

miRNA-375 regulates the invasion and metastasis of human lung cancer A549 cells by targeting EMT

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Abstract: To investigate whether abnormally high expression of miRNA-375 is associated with epithelial cell mesenchymal transition (EMT) in lung cancer cells. The stable overexpression cell line was constructed by lentivirus and transfected into empty vector as a control group. The expression of miRNA-375 was detected by real-time fluorescence quantitative PCR. The protein expression of EMT-related protein E-cadherin and Vimentin were detected in three groups of cells by western blot. The cell invasion and metastasis were evaluated by transwell methods and wound healing. EMT of lung cancer A549 cells was characterized by mesenchymal cell cytoskeleton and morphology, and the expression of EMT-related marker protein E-cadherin was down regulated while the expression of Vimentin was up regulated. Transwell methods and wound healing showed that aberrant expression of miRNA-375 in A549 cells was significantly increased ($P < 0.05$). The abnormal expression of microRNA-375 is associated with EMT in lung cancer A549 cells.

Keywords: MicroRNA-375; Epithelial mesenchymal transition; Lung cancer; A549 cell

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1. Introduction

Lung cancer is the most common solid tumor and is one of the most rapidly growing and morbidly malignant tumors with the highest risk to human health and life[1,2]. Lung cancer is divided into two broad categories, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)[2]. The incidence of lung cancer gradually increases and more than 80% of non-small cell lung cancer[3]. Chemotherapy is the primary treatment for lung cancer. Although non-small cell lung cancer grows slowly and spreads relatively late, chemotherapy generally fails to cure non-small cell lung cancer and only prolongs patient survival and improves quality of life[4-5]. Therefore, we need an effective means of detection for the early diagnosis and treatment of lung cancer.

MicroRNA plays an oncogene or tumor suppressor gene function, and it also plays an important role in the EMT process of tumor cells, which can promote or inhibit tumor cell invasion and metastasis. The aim of this study was to investigate whether abnormally high expression of microRNA-375 positively correlates with the malignant degree of lung adenocarcinoma A549. The role and mechanism of microRNA in invasion and metastasis of lung cancer were studied by western blot, scratch assay and transwell methods. Exploration further, improve the mechanism of lung cancer metastasis for the clinical diagnosis and treatment of lung cancer.

2. Material and methods

2.1. Reagents and antibodies

RPMI 1640 medium was purchased from Hyclone;

Fetal bovine serum (FBS) was purchased from Gibco; Primary antibodies to E-cadherin, Vimentin, β -actin and corresponding secondary antibodies were obtained from Abcam. Immunoblot chemiluminescence was purchased from Syngene. mir-375 mimics were purchased from Biomics, Trizol, reverse transcription kits, PCR primers, Lipofectamine RNAi max were purchased from the United States Invitrogen company. Transwell plates were purchased from Corning Corporation; lung cancer cell line A549 was stored in our laboratory.

2.2. Cell culture and transfection

Human lung adenocarcinoma cell line A549 cells were cultured in DMEM medium containing 10% fetal bovine serum, 100U/ml penicillin and 100U/ml streptomycin at 37 °C under 5% CO₂ saturated humidity. After the cells were grown to logarithmic growth phase, the lentivirus (Lv-miR-375) and the control lentivirus (Lv-NC) were expressed with 25MOI of mir-375 at 37 °C for 10 hours according to the instructions of the transfection kit, A549 cells were transfected.

2.3. RNA extraction and Real time-PCR

The total RNA was extracted with Iso plus RNA and subjected to reverse transcription reaction using PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time). The reverse transcription primer for miR-375 was upstream primer 5'- CGC GCT TTG TTC GTT CGG CTC-3', downstream primer 5'-ATC CAG TGC AGG GTC CGA GTG-3'; real-time PCR primer sequence: upstream primer 5'-CAT GTA CGT TGC TAT CCA GGC-3', downstream primer 5'-CTC

CTT AAT GTC ACG CAC GAT-3'. U6 small nuclear RNA for endogenous reference miRNA, β -actin for target gene mRNA endogenous reference, the use of SYBR Green dye reaction method, 95 °C denaturation 10min, 95 °C denaturation 5s, 60 °C annealing 34s, 74 °C fluorescence detection 3s, a total of 40 cycles. The difference between the two is compared with the $2^{-\Delta\Delta Ct}$ value. Set up three duplicate holes in each group.

2.4. Western Blot Analysis

Western Blot test protein expression levels. A549 cells were infected with recombinant adenovirus after 48h, with reference to the experimental manual, the total protein lysate was extracted, stored at -80 °C for later use. The concentration of the protein was measured by BCA method according to the instructions of the kit, and the protein concentration was adjusted with $5 \times$ loading buffer and denatured at 95 °C, for 10min. The protein was separated by electrophoresis on 50 μ g of the protein and electrophoresed to transfer the protein to PVDF membrane. 5% skim milk powder shaker at room temperature closed 1h, the band into the primary antibody, incubated overnight at 4 °C. Wash with TBST three times, 10min/time, incubate the secondary antibody at room temperature 2h, washed three times with TBST, 10min/times, soaked with ECL luminescent liquid, the strip into the dark box to darkroom exposure. Using Photoshop CS6 grayscale analysis, and statistical gray value.

2.5. Wound Healing Assay

Using a marker pen behind the 6-well plate, draw a ruler approximately 1cm apart across a hole, across the uniform crossbar. Draw at least 3 straight lines per hole. A549, Lv-A549 and Lv-miR-375-A549 cells were planted in three 6-well plates, and about 500,000 cells were inoculated into each well. The cells were planted in a six-well plate and cultured. At the bottom of the plate, 3 lines are drawn longitudinally on the cell monolayer in each well using a 20 μ L pipette tip. After washing the cells three times with PBS, a culture of RPMI-1640 was added to the culture. At 0, 24, and 48h after injury, images were taken with an inverted microscope, the healing area of the scratch was calculated using Photoshop CS6 software, and the healing rate of scratches was calculated.

2.6. Transwell analysis of cell transfer and invasion

A549 cells were cultured in 12-well plates and transfected at a cell fusion level of 80%. Cells were transfused for 6h, replaced with complete culture solution, cultured for 18h, and then digested with 0.25% trypsin, digested with 2ml complete medium, and centrifuged to adjust the cell density to 3×10^5 cells/ml with Opti-MEM Single cell suspension. Lv-miR-375-A549 and Lv-NC-A549 cells

untransfected with diluted matrigel (coated coated matrigel (1: 9)) suspended in 200 μ l of serum- Total 5×10^4 cell invasion assays) Upper chamber. 20% fetal bovine serum containing PRMI1640 was added to the lower chamber. Incubation in the incubator 4h, each set 3 parallel wells. The chamber was removed, the culture medium in the upper chamber was removed, the cells in the upper chamber were gently scraped off with a sterilized cotton swab, and stained with 0.05% crystal violet in 20% methanol for 5 minutes. Excess stains were removed and cells that passed through the basal lamina were observed under an inverted microscope and counted.

2.7. Statistical analysis

All experiments were repeated 3 times. The experimental data were expressed as mean \pm SD, and SPSS 17.0 statistical software was used for data analysis. One-way analysis of variance (ANOVA) was used to compare among groups. Bonferroni test was used to compare the two groups, $P < 0.05$ was considered as statistically significant.

3. Results

3.1. The expression of mir-375 in lung adenocarcinoma A549 cells

The effect of recombinant adenovirus A549 cells on the expression of mir-375, as shown in Figure 1, showed that the infection rate of A549 cells transfected with miR-375 and NC null plasmid was $\geq 90\%$.

3.2. The expression of mir-375 in lentivirus transfected A549 lung cancer cells

As shown in Figure 2A, compared with the human normal lung epithelial 2B cells, the expression of miR-375 was significantly increased, and in human small lung cancer cells H520 miR-375 was down-regulated ($P < 0.05$). As shown in Figure 2B, transfected miR-375 lentivirus lung cancer A549 cells significantly increased the expression of mir-375 in lung cancer A549 cells. The expression of mir-375 was significantly up-regulated compared with the negative control group and the blank control group ($P < 0.05$), but there was no significant difference between the negative control group and the blank control group ($P > 0.05$).

3.3. The expression of E-cadherin and Vimentin protein in A549 cells

Western blot was used to detect the expression of E-cadherin and Vimentin protein in A549 cells after transfected with recombinant adenovirus. The results showed that compared with A549 and LV-A549, A549 cells transfected with mir-375 recombinant adenovirus E-cadherin protein ($P < 0.05$). The expression of Vimentin protein was significantly increased ($P < 0.05$), but there was no significant difference between LV-A549 and A549 ($P > 0.05$).

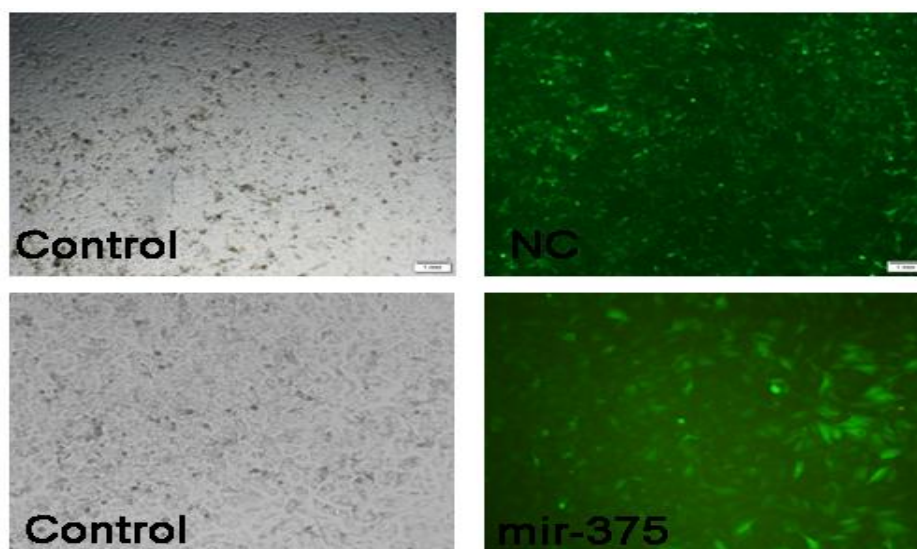


Figure 1. The expression on miRNA-375 in the lung cancer A549 cells after transfection with recombinant adenovirus ($\times 100$).

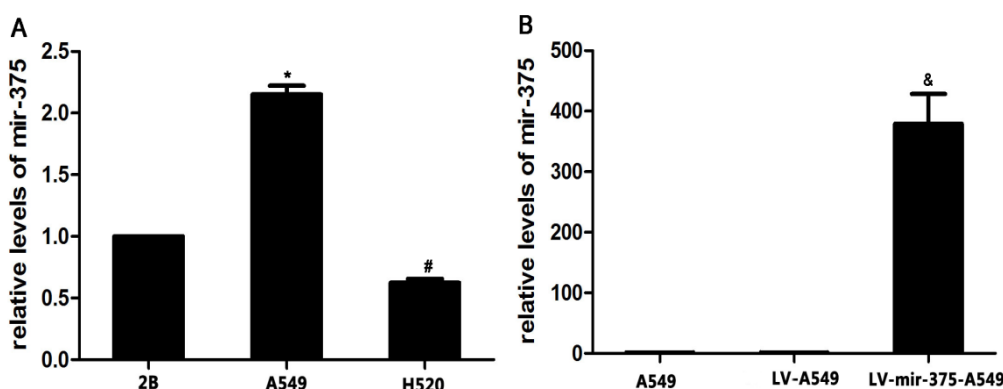


Figure 2. A: miR-375 expression in A549, BEAS-2B, H520 cells. B: miR-375 expression in A549, LV-A549, LV-miR-375-A549 cells. * $P < 0.05$ vs 2B; # $P < 0.05$ vs 2B; & $P < 0.05$ vs LV-A549.

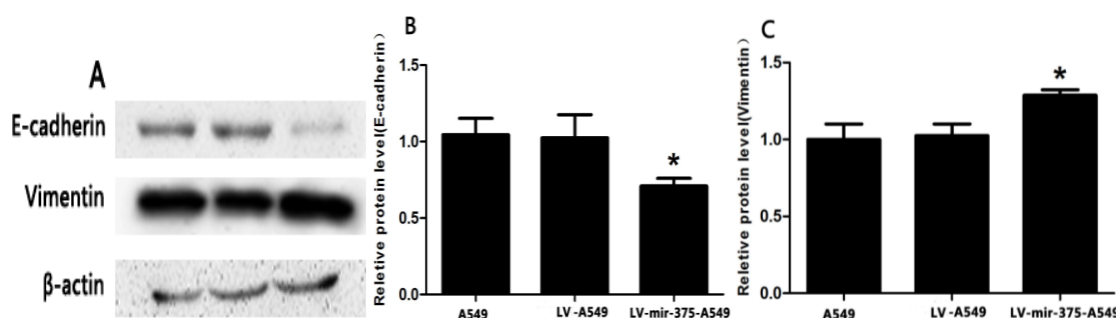


Figure 3. A: The protein expression of EMT-related protein markers in three groups of cells; B: Expression of E-cadherin protein in A549 cells after transfection of recombinant adenovirus; C: Vimentin protein expression in A549 cells after transfection of recombinant adenovirus. * $P < 0.05$ vs A549.

3.4. The migration ability in lung cancer A549 cell

Wound healing test on lung cancer A549 cell migration ability. The results showed that the wound healing rate of LV-miR-375-A549 transfected plasmid group was decreased by 35.76% and 34.35%

respectively compared with LV-A549 empty vector group and A549 blank control group, but LV-A549 empty vector group and A549 blank The difference between the control group was not statistically significant, shown in Figure 4.

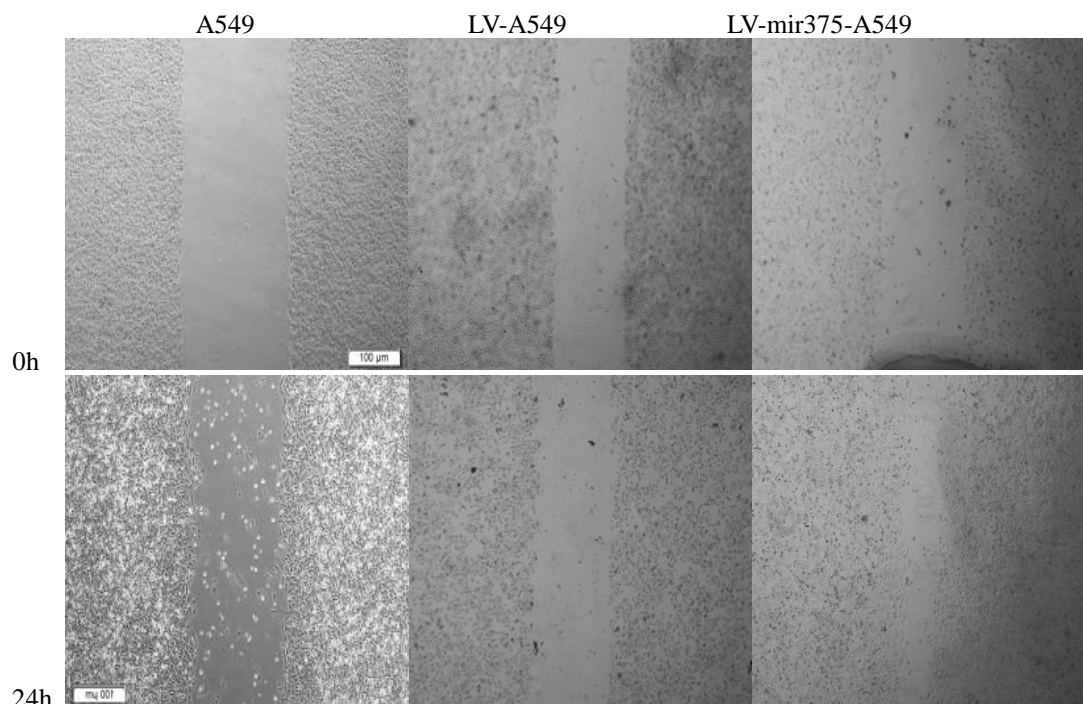


Figure 4. The effect of mir-375 on metastasis of lung cancer A549 cells.

3.5. The invasiveness in lung cancer A549 cells

Transwell chambers were used to compare the invasiveness of A549 cells transfected with LV-miR-375. The results showed that the number of transmembrane cells transfected with recombinant plasmid LV-miR-375-A549 was (109.60 ± 6.25) . The numbers of transmembrane cells in A549 empty vector group and A549 blank control group were (48.00 ± 4.82) and (47.50 ± 4.97) , respectively (Figure 5) The number

of transmembrane cells in empty vector group was significantly increased compared with A549 blank control group ($P < 0.05$), but there was no significant difference between untransfected group and negative control group ($P > 0.05$). The above results show that, miR-375 can promote the invasive ability of A549 cells.

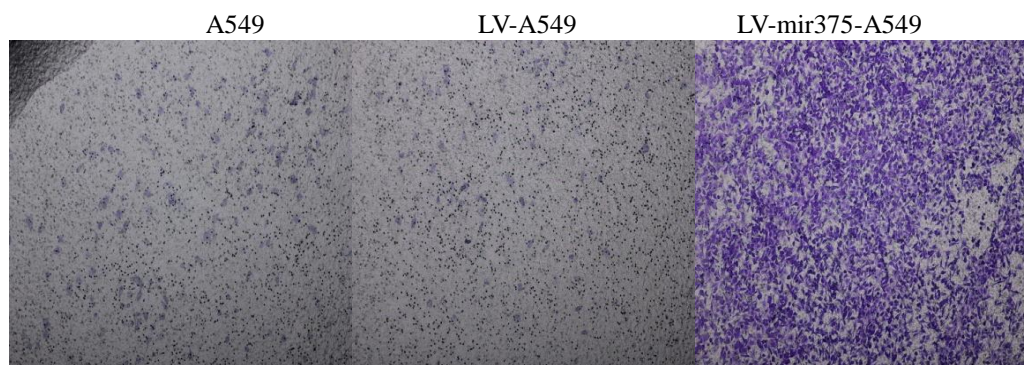


Figure 5. The effects of miR-424 on migration abilities of A549 by crystal violet staining.

4. Discussion

Lung cancer is one of the malignant tumors that seriously affect human health. The increasing incidence of lung cancer causes widespread concern worldwide. With the continuous research of lung cancer in the world, more and more researches show that it is very important to diagnose and treat the occurrence and development of lung cancer in the early stage[6-8].

MicroRNAs are recently discovered a class of

highly conserved, short-sequence, endogenous, non-coding single-stranded small RNAs that are widely present in animals, plants and viruses and are involved in the regulation of cell proliferation, apoptosis, Differentiation and other life processes[9]. In recent years, studies have shown that microRNA plays a key role in tumorigenesis. In 2008, Chinese researchers first discovered that serum microRNA can be used as a specific biomarker for the diagnosis of lung cancer[10]. A large number of studies have shown that miRNA-375 plays an important role in the

proliferation, differentiation, apoptosis and cell cycle regulation of malignant tumors. MicroRNA-375 is expected to become a new target for tumor therapy and may become an important organism for disease diagnosis and prognosis Markers[11,12]. As a member of the miRNA family, mir-375 has drawn more and more attention in recent years[13]. Mir-375 participates in many cellular processes, including cell proliferation, cell differentiation, cell metabolism, apoptosis and angiogenesis by regulating target genes[14,15]. However, the current research on the signaling pathway of mir-375 in lung cancer related to EMT is not yet clear. In this study, we further clarified the mechanism of mir-375 in lung cancer by analyzing the relationship between mir-375 and EMT signaling molecules.

Epithelial-mesenchymal transition (EMT) refers to the biological process by which epithelial cells are transformed into mesenchymal cells by a specific procedure. EMT is an important process in tumorigenesis and development and is also one of the important mechanisms of tumor invasion and migration and secondary metastasis. This change is usually accompanied by an increase in the decrease of intercellular adhesion molecules such as E-cadherin accompanied by an increase in interstitial cell markers such as Vimentin and fibronectin[16-18]. E-cadherin is a key marker protein for EMT[19]. EMT plays a very important role in a variety of physiological and pathological processes, such as wound healing, chemoresistant cancer resistance, and tumor invasion and migration[20]. To study the role of EMT in the invasion and metastasis of lung cancer is helpful for the prevention and treatment of lung cancer.

However, some studies have found that mir-375 is overexpressed in lung adenocarcinoma and small-cell lung cancer and low in lung squamous cell carcinoma[21]. The differences between the above results may be related to the type of lung cancer, cell line differences, cell species, and sample size and other factors. The results showed that mir-375 can significantly reduce E-cadherin protein level and up-regulate Vimentin protein level in lung cancer A549 cells. It suggested that mir-375 may further affect the invasion and migration of lung cancer by down-regulating the EMT signaling pathway and affecting the epithelial-mesenchymal transition of A549 lung cancer cells. In summary, the experiment by cell transfection lung cancer A549 cells mir-375 low expression, analysis of lung cancer cell migration, invasion and other biological behavior changes, and explore the possible mechanism of this change. The experimental results show that mir-375 can inhibit lung cancer A549 cell migration and invasion, while inhibiting the EMT signaling pathway. We will also continue to investigate the target gene of mir-375 by gene knockout or overexpression of the target gene, and then to construct a stable cell line, analyzing the interaction between the EMT signaling pathway and the target gene of mir-375 used in clinical research to

provide the basis for lung cancer.

References

- [1] Nishikawa E, Osada H, Okazaki Y, et al. miR-375 is activated by ASH1 and inhibits YAP1 in a lineage-dependent manner in lung cancer[J]. *Cancer Res*, 2011, 71(19): 6165-6173.
- [2] Wang L, Qu J, Zhou L, et al. MicroRNA-373 inhibits cell proliferation and invasion via targeting brf2 in human non-small cell lung cancer a549 cell line[J]. *Cancer Res Treat*, 2017, 2(1): 15-18.
- [3] Al-Saleh K, Quinton C and Ellis PM. Role of pemetrexed in advanced non-small-cell lung cancer: meta-analysis of randomized controlled trials, with histology subgroup analysis[J]. *Curr Oncol*, 2012, 19(1): 9-15.
- [4] Turner RM, Chen YW and Fernandes AW. Validation of a case-finding algorithm for identifying patients with non-small cell lung cancer (nslc) in administrative claims databases[J]. *Front Pharmacol*, 2017, 8: 883-889.
- [5] Indovina P, Marcelli E, Maranta P, et al. Lung cancer proteomics: recent advances in biomarker discovery[J]. *Int J Proteomics*, 2011, 201(7): 26-29.
- [6] Xia H, Ooi LL, Hui KM. MicroRNA 216a/217 induced epithelial mesenchymal transition targets PTEN and SMAD7 to promote drug resistance and recurrence of liver cancer[J]. *Hepatology*, 2013, 58(2): 629-641.
- [7] Joo MJ, Au DH, Fitzgibbon ML, et al. Inhaled corticosteroids and risk of pneumonia in newly diagnosed COPD[J]. *Respir Med*, 2010, 104(2): 246-252.
- [8] Barnes PJ and Hansel TT. Prospects for new drugs for chronic obstructive pulmonary disease[J]. *The Lancet*, 2004, 364(9438): 985-996.
- [9] Chen LJ, Li XY, Zhao YQ, et al. Down-regulated microRNA-375 expression as a predictive biomarker in non-small cell lung cancer brain metastasis and its prognostic significance[J]. *Pathology Research and Practice*, 2017, 213(13): 882-888.
- [10] Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases[J]. *Cell Res*, 2008, 18(10): 997-1006.
- [11] Jin Y, Liu Y, Zhang J, et al. The Expression of miR-375 Is Associated with Carcinogenesis in Three Subtypes of Lung Cancer[J]. *PLoS One*, 2015, 10(12): 144-147.
- [12] Li X, Han J, Zhu H, et al. miR181b5p mediates TGFbeta1 induced epithelial to mesenchymal transition in non-small cell lung cancer stem-like cells derived from lung

- adenocarcinoma A549 cells[J]. *Int J Oncol*, 2017, 51(1): 158-168.
- [13] Pacurari M, Addison JB, Bondalapati N, et al. The microRNA-200 family targets multiple non-small cell lung cancer prognostic markers in H1299 cells and BEAS-2B cells[J]. *Int J Oncol*, 2013, 43(2): 548-560.
- [14] Wang L, Song G, Liu M, et al. MicroRNA-375 over expression influences P19 cell proliferation, apoptosis and differentiation through the Notch signaling pathway[J]. *Int J Mol Med*, 2016, 37(1): 47-55.
- [15] Ding L, Xu Y, Zhang W, et al. MiR-375 frequently down regulated in gastric cancer inhibits cell proliferation by targeting JAK2[J]. *Cell Res*, 2010, 20(7): 784-793.
- [16] Adam L, Zhong M, Choi W, et al. miR-200 expression regulates epithelial to mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy[J]. *Clin Cancer Res*, 2009, 15(16): 5060-5072
- [17] Men D, Liang Y and Chen L. Decreased expression of microRNA-200b is an independent unfavorable prognostic factor for glioma patients[J]. *Cancer Epidemiol*, 2014, 38(2): 152-156.
- [18] Wang Z, Li Y, Ahmad A, et al. Targeting miRNAs involved in cancer stem cell and EMT regulation: An emerging concept in overcoming drug resistance[J]. *Drug Resist Updat*, 2010, 13(4-5): 109-118.
- [19] Burk U, Schubert J, Wellner U, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells[J]. *EMBO Rep*, 2008, 9(6): 582-589.
- [20] Peng Z, Wang CX, Fang EH, et al. Role of epithelial-mesenchymal transition in gastric cancer initiation and progression[J]. *World J Gastroenterol*, 2014, 20(18): 5403-5410.
- [21] Shao Y, Geng Y, Gu W, et al. Prognostic significance of microRNA 375 down regulation in solid tumors: a meta-analysis[J]. *Dis Markers*, 2014, 20(6): 261-265.