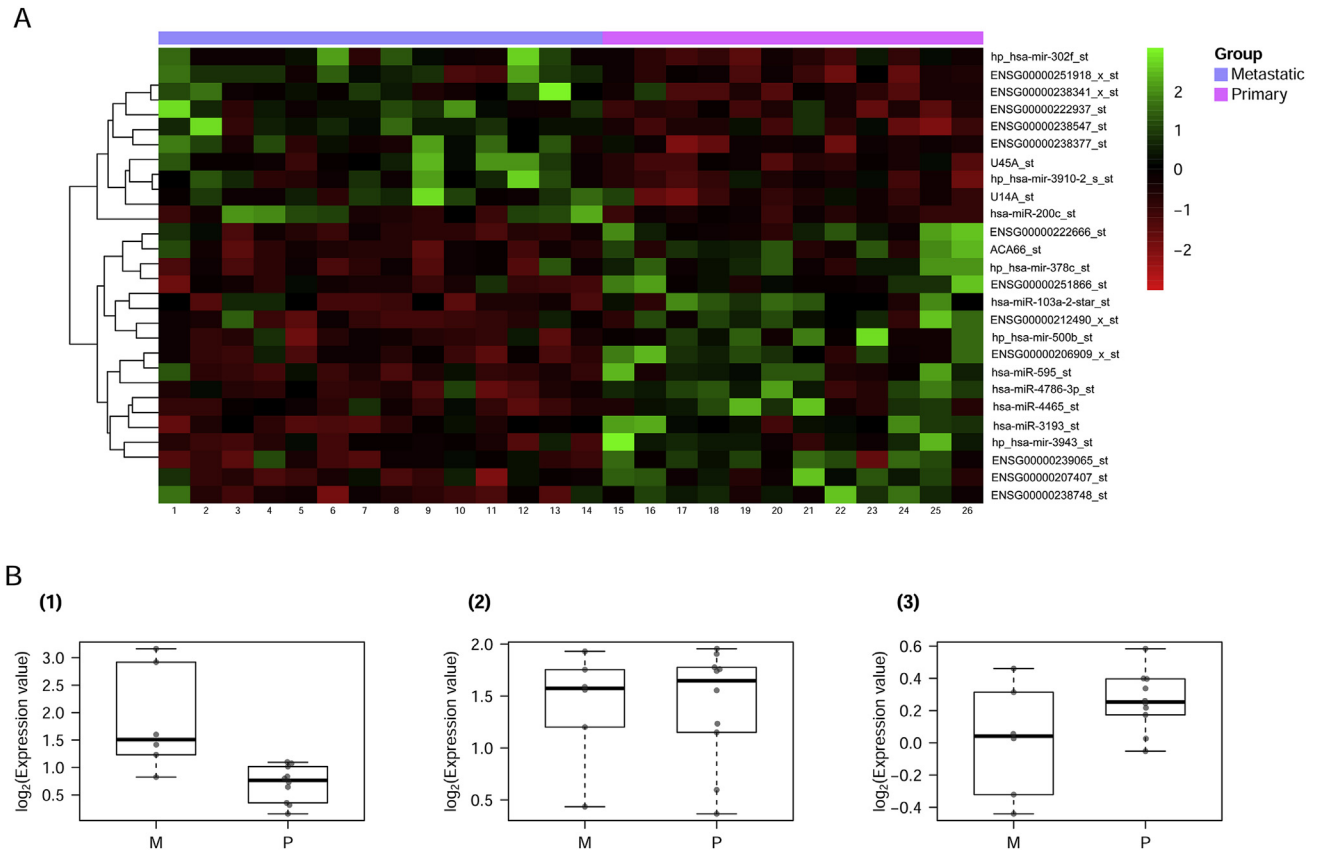

Figure 2 – miR-200c expression associates with osteosarcoma pulmonary metastasis. (A) Heatmap of selected miRNAs by the Elastic Net regression model in a cohort of 26 osteosarcomas. Each row shows data for a specific miRNA in every osteosarcoma case and each column represents the miRNA expression profile for every osteosarcoma. Rows have been ordered according to the results of the Ward hierarchical clustering algorithm. Specific values in each row have been standardized to z-scores (scale on the top right) based on the variable means for each miRNA. Primary or metastatic condition for the osteosarcoma cases are indicated above the heatmap. (B) miRNA validation in a new cohort of 16 osteosarcomas. Boxplots of differential expression between 6 primary (P) and 10 metastatic (M) osteosarcomas in (1) mirR200c, $p = 0.003$; (2) miR4786-3p, $p = 0.96$; and (3) ENSG00000212490_x, $p = 0.23$.

3.4. *Exogenous expression of miR-200c in osteosarcoma cells enhances cellular migration and proliferation*

In contrast to epithelial cancer cells where miR-200c expression blocks EMT, proliferation, and metastasis (Gregory et al., 2008; Hur et al., 2013; Ibrahim et al., 2015; Liu et al., 2014; Song et al., 2015; Tang et al., 2013; Yu et al., 2010), our analysis of osteosarcoma tumors indicate a robust correlation between miR-200c expression and metastasis. To further test this novel paradigm, we performed a series of *in vitro* experiments with U2-OS cells, a human cell line originally derived from a primary osteosarcoma. Transfection of miR-200c mimic into U2-OS cells increased significantly cellular migration (Figure 4). We next evaluated the role of miR-200c in cellular proliferation by analyzing the growth of U2-OS cells. Cultures transfected with miR-200c mimic displayed a higher proliferation ratio than controls (Figure 5A). Moreover, the enhanced proliferation correlated with higher expression levels of cyclin A expression (Figure 5B), a hallmark of proliferative cells. Interestingly, miR-200c mimic transfection resulted in lower basal pAKT levels in U2-OS cells, a result that also supports its long-term role in MET. Based on this result, it is possible that pAKT and miR-200c are inversely correlated *in vivo* in osteosarcoma tumors. However, using our cohorts of patients, we could not establish a significant correlation between pAKT and miR-200c ($R = -0.207$, $p = 0.34$), perhaps owing to the reduced number of cases where data for both biomarkers was available ($n = 23$). Alternatively, pAKT and miR-200c may reflect two distinct mechanisms which culminate in metastasis: cells that use pAKT pathway for metastasis do not necessarily require miR-200c expression, whereas cells that use miR-200c overexpression will display reduced pAKT.

3.5. *Pulmonary osteosarcoma metastases express epithelial marker E-cadherin*

Based on these results, our working hypothesis is that overexpression of miR-200c is one of the mechanisms underlying lung metastasis in osteosarcoma. To test this hypothesis, we analyzed E-cadherin expression in metastatic osteosarcoma tumors. As expected, some cells within each tumor stained positive for E-cadherin (Figure 5C). Immunohistochemistry may not be sensitive enough to detect E-cadherin in cells

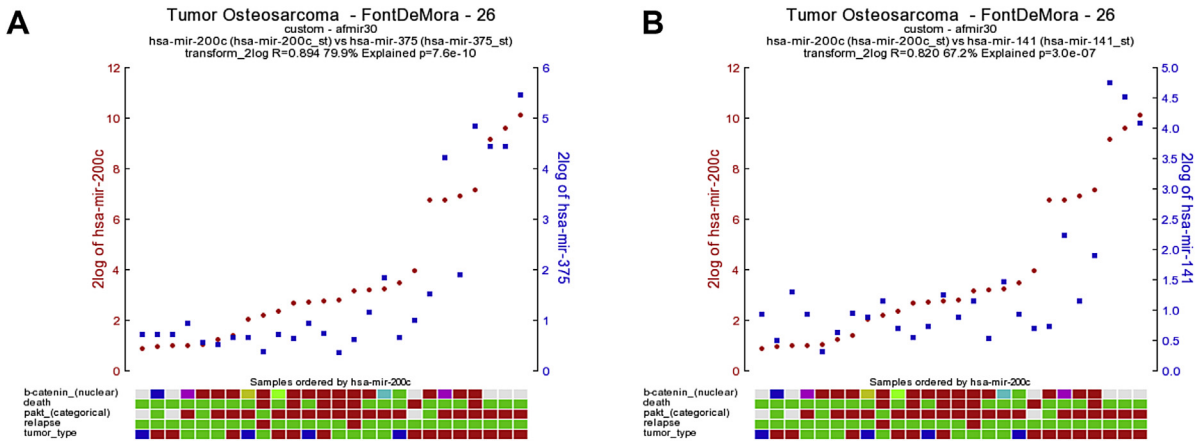

Figure 3 – Correlation between the expression levels of miR-200c and miR-375 or miR-141 in osteosarcomas. Red dots represent the expression levels of miR-200c and blue dots represent the expression levels of (A) miR-375 ($R = 0.894$, $p < 0.001$), or (B) miR-141($R = 0.82$, $p < 0.001$). Each pair of red-blue dots represents a patient for which clinical and histological data are indicated at the bottom. Data was uploaded and analyzed in R2: microarray analysis and visualization platform (http://r2.amc.nl) revealed a very strong correlation between the expression of miR-200c with (A) miR-375 and (B) miR-141.

with low levels of expression which require other techniques such as RT-PCR or microarray analysis. Indeed, *in silico* analysis using Guenther expression data available at R2 (http://r2.amc.nl) revealed a strong increase of E-cadherin expression in osteosarcoma lung metastases vs. primary tumors (Figure 5D), further supporting our hypothesis that osteosarcomas frequently undergo MET to metastasize to the lung.

4. Discussion

In the present study, we compared expression patterns in primary osteosarcomas with lung metastases in an effort to identify molecular pathways that potentially mediate metastasis of this cancer. Our findings reveal that phosphorylated AKT is enhanced in primary tumors whereas expression of miR-200c is upregulated in lung metastases. Relapsed osteosarcoma is characterized by complex genes and signaling pathways driving its development and metastasis (Moriarity et al., 2015). Among the different pathways, activation of AKT has been implicated in pulmonary metastasis using cell line models of osteosarcoma (Fukaya et al., 2005). However, to our knowledge, this is the first report that activation of AKT correlates with outcome in primary human osteosarcoma tumors. PI3K-AKT signaling pathway regulates pleiotropic effects in the cell and is a major target to prevent progression. Upstream targets such as FGFR1 (Weekes et al., 2015) or IGF-IR (Wang et al., 2012) have also suggested to be efficient therapies in osteosarcoma. Nevertheless, targeted therapy combining PI3K-AKT inhibitors plus other drugs to match additional alterations present in the patient was not sufficient to prevent progression (Subbiah et al., 2015). Therefore, other intracellular signaling pathways not explored before are critical for the pulmonary metastasis of osteosarcoma.

The findings of the present study demonstrate a new role for miR-200c in mediating the lung metastasis associated with osteosarcoma. Our findings contrast the published role of miR-200c in tumors of epithelial origin where it functions as a tumor suppressor by inhibiting epithelial to mesenchymal transition (EMT) through the downregulation of ZEB1 (Burk et al., 2008; Gregory et al., 2008; Park et al., 2008). In two independent cohorts of osteosarcoma patients, we observed that miR-200c is overexpressed in lung metastasis. In addition to ZEB1, miR-200c also targets the transcription factors BMI1, E2F3 (Liu et al., 2014) and SIP1 (Gregory et al., 2008), all of which are involved in regulation of EMT. Given the apparent contrast of its expression pattern between epithelial tumors and lung metastasis, overexpressed miR-200c may facilitate osteosarcoma progression by promoting MET rather than EMT in tumor cells. Interestingly, expression of miR-141, another member of the miR-200 family that clusters with miR-200c in chromosome 12p13.31, also correlated with miR-200c expression in our studies of pulmonary metastasis. This result suggests a common regulatory element shared by both miRNAs in the regulation of cellular differentiation. Based on the emerging evidence that supports a role for miR-200c in promoting EMT, our results suggest the novel concept that miR-200c facilitates osteosarcoma metastasis by facilitating the reverse process of MET. These results may also explain why osteosarcomas metastasize mainly in the epithelial niche of lungs (where nearly any cancer can spread) and not in bone marrow or other sites.

The WNT/β-catenin pathway has been associated with bone cancers (reviewed in Tian et al., 2014) and its upregulation promotes EMT in osteosarcoma cells (Lv et al., 2016). In the present study, we demonstrate that activation of β-catenin in primary osteosarcomas after chemotherapy associates with poorer prognosis. Both AKT and β-catenin activation promote EMT and are prognostic biomarkers in primary tumors. However, many lung metastases show increased expression of the epithelial markers CDH1 and miR-200c. These results suggest that during osteosarcoma progression a shift to MET occurs, probably due to the gain of additional genetic or epigenetic alterations.

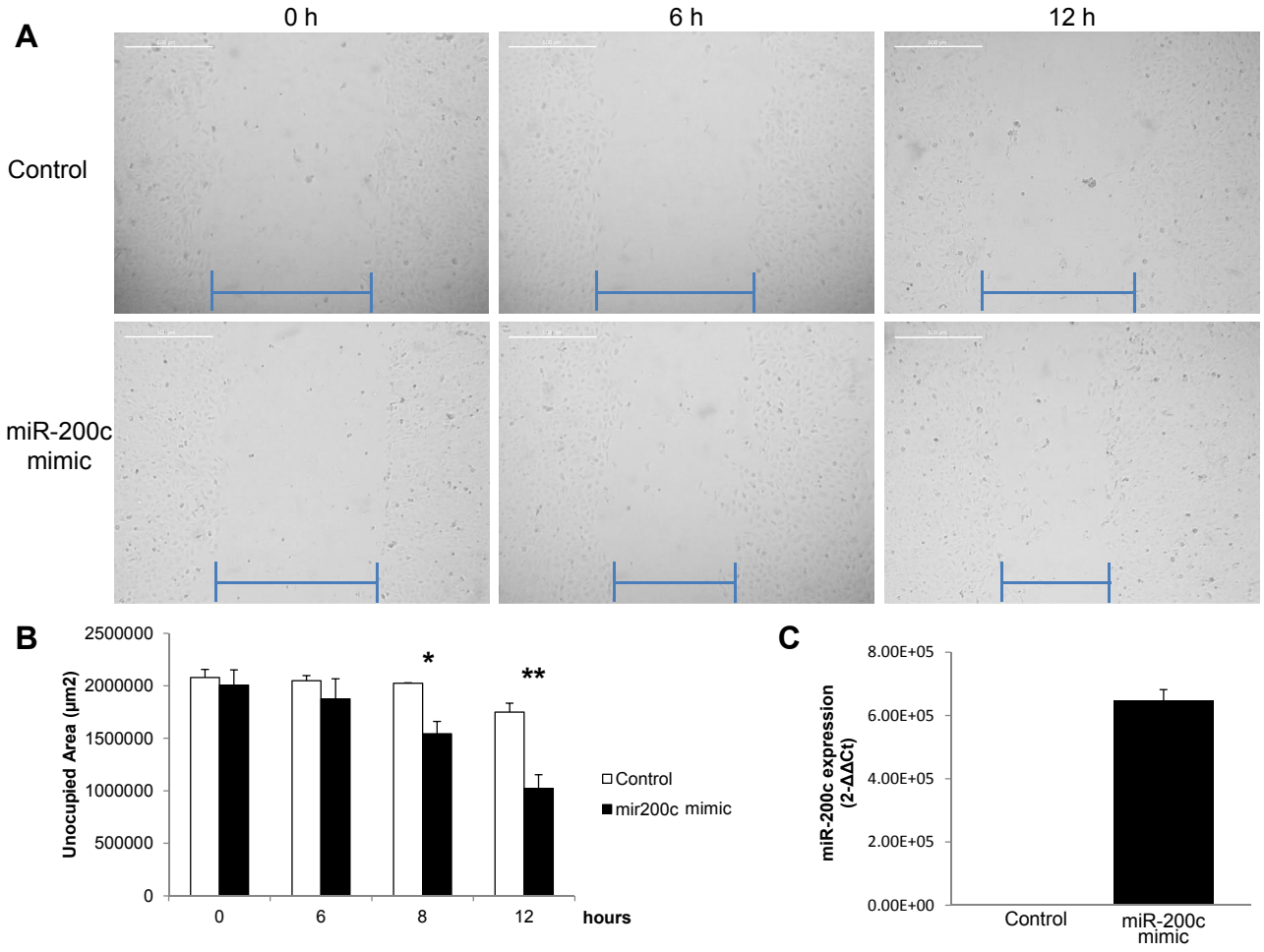

Figure 4 – Expression of miR-200c mimic in U2-OS cells enhances cellular migration. (A) Representative images of U2-OS cells transfected with miR-200c mimic or control. Images were taken at the indicated times after removing the wound field inserts. Migration was measured by wound healing assay. White bars correspond to 500 µm. Bottom bars indicate the average unoccupied distance for each image. (B) Unoccupied areas corresponding to wound fields were measured on triplicate images using *ImageTool* v3.0 software. Bars correspond to means of three independent experiments ± standard error. *p = 0.03; **p = 0.04. (C) Quantitative PCR analysis of U2-OS cells to show the efficiency of the transfection with mir200c two days later.

One potential explanation for the increased expression of miR-200c in the lung metastasis as compared to primary tumors is the possibility that miR200c appears late in osteosarcoma progression. Therefore, circulating cells derived from the primary tumor would contain high levels of miR-200c and this enhanced expression would favor their retention and growth in the lung. A recent analysis of pre-chemotherapy biopsy samples reported expression of miR-27a and miR-181c* in patients who later developed clinical metastatic disease (Jones et al., 2012). However, miR-200c expression was not detected in these primary tumors, supporting our hypothesis that miR200c expression occurs during later stages of osteosarcoma progression. In our study, miR-200c was overexpressed in 50% of all metastases, perhaps indicating that molecules other than miR-200c also regulate osteosarcoma progression. Further studies will be needed to elucidate additional mechanisms that regulate lung metastases. We cannot exclude the remote possibility that detection of miR200c expression in lung metastases is the result of epithelial contamination by pulmonary cells. However, the areas of the tumor used for miRNAs analysis were carefully selected by skilled pathologists in each cohort. Additionally, 6 out of 12 primary osteosarcomas also displayed miR200c expression (see middle samples with green and blue dots under "tumor_type" in Figure 5), suggesting that its detection is not exclusive to lung metastases. Moreover, lung metastases express higher E-cadherin levels than primary osteosarcomas (Figure 5D), further supporting the notion that osteosarcoma metastases undergo MET for which miR200c expression is required.

We also found a strong correlation between miR-200c expression and miR-375 in osteosarcomas. miR-375 negatively regulates PDK-1 expression and PI3K-AKT signaling (Garikipati et al., 2015). Interestingly, miR-375 was able to inhibit osteogenic differentiation via the regulation of RUNX2 expression (Du et al., 2015). Several experimental approaches have led to the proposal that RUNX2 expression in breast cancers may explain their metastatic preference for bone (Taipaleenmaki et al., 2015; Zhang et al., 2015). Following

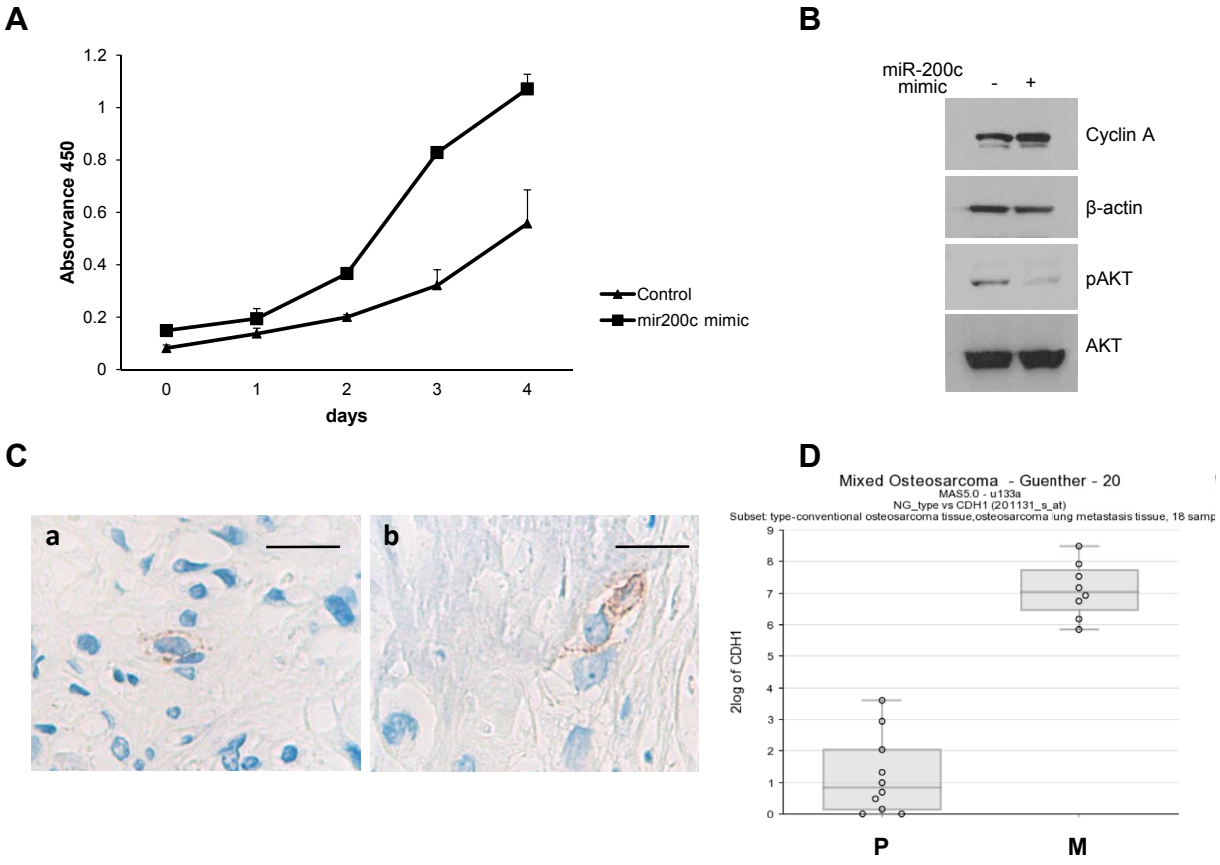

Figure 5 – miR-200c mimic increases proliferation ratio in U2-OS cells. (A) U2-OS cells were transfected with miR-200c mimic or control and next day cells were collected and seeded at 10^3 cells per well in 96 well plates. Proliferation was determined at the indicated times by XTT colorimetric assay. Bars correspond to means of three independent experiments ± standard error. (B) Western blot analysis with the indicated antibodies of U2-OS cells transfected two days before with miR-200c mimic or control. (C) Immunohistochemical staining for E-cadherin reveals a weak and heterogeneous expression in lung metastatic cells. Centered cells in (a) and (b) show membrane expression of E-cadherin in two different tumors with highest miR-200c expression (see Figure 3). Bars correspond to 20 μm. (D) Boxplot representation of E-cadherin (CDH1) differential expression between primary osteosarcomas (P) and lung metastases (M). One way analysis of variance (ANOVA, $p < 0.001$). Total RNA was isolated from osteosarcoma tumors and used to hybridize Affymetrix arrays (GeneChip Human Genome u133a array). Data obtained from Guenther study at R2: microarray analysis and visualization platform (http://r2.amc.nl).

this line of reasoning, it is also possible that miR-375 expression in osteosarcoma may reduce preferences for bone metastases by targeting RUNX2. Therefore, miR-200c, miR-141 and miR-375 may promote lung metastasis by regulating different pathways in osteosarcoma cells. Collectively, our results support the idea that miR-200c signaling is a potential therapeutic target for preventing osteosarcoma metastasis and therefore, combination therapy directed against the PI3K-AKT-mTOR pathway and the miR-200c pathway may constitute a more efficient strategy for blocking osteosarcoma progression.

Acknowledgments

We are very grateful to Empar Mayordomo for helpful discussion with immunostainings and Almudena Vidal for technical assistance. We are also grateful to María Pilar Marín at Microscopy Unit IIS La Fe for acquisition of images. We are thankful for José Luis Zugaza for reviewing the manuscript. Pablo Berlanga was supported by a post-resident grant by IIS La Fe. This study was funded by grant SAF2012-37330 from Spanish Ministry of Economy and Competitivity.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2016.04.004.

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