European Review for Medical and Pharmacological Sciences 2018; 22: 2985-2990

MiR-519d inhibits prostate cancer cell proliferation, cycle and invasion via targeting NRBP1

C.-Q. YAN[1], Y.-H. LU[2], S.-M. TANG[3], W.-X. FAN[4]

[1]Department of Urology, Tangshan Gongren Hospital, Tangshan, China
[2]Department of Pathology, The Hospital of Heilongjiang Province, Harbin, China
[3]Department of Internal Medicine, The Tumor Hospital of Qingdao City, Qingdao, China
[4]Department of Nephrology, The First Affiliated Hospital of Kunming Medical University, Kunming, China

Chengquan Yan and Yanhong Lu contributed equally to this work

Abstract. – OBJECTIVE: To investigate the effects of miR-519d on the proliferation, cycle and invasion of human prostate cancer PC3 cells and its possible molecular mechanism.

MATERIALS AND METHODS: The proliferation, cycle, and invasion of human prostate cancer PC3 cells were detected via cell counting kit-8 (CCK-8) and transwell assay. The expression of NRBP1 mRNA was detected *via* reverse transcription-polymerase chain reaction (RT-PCR). Western blotting was used to detect the expression of NRBP1, cyclin D1, and epithelial-mesenchymal transition (EMT) markers.

RESULTS: The expression of miR-519d in prostate cancer was decreased, which was correlated with tumor size, metastasis, and staging. Proliferation, cycle, and invasion of PC3 cells were significantly decreased after overexpression of miR-519d. Bioinformatics analysis and Western blotting showed that there was a potential miR-519d binding site in NRBP1 3'-UTR, and overexpression of miR-519d significantly inhibited the expression of NRBP1. The expression of E-cadherin in PC3 cells overexpressing miR-519d was up-regulated, and the expressions of N-cadherin, cyclin D1, vimentin, fibronectin, and Snail were down-regulated.

CONCLUSIONS: MiR-519d can repress the proliferation, cycle, and invasion of prostate cancer PC3 cells by inhibiting NRBP1.

Key Words
MicroRNAs, Prostate cancer, Cell proliferation, Cell invasion.

Introduction

MicroRNA (miRNA) is a kind of long single-stranded RNA molecule with 19-25 nucleotides in length that binds to the target mRNA 3'-untranslated region (3'-UTR) by incomplete matching, resulting in degradation of the target mRNA or inhibition of protein translation. MiRNAs participate in cell differentiation, proliferation, apoptosis, and other biological processes[1,2]. Scholars[3,4] have shown that miRNAs are abnormally expressed in a variety of tumors and play important roles in tumor proliferation, differentiation, invasion, metastasis, and therapeutic response.

Prostate cancer (PC) is a kind of malignant tumor in male prostate tissue, whose incidence and mortality rates are increasing year by year. Bone metastasis is an important clinical feature of PC. MiRNA-205 inhibits cancer cell migration and invasion *via* regulating centromere protein F regulating pathways in prostate cancer[5]. MiR-519d-mediated down-regulation of STAT3 inhibits the progression of breast cancer[6]. MiR-519d facilitates cervical cancer progression *via* regulating Smad7[7]. For ovarian cancer, miR-519d suppresses cell proliferation and enhances cisplatin-mediated cytotoxicity *via* directly targeting XIAP[8]. MiR-519d targets MKi67 and suppresses cell growth in the hepatocellular carcinoma cell line[9]. MiR-519d suppresses the epithelial-mesenchymal transition (EMT) in gastric cancer *via* Twist1 and inhibits Wnt/β-catenin signaling pathway[10].

The primary purpose of this study was to investigate the possible mechanism of miR-519d transfection into human prostate cancer PC3 cells *in vitro* and to explore its possible mechanism. The relationship between miR-519d and prostate cancer was studied, so as to provide the preliminary theoretical basis and experimental basis for further research.

Materials and Methods

Material Sources

Clinical samples (cancer tissue and its matching adjacent tissue) were collected from patients with prostate cancer in Tangshan Gongren Hospital, and then, immediately stored in the liquid nitrogen. Characteristics of the patients enrolled in this study

Corresponding Author: Wenxing Fan, MD; e-mail: fanwx1999@163.com

were shown in Table I. This study was approved by the Ethics Committee of Tangshan Gongren Hospital. Signed written informed consents were obtained from the patients and/or guardians.

Human prostate cancer PC3 cells were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and trypsin were purchased from Gibco (Rockville, MD, USA); transfection reagent LipofectamineM2000 was purchased from Invitrogen (Carlsbad, CA, USA); enhanced chemiluminescence (ECL) substrate kit was purchased from Pierce (Rockford, IL, USA); Quick Start Bradford protein quantitative reagent was purchased from Bio-Rad (Hercules, CA, USA); transcription kit and reverse transcriptase-polymerase chain reaction (RT-PCR) kit were purchased from TaKaRa (Otsu, Shiga, Japan). Transwell chamber was purchased from Corning (Acton, MA, USA). Cell counting kit-8 (CCK-8) was purchased from Beyotime Biotechnology Research Institute (Shanghai, China). miRNA negative control and miR-519d mimics were purchased from Shanghai GenePharma Biotech (Shanghai, China). E-cadherin, vimentin, N-cadherin, and fibronectin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Snail antibody was purchased from CST (Danvers, MA, USA); pre-stained protein marker was purchased from Fermentas (Waltham, MA, USA); horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Beijing Ding Biotechnology Co., Ltd. (Beijing, China).

Cell Culture and Transfection

Human prostate cancer PC3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS in an incubator with 5% CO_2 at 37°C. At 1 day before transfection, PC3 cells were inoculated into a 6-well plate and transfected at a cell density of 50% fusion. The old medium was discarded and 500 μL complete medium was added into each well. MiR-519d mimics or miRNA-negative control were added into 250 μL Opti-MEM medium and gently mixed. 250 μL Opti-MEM medium was incubated at room temperature for 20 min to form the transfection complex. The above mixture was then added into the cell culture medium and incubated at room temperature for 5 min. Then, the mixture was mixed gently and incubated with 5% CO_2 at 37°C. The cells were collected at 24 h after MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell migration assays. RNA and protein were extracted at 48 h.

Table I. Characteristics of the patient collective.

Cohort feature	Median	Range	No.
Age (years)	64.5	46.00-72.5	62
Tumor size (cm3)	2.40	0.18-20.00	62
PSA (ng/ml)	7.91	2.4-37.6	56

CCK-8 Test

Prostate cancer cells (8×10^3) transfected with miRNA-negative control and miR-519d mimics were inoculated into a 96-well culture plate (100 mL per well). Five control wells were set in each group and blank group (no cells, but only medium) and cultured in an incubator with 5% CO_2 at 37°C. After incubation for 24 h, 5 mg/mL solution was added for 5 h, and the supernatant was incubated for 48 h in a dark place. Then, the microplate reader was used to measure the optical density (OD) value at a wavelength of 490 nm. The test was repeated for three times.

Cell Cycle Test

The cell suspension was collected, and the cell number was $2 \times 10^5 - 1 \times 10^6$. Then, cells were centrifuged, the supernatant was discarded, and the test tube was tapped. Cells were re-suspended and 1 mL phosphate-buffered saline (PBS) was added at room temperature. Cells were placed into 3 mL ice ethanol (-20°C), and shaken at a high speed. Cells were stored at -20°C for several months. Then, cells were centrifuged and the ethanol was discarded. The tube was tapped to lose the pellet. 2-5 mL PBS was added at room temperature and let stand for 15 min to rehydrate cells. The supernatant was then centrifuged. 1 mL reagent A was added, and mixed for 5-10 s for incubation at room temperature for 30 min. To process living cells, the cell suspension was collected, and the cell number was $2 \times 10^5 - 1 \times 10^6$. Then, cells were centrifuged and the supernatant was discarded. Then, cells were washed once with PBS and the supernatant was discarded. 1 mL reagent A and 10 μL reagent B were added and mixed for 5-10 s for incubation at room temperature for 30 min. Flow analysis: the cell suspension was incubated directly on the machine. Fluorescence microscopy: the incubated cell suspension was centrifuged to remove the supernatant, washed with fresh buffer (such as PBS), and then re-suspended. The cell suspension was added into the glass slide covered with a cover glass, followed by observation.

Transwell Assay

Transwell chamber was placed in a culture plate, and 300 μL pre-heated serum-free medium was added to the upper chamber and let stand at room temperature for 15-30 min to rehydrate the matrix gel. Digestion cells were washed twice with PBS and re-suspended in serum-free medium. The cell density was adjusted to 3×10^5 cells/mL. 200 μL cell suspension was taken and added into a 24-well plate, and 200 μL FBS-containing medium was added into the 24-well plate. After continuous culture for 48 h, cells in the lower chamber were stained with hematoxylin, and they were counted in 5 fields of view. The analysis of its effect on the invasion ability of cells was performed.

Protein Extraction and Western Blotting

Cells were washed with PBS and lysates [50 mmoL/L Tris-Cl (pH 8.0), 150 mmol/L NaCl, 0.2 g/L sodium azide, 100 mg/L Aprotin, 100 mg/L phenylmethylsulfonyl fluoride (PMSF), lg/L sodium dodecyl sulphate (SDS), 10 g/L NP-40, 5 g/L sodium deoxycholate] Ø (Beyotime, Shanghai, China). The supernatant was collected after centrifugation and the protein concentration was determined using Bradford method. The expressions of NRBP1, E-cadherin, vimentin, N-cadherin, Fibronectin, Snail and cyclin D1 were detected, followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 4°C overnight and sealing in 5% skim milk powder at room temperature for 2 h. After washing with phosphate-buffered saline and Tween 20 (PBST), HRP-labeled specific secondary antibody was added at a ratio of 1:5000 for incubation at room temperature for 2 h. Finally, ECL reagent was used to develop X-ray film.

RT-PCR

The total RNA was extracted according to the TRIzol (Invitrogen, Carlsbad, CA, USA) method and RNA was reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to the Primescript RT reagent reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). Primers were designed and synthesized by Shanghai Biotechnology Bioengineering Co., Ltd. (Shanghai, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward primer: 5'-GCACCGTCAAGGCTGAGAAC-3', reverse primer: 3'-TGGTGAAGAcGcCAGTGGA-5'. Real-time PCR system and conditions are based on SYBR Premix Ex TapTM kit (TaKaRa, Otsu, Shiga, Japan), with GAPDH as internal reference. The experiment was repeated for 3 times in each group.

Statistical Processing

The experiment was repeated for 3 times in each group, the GraphPad Prism 5.0 software (Version X; La Jolla, CA, USA) was used for statistical analysis. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

MiR-519d Expression in Prostate Cancer Tissue Was Decreased With Clinical Characteristics

First, the expressions of miR-519d in the prostate cancer tissues and corresponding adjacent samples were detected. Results showed that miR-519d expression in the cancer samples was significantly lower than that in the corresponding adjacent tissues ($p<0.05$, Figure 1A). Moreover, it was found that miR-519d was related to the tumor size, metastasis, and staging (Table II), suggesting that abnormal expression of miR-519d contributes to the progression of prostate cancer.

Effects of miR-519d Overexpression on Proliferation, Cycle and Invasive Ability of Prostate Cancer PC3 Cells

The CCK-8 results showed that the proliferation of miR-519d cells was significantly lower than that in control group ($p<0.05$) (Figure 2B). Furthermore, it was found that miR-519d up-regulation could repress PC3 cells cycle (Figure 2A). The number of cells passing through the membrane in overexpression miR-519d group was significantly smaller than that in control group ($p<0.05$). The number of cells passing through the membrane in control group was (322±15)/field, while that in miR-519d mimics-treated group was (97±11)/field (Figure 2B). The results suggest that miR-519d can inhibit cell proliferation, cycle, and invasion of PC3 cells.

Effects of miR-519d Overexpression on PC3 Cell Cycle and Expression of EMT Markers in Prostate Cancer Cells

The potential miR-519d binding site was found in NRBP1 3'-UTR using miRBase software predictions and bioinformatics analysis (Figure 3A). Fluorescence quantitative PCR analysis showed that NRBP1 expression was significantly inhibited by miR-519d mimics transfection (Figure 3B). Western blotting was used to detect the expres-

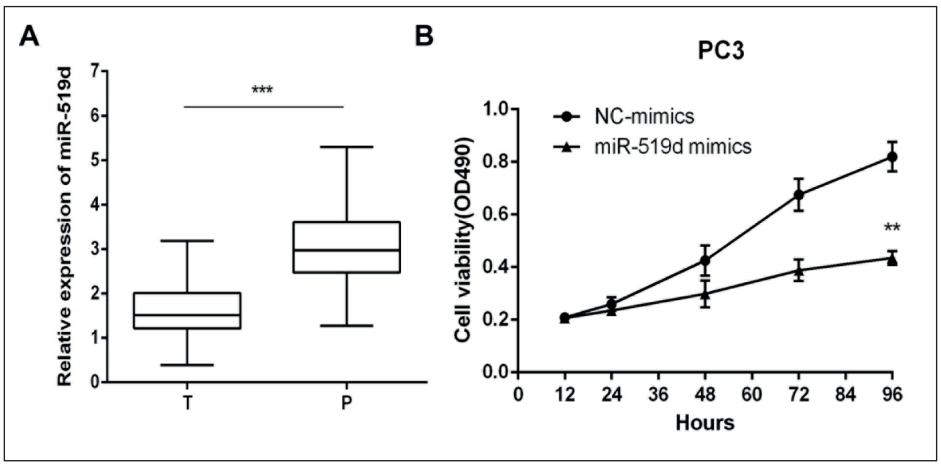

Figure 1. miR-519d is decreased in tumor tissues and inhibits cell proliferation. **A**, miR-519d expressions in tumor tissues (T) and corresponding adjacent tissues (P) are detected by RT-PCR. **B**, The CCK-8 assay is performed to detect cell proliferation in NC mimics and miR-519d mimics. NC-mimic is regarded as the control group; *$p<0.05$.

sions of NRBP1, E-cadherin, vimentin, N-cadherin, fibronectin (FN), Snail and cyclin D1 in miR-519d overexpression group. The expressions of NRBP1, vimentin, N-cadherin, fibronectin, and Snail were inhibited and the protein expression of E-cadherin was promoted. Moreover, compared with that in control group, the expression of cyclin D1 in PC3 cells with miR-519d overexpression was down-regulated (Figure 3C and D).

Discussion

In recent years, miRNA, as an important regulatory factor in the post-transcriptional regulatory network, has been attracting more and more attention in the development, progression and metastasis of tumors[3,11-14]. Overexpression of miR-519d in lung adenocarcinoma inhibits cell proliferation and invasion *via* the association of eIF4H[15]. C14orf28 down-regulated by miR-519d contributes to oncogenicity and regulates apoptosis and EMT in colorectal cancer[16]. MiRNA-519d promotes melanoma progression by down-regulating EphA4[17]. We found that miR-519d was overexpressed in prostate cancer PC3 cell lines, the cell proliferation and cycle were significantly inhibited, and the cell invasion was also weakened.

Cell cycle out of control can lead to cell proliferation abnormality, which is an important part of tumor occurrence and development. The process of the cell cycle is regulated by multiple control points, among which the regulation points G1/S and G2/M are the most important. Cyclin D1 is the most critical protein that regulates G1/S detection point, which binds to cyclin D1-CDK4 to activate CDK4, and leads to cell expression through G1/S. Abnormal expression of Cyclin D1 can lead to G1/S detection points[18]. We found that overexpression of miR-519d significantly inhibited the expression of cyclin D1, suggesting that the overexpression of miR-519d may inhibit cell proliferation by down-regulating cyclin D1 through the G1/S restriction point.

EMT is a unique step in the acquisition of some interstitial cells in the interaction process of epithelial cells with surrounding stroma, which is currently considered as a key step in a variety of tumor invasion and metastasis process[19]. MiRNAs are regulators of cancer metastasis and EMT[20]. MiR-519d has a binding site to the 3'-UTR of NRBP1, suggesting that it has the possibility of inhibiting the expression of NRBP1. It is further demonstrated that miR-519d can down-regulate NRBP1 mRNA and protein expressions. We found that the expression of miR-519d could inhibit the expressions of N-cadherin, E-cadherin, vimentin, N-cadherin, fibronectin and Snail, and promote the expression of E-cadherin, suggesting that miR-519d may inhibit NRBP1 expression and negatively regulate EMT process in prostate cancer cells.

Table II. Characteristics of tumor characteristics.

Tumor characteristics	No. of samples (%)
Node-positive (N1) cases	5 (8.06)
Distant metastasis (M1)	0 (0)
Lymphatic vessel invasion (L1)	7 (11.2)
Vascular invasion (V1)	1 (1.6)
Stage	
pT2a 10 (8.93)	6 (9.68)
pT2b 1 (0.89)	1 (1.6)
pT2c 72 (96.29)	36 (58.1)
pT3a 14 (12.50)	10 (16.1)
pT3b 13 (11.61)	8 (12.9)
pT4	1 (1.6)

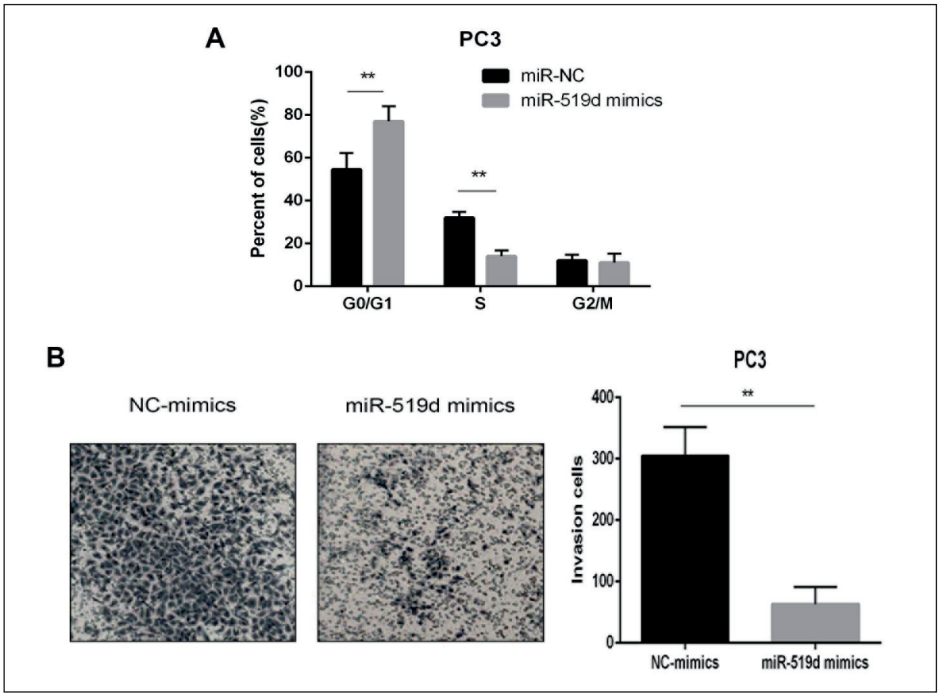

Figure 2. miR-519d inhibits cell cycle and invasion. **A**, The effect of miR-519d expression on cell cycle is detected at G0/G1, S, G2/M. **B**, The effect of miR-519d expression on cell invasion is detected by transwell assay; *p<0.05.

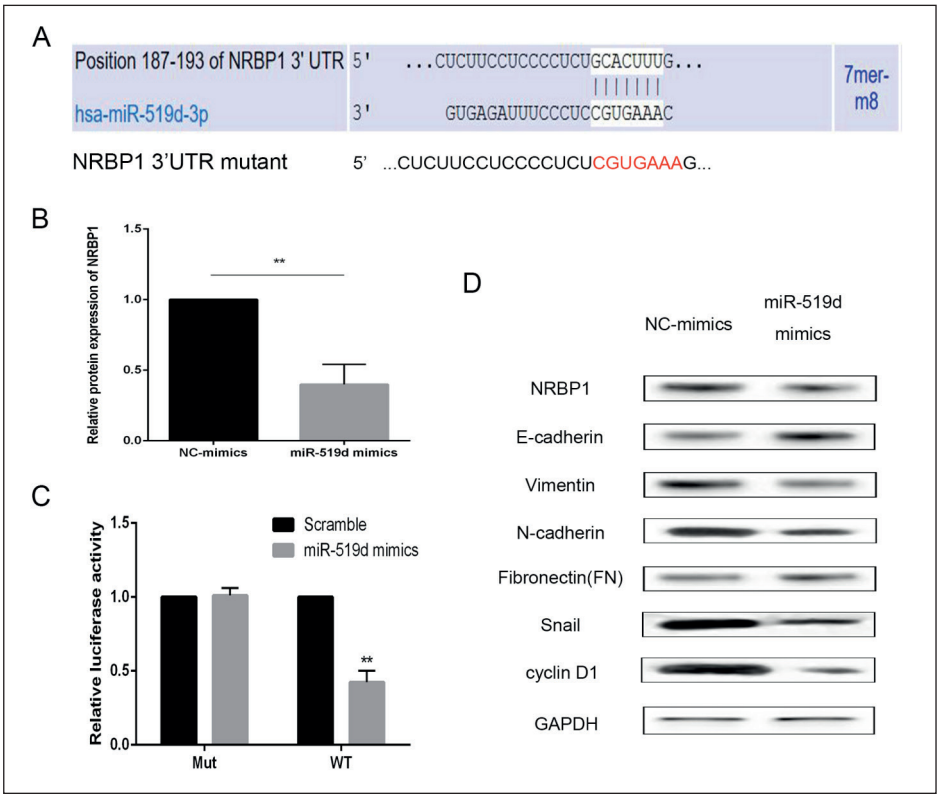

Figure 3. miR-519d negatively regulates NRBP1 by binding to its 3'-UTR, and inhibits EMT process. **A**, **B** and **C**, The potential binding site is predicted through miRBase software predictions and bioinformatics analysis, and then confirmed by luciferase reporter and Western blotting. **D**, The protein expression levels of NRBP1, E-cadherin, vimentin, N-cadherin, fibronectin, Snail and cyclin D1 are detected via Western blotting; *p<0.05.

Conclusions

We found that miR-519d can inhibit the proliferation, cycle, and invasion of PC3 cells in prostate cancer, providing an important theoretical basis in treatment for prostate cancer.

Conflict of Interest:

The authors declared no conflict of interest.

References

1) Garzon R, Fabbri M, Cimmino A, Calin GA, Croce CM. MicroRNA expression and function in cancer. Trends Mol Med 2006; 12: 580-587.
2) Zhou J, Zhang M, Huang Y, Feng L, Chen H, Hu Y, Chen H, Zhang K, Zheng L, Zheng S. MicroRNA-320b promotes colorectal cancer proliferation and invasion by competing with its homologous microRNA-320a. Cancer Lett 2015; 356(2 Pt B): 669-675.
3) Liu H, Li SR, Si Q. Regulation of miRNAs on c-met protein expression in ovarian cancer and its implication. Eur Rev Med Pharmacol Sci 2017; 21: 3353-3359.
4) Shen J, Niu W, Zhou M, Zhang H, Ma J, Wang L, Zhang H. MicroRNA-410 suppresses migration and invasion by targeting MDM2 in gastric cancer. PLoS One 2014; 9: e104510.
5) Nishikawa R, Goto Y, Kurozumi A, Matsushita R, Enokida H, Kojima S, Naya Y, Nakagawa M, Ichikawa T, Seki N. MicroRNA-205 inhibits cancer cell migration and invasion via modulation of centromere protein F regulating pathways in prostate cancer. Int J Urol 2015; 22: 867-877.
6) Deng X, Zhao Y, Wang B. MiR-519d-mediated downregulation of STAT3 suppresses breast cancer progression. Oncol Rep 2015; 34: 2188-2194.
7) Zhou JY, Zheng SR, Liu J, Shi R, Yu HL, Wei M. MiR-519d facilitates the progression and metastasis of cervical cancer through direct targeting Smad7. Cancer Cell Int 2016; 16: 21.
8) Pang Y, Mao H, Shen L, Zhao Z, Liu R, Liu P. MiR-519d represses ovarian cancer cell proliferation and enhances cisplatin-mediated cytotoxicity in vitro by targeting XIAP. Onco Targets Ther 2014; 7: 587-597.
9) Hou YY, Cao WW, Li L, Li SP, Liu T, Wan HY, Liu M, Li X, Tang H. MicroRNA-519d targets MKi67 and suppresses cell growth in the hepatocellular carcinoma cell line QGY-7703. Cancer Lett 2011; 307: 182-190.
10) Yue H, Tang B, Zhao Y, Niu Y, Yin P, Yang W, Zhang Z, Yu P. MIR-519d suppresses the gastric cancer epithelial-mesenchymal transition via Twist1 and inhibits Wnt/beta-catenin signaling pathway. Am J Transl Res 2017; 9: 3654-3664.
11) Li XJ, Ren ZJ, Tang JH. MicroRNA-34a: a potential therapeutic target in human cancer. Cell Death Dis 2014; 5: e1327.
12) Wang YW, Shi DB, Chen X, Gao C, Gao P. Clinicopathological significance of microRNA-214 in gastric cancer and its effect on cell biological behaviour. PLoS One 2014; 9: e91307.
13) Pan Y, Liang H, Chen W, Zhang H, Wang N, Wang F, Zhang S, Liu Y, Zhao C, Yan X, Zhang J, Zhang CY, Gu H, Zen K, Chen X. MicroRNA-200b and microRNA-200c promote colorectal cancer cell proliferation via targeting the reversion-inducing cysteine-rich protein with Kazal motifs. RNA Biol 2015; 12: 276-289.
14) Haigl B, Vanas V, Setinek U, Hegedus B, Gsur A, Sutterluty-Fall H. Expression of microRNA-21 in non-small cell lung cancer tissue increases with disease progression and is likely caused by growth conditional changes during malignant transformation. Int J Oncol 2014; 44: 1325-1334.
15) Bai Y, Lu C, Zhang G, Hou Y, Guo Y, Zhou H, Ma X, Zhao G. Overexpression of miR-519d in lung adenocarcinoma inhibits cell proliferation and invasion via the association of eIF4H. Tumour Biol 2017; 39: 1393395098.
16) Yang X, Hu Y, Liu Y, Liu W, Zhao X, Liu M, Tang H. C14orf28 downregulated by miR-519d contributes to oncogenicity and regulates apoptosis and EMT in colorectal cancer. Mol Cell Biochem 2017; 434: 197-208.
17) Hua KT, Hong JB, Sheen YS, Huang HY, Huang YL, Chen JS, Liao YH. MicroRNA-519d promotes melanoma progression by downregulating EphA4. Cancer Res 2017: 1933-2017.
18) Malumbres M. Cyclins and related kinases in cancer cells. J Buon 2007; 12 Suppl 1: S45-S52.
19) Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002; 2: 442-454.
20) Ding XM. MicroRNAs: regulators of cancer metastasis and epithelial-mesenchymal transition (EMT). Chin J Cancer 2014; 33: 140-147.