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Original Article

Low miR-491-5p is an unfavorable prognostic marker for osteosarcoma

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Abstract: Osteosarcoma is one of the most leading causes of cancer-related mortality in children and adolescence around the world. The aim of this study was to determine the clinical value of miR-491-5p in osteosarcoma. Quantitative real-time PCR was used to evaluate miR-491-5p level in the tissue/serum samples from patients with osteosarcoma. Then the clinical significance of tissue/serum miR-491-5p was analyzed. Our resulted showed that the expression level of miR-491-5p was significantly decreased in cancer tissues compared to adjacent normal tissues. Serum miR-491-5p was downregulated in osteosarcoma patients and was able to differentiate the osteosarcoma patients from healthy volunteers. In addition, tissue and serum miR-491-5p was highly correlated. Tissue/serum miR-491-5p was significantly associated with various clinicopathological parameters such as distant metastasis, clinical stage and histological grade. Moreover, osteosarcoma patients with lower levels of tissue/serum miR-491-5p suffered worse 5 year overall/disease free survival. Finally, both tissue and serum miR-491-5p were independent prognostic factors for osteosarcoma. This study demonstrated that miR-491-5p had strong potential to serve as a noninvasive biomarker for osteosarcoma diagnosis and predicting prognosis.

Keywords: Biomarker, diagnosis, mir-491-5p, osteosarcoma, prognosis

Introduction

Osteosarcoma (OS) comprises about 20 percent of all primary bone malignancy and is the common cause of cancer related death in children and adolescence [1, 2]. The therapeutic strategies for osteosarcoma includes tumor excision, chemotherapy and radiotherapy. Although great advance has been made in the treatment of this malignancy, the clinical outcome of osteosarcoma patients in the late stage remains poor [3]. The molecular mechanisms accounting for the pathogenesis of osteosarcoma is unclear. Thus it is very important to find out novel targets for osteosarcoma diagnosis and prognosis.

MicroRNAs (miRNAs) are a group of small, highly conserved non-coding RNA molecules with 20-22-nucleotide (nt) in length [4]. There is a considerable amount of evidence indicating that miRNAs play critical roles in regulating almost, if not all, biological functions such as cell proliferation, differentiation and development [5, 6]. As deregulated expression of miRNAs is significantly associated with the initiation and progression of cancer, they have been demonstrated to be useful biomarkers for tumor detection, diagnosis and prognosis prediction [7, 8]. miR-542-5p level was significantly increased in osteosarcoma tissues compared to the adjacent normal tissues. In addition, osteosarcoma patients with higher tissue miR-542-5p expression had worse disease free survival, indicating miR-542-5p was a potential prognostic biomarker for osteosarcoma [9]. Serum miR-152 level was able to discriminate patients with osteosarcoma from healthy controls. In addition, decreased miR-152 level was correlated with poor prognosis of osteosarcoma, suggesting that miR-152 might be a predictor for diagnosis and prognosis of osteosarcoma [10].

Aberrant expression of miR-491-5p has been reported in various types of cancers such as breast cancer, cervical cancer and lung cancer [11-13]. However, the clinical significance of miR-491-5p in osteosarcoma was unknown. In this study, we aimed to determine the potential prognosis value of tissue miR-491-5p in osteosarcoma.

Table 1. Relationship between tissue miR-491-5p and the clinicopathological features of osteosarcoma

Parameters	No. of cases	Tissue miR-491-5p		*P*
		High	Low	
Gender				0.633
Male	42	20	22	
Female	30	16	14	
Age				0.448
<20	49	26	23	
≥20	23	10	13	
Tumor site				0.181
Femur/Tibia	53	24	29	
Others	19	12	7	
Tumor size (cm)				0.018
<8	34	22	12	
≥8	38	14	24	
Distant metastasis				0.003
No	46	29	17	
Yes	26	7	19	
Clinical stage				0.023
I	6	4	2	
IIA	27	19	8	
IIB	34	11	23	
III	5	2	3	
Histological grade				0.031
I	20	15	5	
II	34	14	20	
III	18	7	11	

Materials and methods

Patient cohort and sample preparation

A total of 72 patients with osteosarcoma and 40 healthy volunteers were recruited in the present study. None of the patients received chemotherapy or radiotherapy before the surgery. The study was approved by the Ethics Committee of Shanghai Tongji Hospital. Written informed consent was obtained for the acquisition and use of patient tissue/serum samples as well as clinical data. The patients with osteosarcoma was pathologically confirmed and clinically staged based on the International Union Against Cancer (UICC). The clinicopathologcal parameters were collected from the medical records and are summarized in **Table 1**.

For the tissue samples, all specimens were snap-frozen in liquid nitrogen immediately after surgical resection, and then stored at -80°C. For the serum samples, about 3 mL venous blood were drawn and then allow the clot to form. Then the blood was centrifuged at 1100 g for 20 min at room temperature. The serum samples were transferred to labeled cryovials. The specimens were stored at -80°C until RNA extraction.

Real-time polymerase chain reaction

The total RNA was extracted from tissue and serum samples using Trizol and miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany) according to manufacturer's instructions respectively. The quality of the RNA were determined using UV spectrophotometry (A260/A280 ratio of 1.8-2.0). The miScript II RT Kit (Qiagen) was used to generate cDNA. RT-qPCR was performed to detect miR-491-5p expression levels using the SYBR Premix DimerEraser (Takara, Dalian, China) on a ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). U6 snRNA was used as internal control and relative expression was calculated according to the Ct method ($2^{-\Delta\Delta Ct}$).

Statistical analysis

All the data was analyzed with GraphPad Prism 5 (La Jolla, CA, USA). Mann-Whitney U test was performed to compare the differences of tissue/serum miR-491-5p between experimental groups. The linear correlation coefficient was used to estimate the relationship between tissue and serum miR-491-5p level. The association between tissue/serum miR-491-5p and various clinicopathological parameters of osteosarcoma was analyzed by Chi-square test. The receiver operating characteristic (ROC) curves were established for evaluating the diagnostic value of serum miR-491-5p. Survival data was analyzed using the Kaplan-Meier method. The Cox proportional hazards model were employed to find out the independent prognostic factor for osteosarcoma. A *p*-value of <0.05 was considered statistically significant.

Results

The expression of tissue and serum miR-491-5p in patients with sarcoma

The tissue expression level of miR-491-5p was detected in 72 osteosarcoma samples and

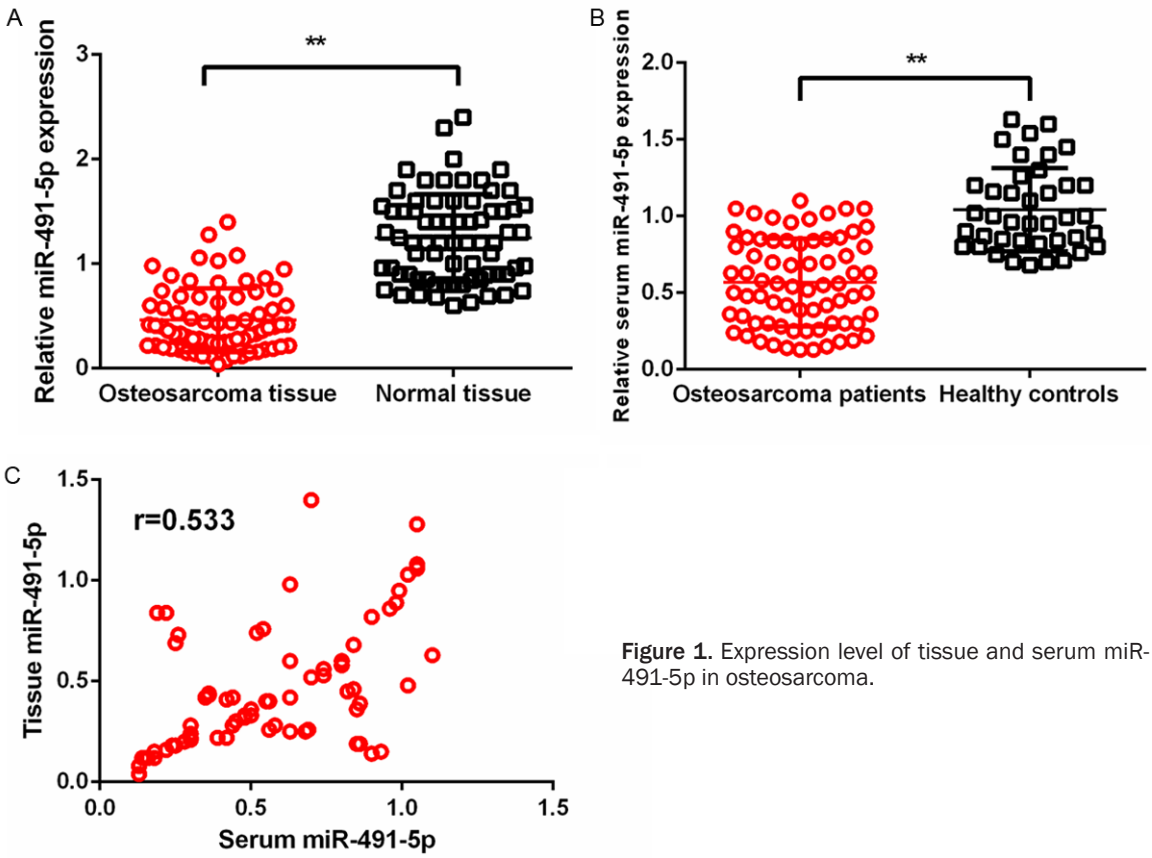

Figure 1. Expression level of tissue and serum miR-491-5p in osteosarcoma.

matched normal tissues. The real-time PCR results showed that tissue miR-491-5p was significantly downregulated in the cancer tissues compared to the adjacent normal tissues (**P<0.01) (**Figure 1A**). Similarly, the serum expression level of miR-491-5p was examined in 72 osteosarcoma patients and 40 healthy controls. We observed that significantly lower serum miR-491-5p in the osteosarcoma patients in comparison with the healthy volunteers (**P<0.01) (**Figure 1B**). Then the correlation between tissue and serum miR-491-5p was evaluated. The data suggested that tissue and serum miR-491-5p was highly correlated (r=0.533) (**Figure 1C**).

The diagnostic value of serum miR-491-5p in osteosarcoma

ROC curve analysis demonstrated that serum miR-491-5p was able to discriminate the osteosarcoma patients from healthy controls and the area under the curve (AUC) value was 0.834. The sensitivity and specificity was 72% and 86% respectively (**Figure 2**).

Tissue/serum miR-491-5p level was associated with clinicopathological parameters of osteosarcoma

The Chi-squared analysis showed that tissue miR-491-5p was significantly associated with tumor size (P=0.018), distant metastasis (P=0.003), clinical stage (P=0.023) and histological grade (P=0.031). However, its level was not correlated with age, gender and tumor site (**Table 1**).

For the serum miR-491-5p, the results showed that low serum miR-491-5p occurred more frequently in osteosarcoma patients with distant metastasis (P=0.006), advanced clinical stage (P=0.009) and histological grade (P=0.033), while no significant association between serum miR-491-5p and age, gender, tumor site and tumor size (**Table 2**).

Prognostic value of tissue/serum miR-491-5p in osteosarcoma

The survival analysis showed that the osteosarcoma patients with lower tissue miR-491-5p

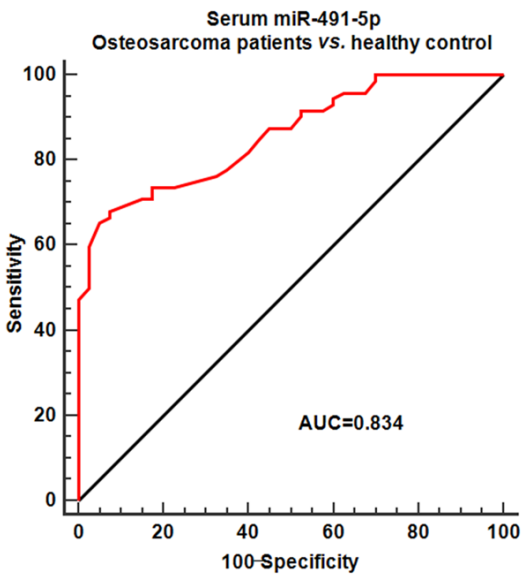

Figure 2. Diagnostic value of serum miR-491-5p.

Table 2. Relationship between serum miR-491-5p and the clinicopathological features of osteosarcoma

Parameters	No. of cases	Serum miR-491-5p		*P*
		High	Low	
Gender				0.217
Male	42	23	19	
Female	30	12	18	
Age				0.551
<20	49	25	24	
≥20	23	10	13	
Tumor site				0.508
Femur/Tibia	53	27	26	
Others	19	8	11	
Tumor size (cm)				0.101
<8	34	20	14	
≥8	38	15	23	
Distant metastasis				0.006
No	46	28	18	
Yes	26	7	19	
Clinical stage				0.009
I	6	6	0	
IIA	27	16	11	
IIB	34	12	22	
III	5	1	4	
Histological grade				0.033
I	20	14	6	
II	34	16	18	
III	18	5	13	

had a shorter overall survival (OS) (P=0.004) and disease free survival (DFS) (P=0.042) time than the patients with higher tissue miR-491-5p (**Figure 3A**). Similarly, the osteosarcoma patients in the low serum miR-491-5p group suffered more unfavorable overall (P<0.001) and disease free survival (P=0.002) compared to those in the high serum miR-491-5p group (**Figure 3B**).

A multivariate Cox regression analysis was performed to determine the prognostic value of miR-491-5p in osteosarcoma. The results showed that distant metastasis (OS: HR=3.65, 95% CI=1.52-6.48, P=0.005; DFS: 3.78, 95% CI=1.54-7.93, P=0.004), clinical stage (OS: HR=2.86, 95% CI=1.34-5.15, P=0.016; DFS: 2.71, 95% CI=1.30-5.42, P=0.019), tissue miR-491-5p (OS: HR=2.55, 95% CI=1.28-4.64, P=0.039; DFS: 2.44, 95% CI=1.21-4.51, P=0.030) and serum miR-491-5p (OS: HR=2.72, 95% CI=1.30-4.92, P=0.028; DFS: 2.64, 95% CI=1.27-5.02, P=0.022) were independent prognostic factors for overall and disease free survival of osteosarcoma patients (**Table 3**).

Discussion

Osteosarcoma remains a significant public health concern. The survival rates for this malignancy have remained unchanged at 60-65% for the past few decades [14]. Thus identification of novel diagnostic and prognostic biomarkers might not only contribute to early detection of osteosarcoma, but also are beneficial for improving the cure rates for osteosarcoma. miRNAs has been proved to be closely correlated with tumorigenesis and promising biomarkers for cancer diagnosis and prognosis [15, 16]. miR-491 is commonly co-deleted with cyclin dependent kinase inhibitor 2A in many types of cancer [17]. miR-491 encodes two mature miRNA namely miR-491-3p and miR-491-5p. Various studies have shown that the downstream targets of these two molecules are oncogenes. For instance, miR-491-5p directly targets EGFR, CDK6 and Bcl-xL, whereas miR-491-3p targets IGFBP2 and CDK6 [18], indicating reduced miR-491-5p might promote the carcinogenesis.

Our work demonstrated that miR-491-5p was significantly reduced in the tissue and serum samples from patients with osteosarcoma. In addition, serum miR-491-5p was able to differ-

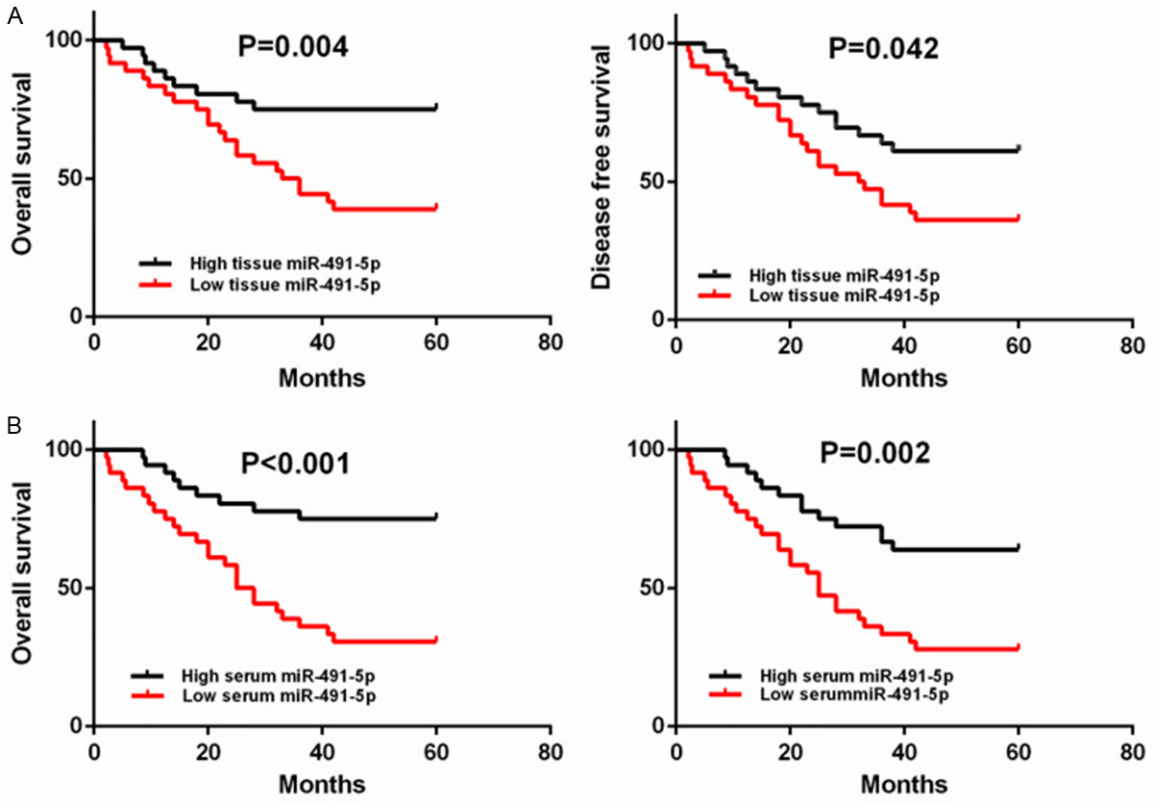

Figure 3. Association between tissue/serum miR-491-5p levels and overall/disease free survival.

Table 3. Multivariate analysis of parameters associated with overall survival and disease free survival

Parameters	Overall survival			Disease free survival		
	HR	95% CI	P	HR	95% CI	P
Distant metastasis	3.65	1.52-6.48	0.005	3.78	1.54-7.93	0.004
Clinical stage	2.86	1.34-5.15	0.016	2.71	1.30-5.42	0.019
Tissue miR-491-5p	2.55	1.28-4.64	0.039	2.44	1.21-4.51	0.030
Serum miR-491-5p	2.72	1.30-4.92	0.028	2.64	1.27-5.02	0.022

entiate osteosarcoma from healthy volunteers with high accuracy. Reduced tissue/serum miR-491-5p expression were both significantly associated with worse clinical outcome of osteosarcoma. Furthermore, tissue/serum miR-491-5p were proved to be independent prognostic factors for osteosarcoma, suggesting that miR-491-5p played a tumor suppressive role in osteosarcoma and its downregulation might promote its progression. Consistent with our findings, Yin et al reported that miR-491-5p was downregualted in osteosarcoma tissues and cell lines. In addition, miR-491-5p overexpression inhibited proliferation capacity of osteosarcoma cells and induced apoptosis by targeting forkhead-box P4 [19]. This study further confirmed our results that miR-491-5p might acted as a tumor suppressor in osteosarcoma.

miR-491-5p has been reported to be a tumor suppressor in various types of cancers. The expression level of miR-491-5p was remarkably reduced in colon cancer tissues especially those from aged patients. In addition, patient with higher miR-491-5p had favorable survival time than those with lower miR-491-5p, suggesting miR-491-5p was a potential prognostic marker for colon cancer [20]. Li et al showed that both miR-491-5p and miR-491-3p was decreased in glioblastoma tissues when compared to normal controls. Moreover, overexpression of miR-491-5p suppressed the oncogenic behaviors of glioma cells both *in vitro* and *in vivo*, and opposite findings were observed when miR-491-5p was underexpressed, indicating miR-491-5p was a tumor suppressor gene in glioblastoma [18]. Similarly, reduced miR-491-5p expression was

significantly associated with worse overall survival of patients with oral squamous cell carcinoma. Ectopic expression of miR-491-5p suppressed the invasive capacity of oral cancer cells both *in vitro* and *in vivo. F*urthermore, G-protein-coupled receptor kinase-interacting protein 1 was demonstrated to be a downstream target of miR-491-5p [21].

In conclusion, both tissue and serum miR-491-5p were significantly reduced in patients with osteosarcoma. In addition, reduced tissue/serum miR-491-5p was correlated with tumor development and poor prognosis, indicating miR-491-5p might be employed as a promising diagnostic and prognostic biomarker for osteosarcoma.

Disclosure of conflict of interest

None.

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References

[1] Geller DS, Gorlick R. Osteosarcoma: a review of diagnosis, management, and treatment strategies. Clin Adv Hematol Oncol 2010; 8: 705-718.

[2] Marina N, Gebhardt M, Teot L, Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist 2004; 9: 422-441.

[3] Luetke A, Meyers PA, Lewis I, Juergens H. Osteosarcoma treatment-where do we stand? A state of the art review. Cancer Treat Rev 2014; 40: 523-532.

[4] Ranganathan K, Sivasankar V. MicroRNAs-biology and clinical application. J Oral Maxillofac Pathol 2014; 18: 229-234.

[5] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[6] Mendell JT. MicroRNAs: critical regulators of development, cellular physiology and malignancy. Cell Cycle 2005; 4: 1179-1184.

[7] Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol Med 2014; 20: 460-469.

[8] Zhang B, Farwell MA. microRNAs: a new emerging class of players for disease diagnostics and gene therapy. J Cell Mol Med 2008; 12: 3-21.

[9] Cheng DD, Yu T, Hu T, Yao M, Fan CY, Yang QC. MiR-542-5p is a negative prognostic factor and promotes osteosarcoma tumorigenesis by targeting HUWE1. Oncotarget 2015; 6: 42761-42772.

[10] Wang NG, Wang DC, Tan BY, Wang F, Yuan ZN. Down-regulation of microRNA152 is associated with the diagnosis and prognosis of patients with osteosarcoma. Int J Clin Exp Pathol 2015; 8: 9314-9319.

[11] Zeng H Chen YL, You WT, Huang XQ, Zhou CY, Li H. miR-491-5p functions as a tumor suppressor by targeting JMJD2B in ERα-positive breast cancer. FEBS Lett 2015; 589: 812-2.

[12] Zhao Q, Zhai YX, Liu HQ, Shi YA, Li XB. MicroRNA-491-5p suppresses cervical cancer cell growth by targeting hTERT. Oncol Rep 2015; 34: 979-986.

[13] Gong F, Ren P, Zhang Y, Jiang J, Zhang H. MicroRNAs-491-5p suppresses cell proliferation and invasion by inhibiting IGF2BP1 in non-small cell lung cancer. Am J Transl Res 2016; 8: 485-495.

[14] Kleinerman E. Maximum benefit of chemotherapy for osteosarcoma achieved-what are the next steps? Lancet Oncol 2016; 17: 1340-1342.

[15] Moore LM, Zhang W. MIR-491: CDKN2A tumor suppressor co-pilot. Oncoscience 2015; 2: 825-826.

[16] Zhang J, Yan YG, Wang C, Zhang SJ, Yu XH, Wang WJ. MicroRNAs in osteosarcoma. Clin Chim Acta 2015; 444: 9-17.

[17] Sampson VB, Yoo S, Kumar A, Vetter NS, Kolb EA. MicroRNAs and potential targets in Osteosarcoma: Review. Front Pediatr 2015; 3: 69.

[18] Li X, Liu Y, Granberg KJ, Wang Q, Moore LM, Ji P, Gumin J, Sulman EP, Calin GA, Haapasalo H, Nykter M, Shmulevich I, Fuller GN, Lang FF, Zhang W. Two mature products of MIR-491 coordinate to suppress key cancer hallmarks in glioblastoma. Oncogene 2015; 34: 1619-1628.

[19] Yin Z, Ding H, He E, Chen J, Li M. Up-regulation of microRNA-491-5p suppresses cell proliferation and promotes apoptosis by targeting FOXP4 in human osteosarcoma. Cell Prolif 2016; [Epub ahead of print].

[20] Tao K, Yang J, Guo Z, Hu Y, Sheng H, Gao H, Yu H. Prognostic value of miR-221-3p, miR-342-3p and miR-491-5p expression in colon cancer. Am J Transl Res 2014; 6: 391-401.

[21] Huang WC, Chan SH, Jang TH, Chang JW, Ko YC, Yen TC, Chiang SL, Chiang WF, Shieh TY, Liao CT, Juang JL, Wang HC, Cheng AJ, Lu YC, Wang LH. miRNA-491-5p and GIT1 serve as modulators and biomarkers for oral squamous cell carcinoma invasion and metastasis. Cancer Res 2014; 74: 751-764.