Tropical Journal of Pharmaceutical Research January 2022; 21 (1): 37-43
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i1.7

Original Research Article

MiR-641 participates in the progression of breast cancer by modulation of RELN expression

Ruixin Qu, Yaoqiang Wu, Daxin Chen, Feng Jiang*
Department of General Surgery, Dandong First Hospital, Dandong, China

For correspondence:* ***Email:* *jf13942511107@163.com;* ***Tel:*** *+86-013942511107*

Sent for review: 16 August 2021 *Revised accepted: 6 January 2022*

Abstract

Purpose: *To examine the role of micro-ribonucleic acid 641 (miR-641) in breast cancer, and to uncover its possible molecular mechanism.*
Methods: *MiR-641 expressions in breast cancer cell lines and tissues were determined using Real-time fluorescence quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and the diagnostic potential value of miR-641 was assessed using receiver operating characteristic (ROC) curves. The survival of the patients was analyzed using Kaplan-Meier, and the cell viability and migration capacity were evaluated using Transwell and cell counting kit-8 (CCK-8) assay, and the downstream target gene of miR-641 was confirmed via dual-luciferase reporter gene assay. Finally, reversal assay was employed to corroborate the molecular mechanism that affects cell proliferation and migration via modulation of RELN.*
Results: *MiR-641 was lowly expressed in breast cancer cell lines and tissues, and its expression in the metastasizing group was lower than that in the matched group ($p < 0.05$). It was also observed that miR-641 expression gradually decreased as the breast cancer advanced. Moreover, lower miR-641 expression revealed a poor prognosis, and up-regulating miR-641 suppressed the proliferative and migrative capacities of breast cancer cells. It was proven that RELN is a target gene of miR-641. RELN expression rose in breast cancer, and it was evidently and negatively correlated with that of miR-641. Finally, miR-641 regulated RELN, and it affected the proliferation and migration of cells.*
Conclusion: *MiR-641 has an obviously decreased expression level in breast cancer, and facilitates the proliferative and migrative capacity of breast cancer cells probably by modulating the RELN expression. This study may provide new targets for the treatment of breast cancer.*

Keywords: *Breast cancer, REELIN, micro-ribonucleic acid 641, Proliferation, Migration*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Breast cancer is a common cancer in women [1], and it remains number one among all female malignancies due to its consistently increasing incidence rate in recent years [2]. Some preliminary outcomes have been obtained in the clinical treatment of breast cancer, but these are still not enough to cater to the demand from patients [3,4]. The leading causes are, the lack of specific targets for diagnosis and treatment, and the inter-patient heterogeneity-induced treatment resistance [5,6]. Although great progress in the treatment of breast cancer has been driven by

the development of modern technologies, the molecular mechanism of breast cancer is still the focus of oncology research. Therefore, research into the pathogenesis and prognosis of breast cancer, especially specific targets is of profound significance for patients.

Micro-ribonucleic acids (miRNAs) are small non-coding RNAs with about 20-23 nucleotides in organisms, and they negatively modulate post-transcriptional gene expression by complementarily binding to the 3' untranslated region (3'-UTR) of the target gene, further degrading the mRNA or inhibiting its translation [7]. The MiRNAs have been widely recognized as tumor suppressor genes or oncogenes in cancers, because they bind to the 3'UTR of the target gene, thereby promoting degradation and inhibiting translation [8]. There have been many reports that abnormal expression profiles of miRNAs have been discovered in a variety of malignant tumors [9,10]. A study found that miR-133b restrains the invasion, migration and proliferation of esophageal squamous carcinoma cells *via* targeted regulation of epidermal growth factor receptor [11]. Besides, miR-20a and miR-486 have been proven to be potential tumor markers for colon cancer [12].

According to a previous study, miR-641 functioned as an anti-tumor gene by targeting MDM2 in lung cancer [13]. However, miR-641 has been relatively, rarely researched in other tumors, particularly breast cancer. In this study, miR-641 expression levels in breast cancer tissues and its effects on the migration and proliferation abilities of breast cancer cell lines were analyzed to examine the specific functions of miR-641 in breast cancer, in the hope of providing a novel target for the early intervention in breast cancer.

EXPERIMENTAL

Clinical samples

A total of 48 pairs of paracancerous tissues (≥ 5 cm away from the cancer tissue margins) and breast cancer tissues were excised from breast cancer patients undergoing surgery. Upon removal, they were immediately preserved in liquid nitrogen for subsequent assays. The patients and their family members gave their informed consent and signed the informed consent document, and this study was conducted with the review and approval of the Ethics Committee of our hospital.

Cell culture and transfection

Breast cancer cell lines (BT-549, MDA-MB-231 and MCF-7) and normal breast cells (MCF-10A) were bought from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in the basal medium DMEM (Invitrogen, Carlsbad, CA, USA) with 10 % fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) under the conditions of 5 % CO_2 and 37 °C. When the density reached about 40 - 60 %, the cultured cells were transfected using miR-641 mimics and reelin (RELN) overexpression plasmids, as well as their negative control (NC) sequences (Shanghai GenePharma Co. Ltd., Shanghai, China) and the transfection reagent Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), at a final transfection concentration of 50 - 100 nM.

Cell counting kit (CCK)-8 assay

The MCF-7 and MDA-MB-231 cells were digested using Tyrisin, and the concentration of cells in the 96-well plate was adjusted to 3,000 cells/200 µL of medium. The cells were transfected with miR-641-NC or miR-641 mimics post culture for 24 h. Then all the cells were maintained at 5 % CO_2 and 37 °C for 1, 2 and 3 days. At each time point, the culture plate was taken out and added with CCK-8 buffer at 10 µL/well. The absorbance was measured using Synergy HTX multi-mode reader (BioTek Instruments, Inc., Biotek Winooski, VT, USA) at 450 nm after incubation for 20 min.

Transwell assay

3×10^4 transfected cells suspened in 200 µL serum-free medium were added to the upper chamber of the transwell plate (BD Bioscience, San Jose, CA, USA). Besides, medium containing 10% FBS was added into the lower chamber. After 24-48 h of culture, the culture plate was taken out from the incubator, and cotton swabs were used to wipe off the cells in the upper chamber, while the lower chamber cells were fixed with 100 % ice-cold methanol and dyed with 0.1 % crystal violet. Finally, the stained and number of migrating cells were observed with a light microscope, and the results were obtained by averaging the number of cells in five randomly selected fields of view.

Real-time fluorescence quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

A total of 100 mg of tissue was taken from each sample, added with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) and fully lysed. Following trypsinization and centrifugation, the cells were harvested from each sample, added with 1 mL of TRIzol and lysed by pipetting up and down. Subsequently, total RNAs were extracted from each group of tissues and cells using the TRIzol method. The MiRNAs were reversely-transcribed into corresponding complementary deoxyribose nucleic acids (cDNAs) using poly(A) polymerase tailing using Mir-X™ miRNA first-strand synthesis and SYBR® RT-qPCR kits providing the forward and reverse primer sequences of miR-641 and U6. The reaction system volume was in total 25 μl, pre-denaturation at 95˚ for 5 min, denaturation at 95˚ for 30 sec, annealing at 60˚ for 45 sec, extension at 72˚ for 3 min, with 35 cycles, and then extension at 72˚ for 5 min. PCR products were stored at 4˚. The relative expression level of miR-641 was calculated using $2^{-\Delta\Delta Ct}$ method with sextuplicate wells set in each group, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was taken as the internal reference of RELN mRNA, with the primer sequences shown in Table 1.

Table 1: Primer sequences for PCR

Gene	Primer sequence
GAPDH	F: 5'-GAAGAGAGAGACCCTCACGCTG-3'
	R: 5'-ACTGTGAGGAGGGGAGATTCAGT-3'
RELN	F: 5'-AAGGGAGAAGAAACTGAGAAGC-3'
	R: 5'-TGGGAAGGTCGTGACTGA A A-3'

Dual-luciferase reporter gene assay

Mutant-type RELN-3'UTR (RELN-3'UTR-MUT) and wild-type RELN-3'UTR (RELN-3'UTR-WT) plasmids were provided by Shanghai GenePharma Co. Ltd. (Shanghai, China). At 1 d prior to transfection, cells were evenly seeded in a 24-well plate at a density of 5×10^4 cells/mL. When the confluence reached 90 %, the cells were transfected with the mixture of Lipofectamine 3000, pRL-TK (20 ng) and luciferase reporter vectors (400 ng) according to the instructions of Lipofectamine™ 3000 and cultured in the incubator. The relative expression level of the reporter gene was calculated based on the ratio of the fluorescence intensity of the reporter gene to that of the internal reference.

Statistical analysis

SPSS statistical analysis software (version 26.0) was enrolled for all statistical analysis. Paired two-tailed Student's t-test or Chi-square test were enrolled to assess the data different between groups. All data are displayed as mean ± standard deviation, and $p<0.05$ was considered to be statistically significant.

RESULTS

MiR-641 exhibited a substantially reduced expression level in breast cancer

According to the detection consequences, miR-641 expressions in the tissues of breast cancer patients was lower compared to that in the paracancerous tissues (Figure 1 A). The miR-641 expression in non-metastasis group was higher than that in metastasis group (Figure 1 B). Moreover, miR-641 expression gradually declined as the tumor stage increased (Figure 1C). The further ROC curve analysis results revealed that the area under the curve (AUC) was 0.8364 and the cutoff value was 0.2907 (Figure 1 D), confirming that miR-641 has the potential to serve as a biomarker. According to the survival analysis results, patients with lowly expressed miR-641 had a distinctly lower survival rate than those with highly expressed miR-641 (Figure 1 E). The MiR-641 expression level in breast cancer cell lines was also determined. It was discovered that miR-641 was generally increased in normal breast cancer cells compared with that in cancer cell lines, and its expression level was relatively lower in MCF-7 and MDA-MB-231 (Figure 1F). So these two cell lines were enrolled for following assays.

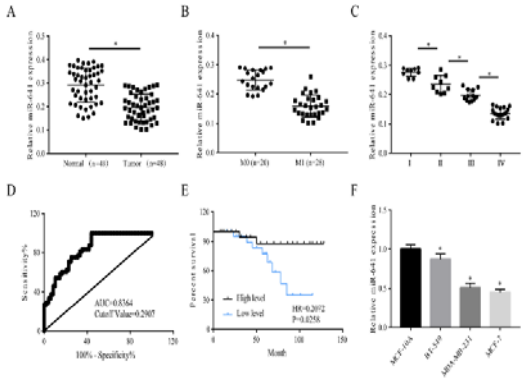

Figure 1: Low-expressed miR-641 in breast cancer. (A) Compared with that in the paracancerous tissues, miR-641 expression significantly decreased in the cancer tissues of breast cancer patients. (B) The patients in metastasis group had a lower miR-641 expression than those in non-metastasis group. (C)

With the rise in tumor stage, miR-641 expression gradually declined. (D) AUC and cut-off values were 0.8364 and 0.2907, respectively. (E) The patients with lowly expressed miR-641 had a lower survival rate than those with highly expressed miR-641. (F) In comparison with that in normal breast cancer cells, miR-641 expression was generally decreased in breast cancer cell lines. (*p<0.05)

Up-regulation of miR-641 notably suppressed the migration and proliferation of breast cancer cells

To further examine the functions of miR-641 in the migrative and proliferative capacity of breast cancer, phenotype experiments were conducted in MCF-7 and MDA-MB-231. The transfection efficiency of miR-641 mimics was first determined in MDA-MB-231, and the outcome suggested that miR-641 expression obviously rose after transfection (Figure 2 A). It was found through the CCK-8 assay that the proliferative ability of MDA-MB-231 was weakened after raising miR-641 expression (Figure 2 B). Besides, the transwell assay results implied that following transfection with miR-641 mimics, the migrative capacity of MDA-MB-231 cells also declined markedly (Figure 2 C). Based on the assay results of MCF-7 cells, the up-regulation of miR-641 substantially suppressed the migration and proliferation of MCF-7 cells (Figures 2 D - F). It can be inferred from these results that miR-641 may affect the migrative and proliferative capacities of breast cancer cells.

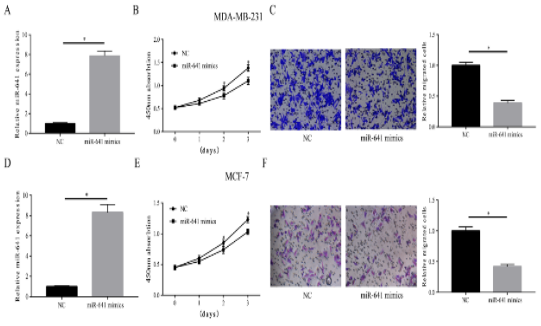

Figure 2: Up-regulation of miR-641 suppressed the migration and proliferation of breast cancer cells. A: The miR-641 expression was significantly raised in the miR-641 mimics transfected MDA-MB-231 cells. B & C) After up-regulation of miR-64, the migration and proliferation abilities of MDA-MB-231 cells were significantly weakened. (magnification: ×40) (D) The miR-641 expression was markedly elevated in the miR-641 mimics-transfected MCF-7 cells. E & F: The proliferation and migrative abilities of MCF-7 cells were significantly weakened following up-regulation of miR-641. (magnification: 40×) (*p < 0.05)

RELN was the potential target gene of miR-641

Since miRNAs usually function by targeting their downstream target genes, the potential target gene of miR-641 was predicted using bioinformatics method. The results revealed that RELN was the potential target gene of miR-641, and possessed potential binding sites (Figure 3 A). Then, dual-luciferase reporter gene assay was conducted to elucidate the action of miR-641 on the 3'UTR of RELN. It was found that the fluorescence intensity obviously declined in RELN-WT group, but there was no distinct change in RELN-MUT group (Figures 3 B and C), indicating the binding relationship between miR-641 and RELN. Moreover, a decline in the expression of RELN was evaluated *via* qRT-PCR after miR-641 was raised in MCF-7 and MDA-MB-231(Figure 3 D), indicating that RELN expression is modulated by miR-641. Subsequently, RELN expression in the breast cancer tissues was determined, and it was discovered that the expression of RELN was significantly decreased in the paracancerous tissues than that in the cancer tissues (Figure 3 E) and negatively correlated with miR-641 expression level in breast cancer tissues (Figure 3 F).

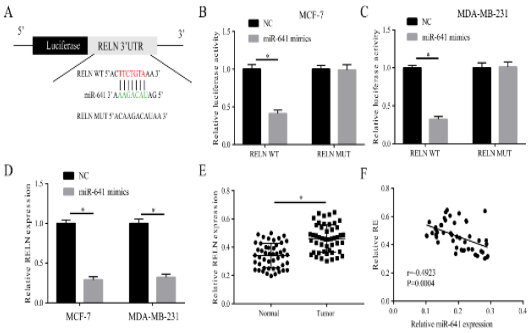

Figure 3: RELN was the potential target of miR-641. A: The potential binding sites between MiR-641 and RELN were predicted using bioinformatics B & C The dual-luciferase reporter gene assay results confirmed the binding sites between miR-641 and RELN. (D) The RELN expression was markedly decreased in the miR-641 up-regulated in MDA-MB-231 cells. (E) The RELN expression significantly rose in breast cancer tissues. (F) MiR-641 expression was significantly negatively correlated with that of RELN in breast cancer tissues (*p<0.05)

MiR-641 worked by regulating RELN

A reversal assay was carried out to verify whether miR-641 exerts effects through down-regulating RELN expression. Based on the results, the expression of RELN was obviously

raised in RELN overexpression transfected MCF-7 and MDA-MB-231 cells (Figure 4 A). Besides, according to the results of CCK-8 and transwell assays, the proliferative and migrative abilities of MCF-7 and MDA-MB-231 cells were enhanced after up-regulation of RELN, but weakened after up-regulation of both miR-641 and RELN (Figure 4 B - D). These findings confirm that miR-641 may modulate the migrative and proliferative capacity of breast cancer cells through degrading RELN.

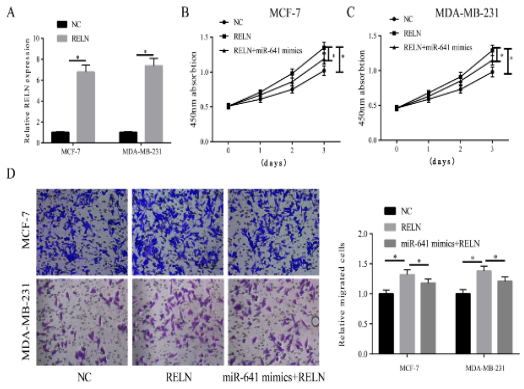

Figure 4: MiR-641 modulates the migration and proliferation of breast cancer via regulating RELN. (A) The expression of RELN was significantly elevated in MCF-7 and MDA-MB-231 cells transfected with RELN overexpression plasmids. (B-D) The proliferation and migration abilities of MCF-7 and MDA-MB-231 cells were enhanced by the up-regulation of RELN, which was partially reversed after up-regulation of both miR-641 and RELN. (magnification: 40×); *$p < 0.05$).

DISCUSSION

Breast cancer is a malignant tumor with high incidence rate. It is also known for its high metastasis and case-fatality rates, and studies in recent years have found that it tends to affect younger people [14,15]. Currently, relevant study results have demonstrated that the highly specific targeted drugs inhibiting breast cancer are far from sufficient to meet the demand of patients, as the pathogenesis of breast cancer is yet to be fully explored.

The division, differentiation and death of cells in either developing body or mature body have to be supported by various safety measures. In cells, there are various regulatory factors guiding the process of turning on or off cell proliferation and differentiation genes, and the mutation or aberrant expression of these regulators, termed as tumor suppressor genes or oncogenes, may lead to uncontrolled cell growth and differentiation, ultimately inducing tumors. The development and progression of tumors is a complex multi-step process involving multiple genes, in which tumor suppressor genes are silenced or oncogenes are activated. The miRNAs discovered in recent years have been widely recognized to regulate these related genes to play a crucial role in tumors [16-18]. In this study research, miR-641 expression was substantially lowly expressed in breast cancer tissues and cells, which was related with poor prognosis of breast cancer. These consequences imply that miR-641 may be implicated in the progress of breast cancer. However, how does it work? Therefore, phenotype experiments were conducted in this present study. The results implied that up-regulated miR-641 expression suppressed the migration and proliferation of breast cancer cells, indicating that miR-641 is likely to act as a crucial role in the progress of breast cancer through modulating the migration and proliferation of breast cancer cells.

According to the actual characteristics of miRNAs, the target gene of miR-641 was predicted by bioinformatics to further explore the potential mechanism of miR-641 in this research. The screening consequences indicated that RELN was the potential target gene of miR-641. It was also confirmed in breast cancer cell lines that RELN expression was modulated by miR-641. REELIN was initially found to be closely correlated with the growth and differentiation of neurons in the central nervous system (CNS) [19]. Although primarily researched in post-mitotic neurons in the CNS, RELN is also widely expressed in other tissues outside the CNS [20]. Existing studies have revealed that RELN also has an important relationship with the development and progression of tumors. For instance, RELN has been discovered to be able to restrain the invasion and proliferation of pancreatic cancer cells [21]. Another research revealed that RELN is highly expressed in multiple myeloma, which is linked with the poor prognosis of myeloma [22]. However, there have been few study reports on RELN in breast cancer, so whether miR-641 functions through the regulation of RELN was further determined in this study.

First, RELN expression in breast cancer was detected, and a substantially high expression was observed, suggesting that RELN may participate in the progress of breast cancer. It was then found through up-regulating RELN expression that RELN obviously promoted the proliferative and migrative capacities of breast cancer cells. Finally, reversal assay confirmed that up-regulation of RELN partly reversed the suppressive effect of up-regulated miR-641 expression on the migration and proliferation of

cells. Therefore, it can be concluded that miR-641 may function through the targeted regulation of RELN.

CONCLUSION

The findings of this study reveal that miR-641 may act as a crucial player in the progress of breast cancer and corroborated the underlying mechanism that miR-641 regulates the downstream target gene RELN to affect the migrative and proliferative capacity of breast cancer cells. Hence, miR-641 is a potential diagnostic or therapeutic target or a prognostic biomarker for breast cancer, which provides new information on the elucidation of the pathogenesis of breast cancer.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

Open Access

REFERENCES

1. *Rankin K, Le D, Sweeny K. Preemptively finding benefit in a breast cancer diagnosis. Psychol Health 2020; 35(5): 613-628.*
2. *Ghoncheh M, Pournamdar Z, Salehiniya H. Incidence and Mortality and Epidemiology of Breast Cancer in the World. Asian Pac J Cancer Prev 2016; 17(S3): 43-46.*
3. *Omarini C, Guaitoli G, Pipitone S, Moscetti L, Cortesi L, Cascinu S, Piacentini F. Neoadjuvant treatments in triple-negative breast cancer patients: where we are now and where we are going. Cancer Manag Res 2018; 10: 91-103.*
4. *Schvartsman G, Kaliks RA. Clinical and Genomic Risk in Adjuvant Therapy for Breast Cancer. N Engl J Med 2019; 381(13): 1289-1291.*
5. *Gaber M, Hany M, Mokhtar S, Helmy MW, Elkodairy KA, Elzoghby AO. Boronic-targeted albumin-shell oily-core nanocapsules for synergistic aromatase inhibitor/herbal breast cancer therapy. Mater Sci Eng C Mater Biol Appl 2019; 105: 110099.*
6. *Low SK, Zembutsu H, Nakamura Y. Breast cancer: The translation of big genomic data to cancer precision medicine. Cancer Sci 2018; 109(3): 497-506.*
7. *Wang B, Doench JG, Novina CD. Analysis of microRNA effector functions in vitro. Methods 2007; 43(2): 91-104.*
8. *Chen J, Zhang L, Liu C, Liang Y. MiR-497-5p promotes osteogenic/odontogenic differentiation of stem cells from the apical papilla by regulation of the TGF-β Smad pathway through Smurf2. Trop J Pharm Res 2021; 20(6): 1113-1118.*
9. *Palmero EI, de Campos SG, Campos M, de Souza NC, Guerreiro ID, Carvalho AL, Marques MM. Mechanisms and role of microRNA deregulation in cancer onset and progression. Genet Mol Biol 2011; 34(3): 363-370.*
10. *Fang Y, Zhou W, Rong Y, Kuang T, Xu X, Wu W, Wang D, Lou W. Exosomal miRNA-106b from cancer-associated fibroblast promotes gemcitabine resistance in pancreatic cancer. Exp Cell Res 2019; 383(1): 111543.*
11. *Zeng W, Zhu JF, Liu JY, Li YL, Dong X, Huang H, Shan L. miR-133b inhibits cell proliferation, migration and invasion of esophageal squamous cell carcinoma by targeting EGFR. Biomed Pharmacother 2019; 111: 476-484.*
12. *Yang Q, Wang S, Huang J, Xia C, Jin H, Fan Y. Serum miR-20a and miR-486 are potential biomarkers for discriminating colorectal neoplasia: A pilot study. J Cancer Res Ther 2018; 14(7): 1572-1577.*
13. *Kong Q, Shu N, Li J, Xu N. miR-641 Functions as a Tumor Suppressor by Targeting MDM2 in Human Lung Cancer. Oncol Res 2018; 26(5): 735-741.*
14. *Partridge AH, Carey LA. Unmet Needs in Clinical Research in Breast Cancer: Where Do We Need to Go? Clin Cancer Res 2017; 23(11): 2611-2616.*
15. *Winters S, Martin C, Murphy D, Shokar NK. Breast Cancer Epidemiology, Prevention, and Screening. Prog Mol Biol Transl Sci 2017; 151: 1-32.*
16. *Peng Y, Shen X, Jiang H, Chen Z, Wu J, Zhu Y, Zhou Y, Li J. miR-188-5p Suppresses Gastric Cancer Cell Proliferation and Invasion via Targeting ZFP91. Oncol Res 2018; 27(1): 65-71.*
17. *Zhang H, Qi S, Zhang T, Wang A, Liu R, Guo J, Wang Y, Xu Y. miR-188-5p inhibits tumour growth and metastasis in prostate cancer by repressing LAPTM4B expression. Oncotarget 2015; 6(8): 6092-6104.*
18. *Fang F, Chang RM, Yu L, Lei X, Xiao S, Yang H, Yang LY. MicroRNA-188-5p suppresses tumor cell proliferation and metastasis by directly targeting FGF5 in hepatocellular carcinoma. J Hepatol 2015; 63(4): 874-885.*

19. Kwon IS, Cho SK, Kim MJ, Tsai MJ, Mitsuda N, Suh-Kim H, Lee YD. Expression of Disabled 1 suppresses astroglial differentiation in neural stem cells. Mol Cell Neurosci 2009; 40(1): 50-61.
20. Smalheiser NR, Costa E, Guidotti A, Impagnatiello F, Auta J, Lacor P, Kriho V, Pappas GD. Expression of reelin in adult mammalian blood, liver, pituitary pars intermedia, and adrenal chromaffin cells. Proc Natl Acad Sci U S A 2000; 97(3): 1281-1286.
21. Sato N, Fukushima N, Chang R, Matsubayashi H, Goggins M. Differential and epigenetic gene expression profiling identifies frequent disruption of the RELN pathway in pancreatic cancers. Gastroenterology 2006; 130(2): 548-565.
22. Priyadarshi S, Panda KC, Panda AK, Ramchander PV. Lack of association between SNP rs3914132 of the RELN gene and otosclerosis in India. Genet Mol Res 2010; 9(3): 1914-1920.