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Letter to the Editor

MIR-10b IS inversely correlated with higher tumor grade in osteosarcoma

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To the Editor

In a recent study published in *Clinica Chimica Acta*, Huang and colleagues [1] performed a systematic assessment and meta-analysis that reinforced the up-regulation of miR-10b as an unfavorable independent factor in several adult tumors, including gliomas, breast cancer, and carcinomas from the digestive and respiratory systems. Nonetheless, despite the consensus oncogenic potential, a tumor suppressor role for this microRNA has been described in gastric [2] and cervical carcinoma [3], pointing to a tumor-dependent behavior.

On this regard, we would like to add more information about the contribution of miR-10b on osteosarcoma (OS), the most common bone tumor that affects children and adolescents [4].

The expression pattern of miR-10b was verified through TaqMan probe-based quantitative PCR (Applied Biosystems, Foster City, CA, EUA, ID 002218) in a cohort of 24 consecutive pediatric primary OS samples and 12 non-tumor bone tissues from age-matched patients used as controls. Clinical data is described in [5]. Samples were obtained after informed consent of patients or their guardians (CAAE n° 45567715.9.0000.5440) at the Clinics Hospital from the Ribeirão Preto School of Medicine - University of São Paulo.

Our results showed that even though miR-10b is not significantly altered in OS samples when compared to the control group, its levels are inversely associated with tumor grade (fold change -1.82). Overall and event-free survival were not affected (Log-Rank test $p = 0.605$ and $p = 0.525$, respectively) (data not shown) and no associations between miR-10b expression and other prognostic factors, such as Huvos grade, metastasis, relapse, or death were found (Table 1).

Further *in vitro* transfection of HOS, SAOS-2 and MG63 cell lines (all of which express even lower levels of mir-10b [-2.41 fold change]) with miR-10b-5p pre-miRNA (mirVanaTM miRNA mimic ID: MC11108, Ambion®) reinforced its probable role as a tumor suppressor. As seen in fig. 1A, augmented levels of miR-10b significantly decreased the colony formation capacity of HOS and SAOS-2 cells (suggesting an involvement of miR-10b in OS survival) and efficiently reduced the migration and invasion rates of all the OS cell lines (Fig. 1B–C). Nevertheless, miR-10b did not alter the expression of invasion/apoptosis related genes, such as *MMP2*, *ROCK1* and *BCL2* (data not shown). Likewise, mir-10b did not decrease the protein levels of its validated target HOXD10 (Fig. 1D) which is an important transcription controller known to activate and repress several genes involved with cell growth and differentiation [6] and whose suppression usually leads to an increase on several pro-metastatic gene products [7].

Nevertheless, the association between lower mir-10b expression and higher tumor grade connotes its involvement with more aggressive tumors. Moreover, even though our results did not show associations with metastasis *per se*, mir-10b levels were 1.55 times lower in tumors that further metastasized.

As stated above, our study is not the first one to oppose to the role of miR-10b as a metastamiR. Drastic reductions on proliferation, migration and invasion were observed in gastric tumors and adenomiotic epithelial cells after transfection with pre-miR-10b [2,8]. Likewise, lower levels of this miRNA were significantly associated to a more aggressive phenotype in cervical cancer [3].

Unexpectedly, mir-10b was recently described as overexpressed in OS samples, and showed to promote invasion by targeting *KLF4* in OS cell lines [9]. Nonetheless, both studies differ on several aspects: number of OS (24 *vs* 15) and non-tumor (12 *vs* 5) samples; the cell lines used; and the methodology implemented. More important, our cohort consisted solely of primary biopsy samples that were microdissected by an experienced pathologist; samples from patients that had underwent any chemotherapy before surgical intervention were emphatically excluded in order to avoid biases on gene expression. Moreover, even though both studies consider OS, the patients involved on each research have different ethnicity, which could contribute with the different findings [10–12]. In gastric cancer, miR-10b has also been described as tumor suppressor and oncomiR, depending on the evaluated cohort [2,13–15].

Taken together, our data pointed to an antitumor role of miR-10b in OS, being able to reduce colony formation and invasion capacity *in vitro* independently of HOXD10 expression. However, as both studies with OS and miR-10b presented opposed conclusions from a reduced number of OS samples (< 30), the inclusion larger cohorts would be important to confirm our results and better determine the exact role of miR-10b in this tumor.

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Table 1
miR-10b levels are inversely associated with tumor grade in osteosarcoma samples. qRT-PCR was performed using Taqman® assays according to the manufacturer's protocol on a 7500 Real Time PCR System (Applied Biosystems, Waltham, MA, USA). Small nuclear RNU6B and RNU48 were used as internal controls and the MRC5 cell line was used as calibrator. Relative expression was calculated by $2^{-\Delta\Delta CT}$ analysis method. All tests were carried out for $\alpha = 0.05$. Data was analyzed through Mann-Whitney tests performed using the SPSS 21.0 software (SPSS Inc., IL, USA). *p value compared to non-tumor tissue.

	n (%)	miR-10b expression		p value
		Median	Min–max value	
Samples				
Non-tumor tissue	12	35.61	8.47–91.61	0.107*
Osteosarcoma	24	23.21	8.81–45.07	
Cell lines	3	14.77	6.43–25.8	0.112*
Clinical features				
Age-years (%)				
< 15 years	13 (54.2%)	25.52	8.96–37.63	0.586
> 15 years	11 (45.8%)	20.24	8.81–45.07	
Sex (%)				
Female	14 (58.3%)	23.54	8.81–45.07	0.661
Male	10(41.7%)	23.21	11.58–37.63	
Malignancy grade (%)				
Grade I and II	5 (20.8%)	34.31	26.84–45.07	**0.023**
Grade III and IV	19 (79.2%)	19.96	8.81–37.63	
Huvos level (%)				
Bad responder	14 (82.4%)	22.74	8.96–45.07	0.705
Good responder	3 (17.6%)	20.24	17.06–37.24	
Metastasis (%)				
Absence	4 (16.7%)	31.98	19.87–37.24	0.188
Presence	20 (83.3%)	20.57	8.81–45.07	
Relapse (%)				
Absence	13 (54.2%)	20.24	8.81–41.87	0.434
Presence	11 (45.8%)	26.66	8.95–45.07	
Death (%)				
Absence	17 (70.8%)	26.66	11.58–45.07	0.136
Presence	7 (29.2%)	15.42	8.81–35.52	

Statistically significant value is shown in bold.

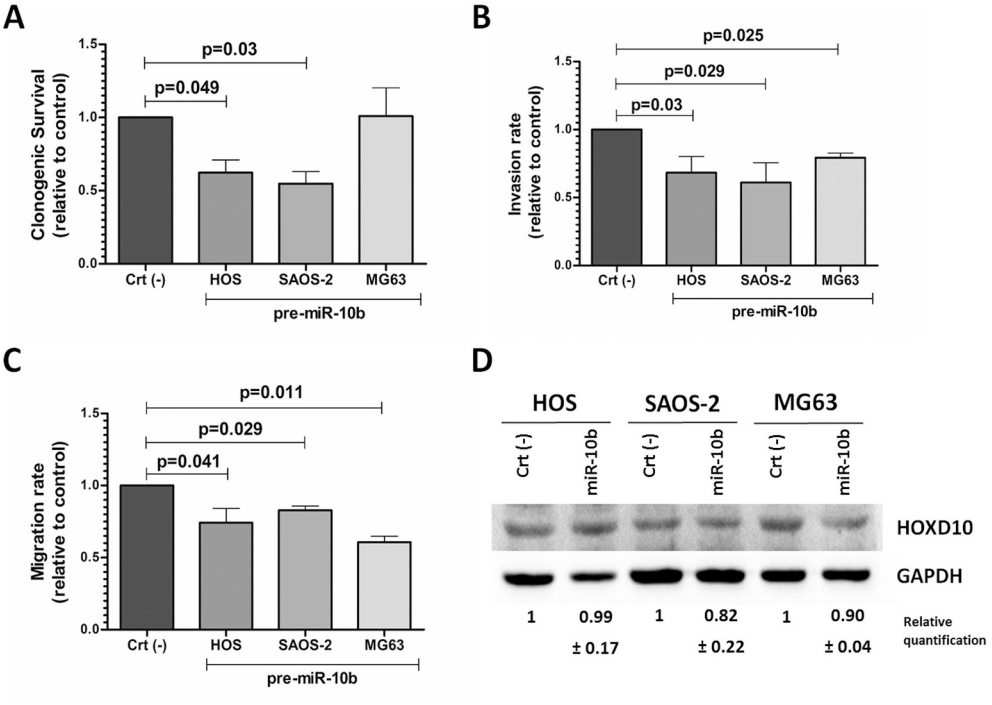

Fig. 1. *In vitro transfection of osteosarcoma cells with miR-10b inhibits clonogenic survival and significantly diminishes their invasive potential.* A) Clonogenicity was significantly reduced in HOS and SAOS-2 cell lines (experiments were performed according with Franken et al. [16], and only colonies with > 50 cells were counted); B) Transwell invasion assays through Matrigel-coated inserts (Becton Dickinson & Co., NJ, USA) showed diminished invasion in transfected osteosarcoma cells after 24 h; C) Similar results were obtained through the wound healing assay; D) miR-10 transfection does not alter the protein levels of HOXD10. The antibodies (Abcam Cambridge, UK) included primary rabbit monoclonal anti-HOXD10 (ab172865) (at 1:100 dilution in non-fat milk 5%) and rabbit monoclonal anti-GAPDH antibody (ab128915) (at 1:10,000 dilution in TBS-T). The immunoblots were developed using goat anti-rabbit secondary antibody (ab6721) (at 1:5000 dilution in non-fat milk 5%) followed by detection with the ECL Western Blotting Substrate Kit (Abcam, Cambridge, United Kingdom) 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 three independent sets of tests. OS cell lines were transfected with pre-miRNA miR-10b-5p and control (mirVanaTM miRNA mimic negative control, Ambion®) using Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At all instances, transfection efficiency was monitored by qRT-PCR and experiments were performed with > 1000X miR-10b increase. Data was 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 $\alpha = 0.05$ and analyses performed using the SPSS 21.0 software (SPSS Inc., IL, USA) and expressed as the mean ± standard deviation.

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Conflict of interest

None.

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