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Original Article

MiR-539 inhibits cell proliferation and invasion by targeting CARMA1 in NSCLC

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Abstract: MicroRNAs (miRNAs) play an important role in the development and progression of non-small cell lung cancer (NSCLC). Recently, several studies have shown that miR-539 is down regulated in various cancers, however, its expression and function in NSCLC remain unclear. In this study, we found that miR-539 was downregulated in NSCLC tissues and cell lines. Overexpression of miR-539 suppressed cell proliferation and invasion in A549 cells. Furthermore, our results also shown miR-539 reduction was associated with increased CARMA1 expression levels in NSCLC tissues and luciferase activity assays showed that CARMA1 is a direct target ofmiR-539. Taken together, our study demonstrates that miR-539 plays important roles in NSCLC pathogenesis, implicating potential application in cancer therapy.

Keywords: Non-small cell lung cancer (NSCLC), miR-539, CARMA1, proliferation, invasion

Introduction

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer related death worldwide, among of which non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers [1, 2]. Despite improvements of surveillance and clinical treatment strategies of NSCLC and its five-year survival rate, the 5-year survival of NSCLC patients after curative resection is reported to be only 30-60% and distant metastasis is still one of the most common failure patterns [3]. Therefore, it is urgent to elucidate the potential mechanism that mediates the initiation and progression of NSCLC. Hence, identification of new molecules involved in tumor metastasis is of crucial importance to reduce morbidity and mortality of this devastating disease.

MicroRNA (miRNA), an abundant group of endogenous non-coding single strand RNAs of 22 nucleotides, regulates their target genes by binding to the 3' untranslated region (3'-UTR) of target messenger RNAs (mRNAs), causing either degradation or inhibition of translation. In this manner, miRNAs play important roles in a range of biological processes including cell proliferation, apoptosis, invasion, migration, differentiation and so on [4-7]. Numerous studies have suggested that miRNAs were deregulated in tumorigenesis [8, 9]. The miRNA expression profiles and specific miRNAs are correlated with disease prognoses and clinical outcomes in lung cancer [10-12]. For example, High expression of miR-21 and miR-155 were reported to predict recurrence and unfavourable survival in non-small cell lung cancer [13]. Decreased miRNA-148a is associated with lymph node metastasis and poor clinical outcomes and functions as a suppressor of tumor metastasis in non-small cell lung cancer [14]. Differential expression of miR-125a-5p and let-7e predicts the progression and prognosis of non-small cell lung cancer [15]. Therefore, miRNA analysis provides a more comprehensive understanding of the pathogenesis of lung cancer [16].

miR-539 represents a novel therapeutic target in the treatment of heart failure by suppresses O-GlcNA case expression [17]. Recently, miR-539 functions as a tumor suppressor and reported to be downregulated in osteosarcoma and thyroid cancer, playing an important role in tumorigenesis and tumor development [18, 19]. However, the expression and function of

miR-539 in NSCLC remain unclear. In this study, we addressed miR-539 as a negative regulator of growth and invasion of NSCLC, and identified the mechanism of miR-539 inhibiting on growth and invasion of NSCLC might be through targeting CARMA1.

Materials and methods

Patients and clinical specimens

Paired NSCLC and adjacent noncancerous lung tissues (pathologically confirmed normal tissues and 2 cm away from the cancer tissues) were obtained from 25 patients who received curative resection of NSCLC Xiangya Hospital of Central South University (Changsha, Hunan Province, China) from January 2011 to December 2013. These tissues were flash-frozen in liquid nitrogen immediately after resection and stored at -80°C until use. None of the patients received neoadjuvant chemotherapy or radiotherapy before surgery. This study was approved by the Research Ethics Committee of our hospital, and written informed consent was obtained from each patient.

Cell culture and transfection

Four NSCLC cell lines (A549, H460, 95D, and HCC827) and normal human bronchial epithelial cell line (16HBE) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen®; Life Technologies Corp, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin G sodium, and 100 μg/mL streptomycin sulfate. All of the cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

For RNA transfection, the cells were seeded into each well of 24-well plates, incubated overnight, and then transfected with mature miR-539 mimics, miR-539 inhibitors, or negative control (miR-NC or anti-miR-NC; GenePharma, Shanghai, People's Republic of China) using Lipofectamine® 2000 (Invitrogen; Life Technologies Corp) according to the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), using the standard method. cDNA synthesis was performed with 2 μg of total RNA, using the miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR was performed on the ABI 7500 cycler (Applied Biosystems, CA, USA), using the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Hsa-RNU6B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as endogenous controls for miRNAs and mRNAs expression, respectively.

Luciferase reporter assay

The pGL3-reporter luciferase vector was used to construct the pGL3-CARMA1 or pGL3-CARMA1-mut vectors. The pGL3-CARMA1-mut vector was built with CARMA1 that underwent site-directed mutagenesis of the miR-539 target site, using a Stratagene Quik-Change® Site-Directed Mutagenesis Kit (Stratagene; Agilent Technologies, Santa Clara, CA, USA). For the luciferase reporter assay, cells were cultured in 24-well plates and transfected with the plasmids and miR-539 mimics using Lipofectamine 2000. At 24 hours after transfection, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega Corporation, Fitchburg, WI, USA). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well.

MTT assay

The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used in the evaluation of cells proliferation. Cells were seeded into 96-well plates at 5×10^3 cells/well. Twenty-four hours later, MTT assay was conducted. Finally, the optical density was determined at 570 nm using the ELISA plate reader (Model 550; Bio-Rad). At least three independent experiments were ensured.

Invasion assay

The capability of cell invasion was examined by transwell invasion assay. Cells were cultivated to 80% confluence on the 12-well plates. Then, we observed the procedures of cellular growth at 72 h. All the experiments were repeated intriplicate. The transwell migration chambers were used to evaluate cell invasion. Then cells invading cells across the membrane were counted under a light microscope.

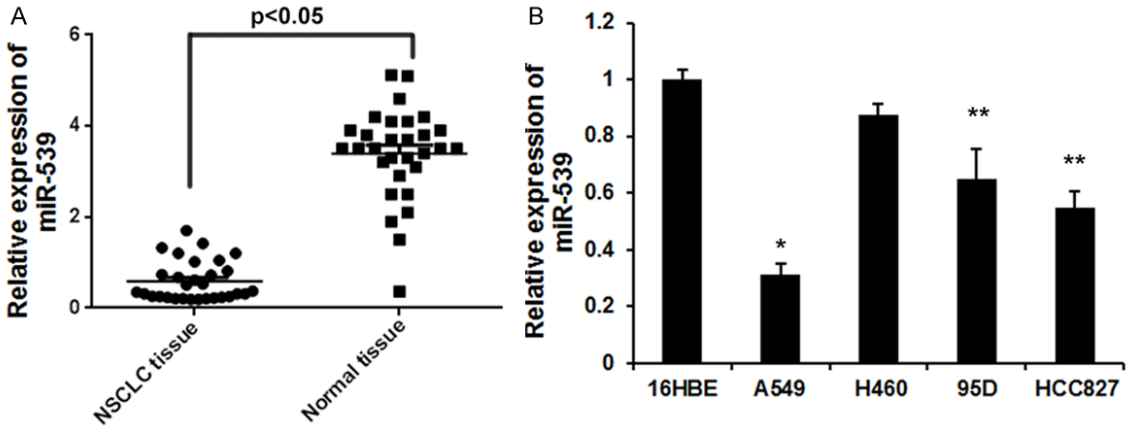

Figure 1. MicroRNA-539 was decreased in NSCLC tissues and cell lines. A. The expression levels of miR-539 in 20 pairs of NSCLC tissues and their matched normal tissues were measured by quantitative real-time PCR. U6 was used as an internal control. B. The expression levels of miR-539 in four NSCLC cell lines (A549, H226, H1299 and H460) and normal human bronchial epithelial cell line (16HBE).

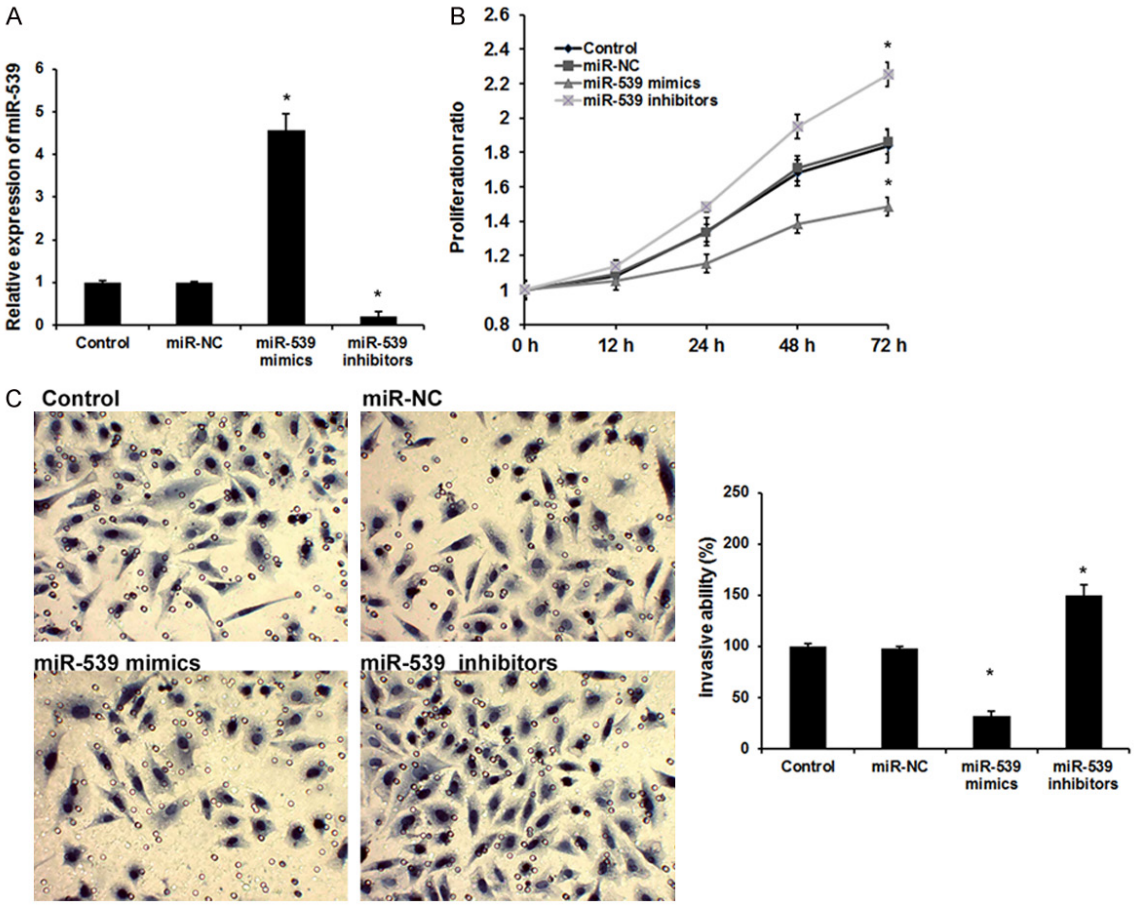

Figure 2. MiR-539 inhibited NSCLC cells proliferation and invasion of NSCLC cell. A549 cells were transfected with miR-539 mimic and inhibitor or negative control mimics, and in vitro migration was assessed by wound healing assay. A. The effects of miR-539 mimic and inhibitor on miR-539 expression. B. MTT assay were performed to detect cell viability. C. miR-539 suppressed the invasion of non-small-cell lung cancer (NSCLC) cells. *P<0.05.

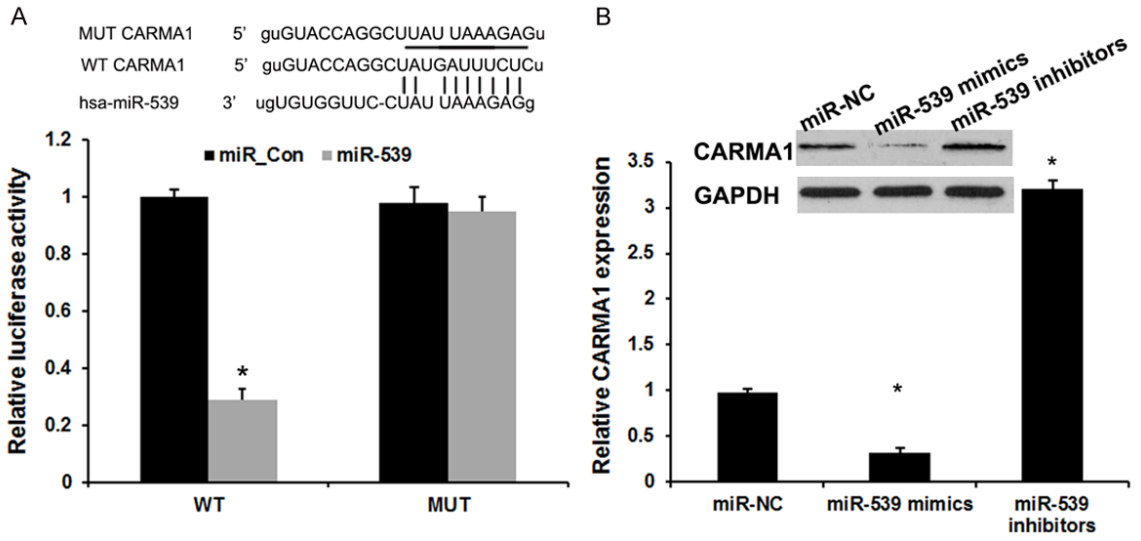

Figure 3. CARMA1 was a direct target of miR-539. A. Schematic representation of wild-type (WT) and mutated (Mut) putative miR-539-binding sites in the 3'-UTR of CARMA1. A549 cells were cotransfected with CARMA1 3'-UTR, pGL-3 control and either miR-539 or negative control mimics. Relative firefly luciferase activity was normalized to Renilla luciferase activity. B. Protein levels were detected by western blot analysis in A549 cells transfected with miR-539 or control mimic and in A549 cells transfected with miR-539 or control inhibitor. GAPDH was used as an internal control. *P<0.05, **P<0.01.

Western blot

Whole cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manual, and then, the protein was quantified by a BCA assay (Pierce, Rockford, IL, USA). Then, the protein samples were separated by SDS-PAGE (10%) and detected by Western blot using polyclonal (rabbit) anti-CARMA1 antibody (Santa Cruz Bio-technology, Santa Cruz, CA, USA). Goat anti-rabbit IgG (Pierce, Rockford, IL, USA) secondary antibody conjugated to horse radisher oxidase and ECL detection systems (Super Signal West Femto, Pierce) were used for detection.

Statistical analysis

All data were shown as mean ± SD. Statistical significance was assessed by T-test for two-group comparison. Differences with p value <0.05 were considered statistically significant.

Results

miR-539 is downregulated in NSCLC tissues and cell lines

The expression level of miR-539 was examined in NSCLC specimens and the corresponding normal tissues. The average expression level of miR-539 was significantly lower in NSCLC specimens compared with adjacent normal tissues (P<0.01) (**Figure 1A**). We also determined the expression level of miR-539 in different human NSCLC cell lines by quantitative RT-PCR. Comparing with the human bronchial epithelial cell line 16HBE, the expression level of miR-539 was consistently down-regulated in four NSCLC cell lines (A549, H226, H1299 and H460) (**Figure 1B**).

MiR-539 inhibited NSCLC cells proliferation and invasion of NSCLC cell

To test the function of miR-539 in NSCLC cells, stable cell lines A549 cells expressing miR-539 and negative control (NC) were established by Lipo-2000 transduction. Then we examined the effect of miR-539 on the proliferation of A549 cells from 0 h to 96 h (P<0.05). Further, we observed that over expression of miR-539 dramatically suppressed the proliferation of A549 cells. In contrast to use a miR-539 inhibitor promoted the proliferation of A549 cell (P<0.05) (**Figure 2A**). To determine the function of miR-539 in NSCLC cells, the cell invasion were determined. Overexpression of miR-539 in NSCLC cells significantly suppressed cell invasion (P<0.01), whereas loss of its expression promoted NSCLC cellular invasion (P<0.01)

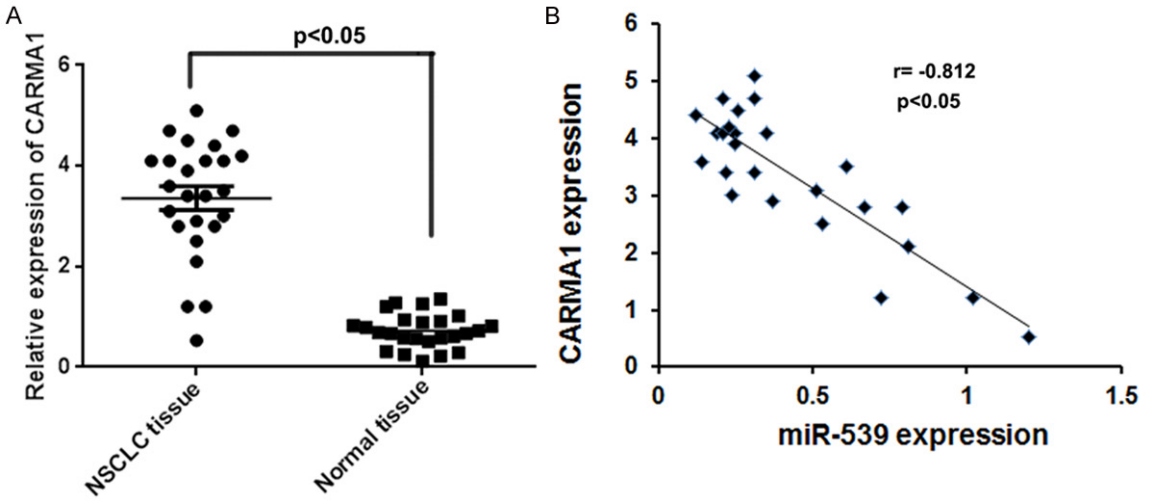

Figure 4. CARMA1 expression levels were inversely correlated with miR-539 in non-small-cell lung cancer tissues. A. CARMA1 expression levels in NSCLC tissues. B. CARMA1 expression levels were negatively correlated with miR-539.

(**Figure 2B**). These observations suggest that miR-539 plays an important role in inhibiting invasion of NSCLC cells.

CARMA1 is a direct target gene of miR-539 in thyroid cancer cells

Since miR-539 inhibits thyroid cancer cell metastasis, we reasoned that the specific genes suppressed by miR-539 should predominantly function in this process. Using computational prediction, we found that the 3'UTR of CARMA1 mRNA contains one miR-539 binding site. To verify the prediction, we constructed wild-type CARMA1 3'-UTR and mutated luciferase reporter plasmids (**Figure 3A**). The luciferase activity assays indicated that miR-539 mimics significantly inhibited and anti-miR-539 significantly increased the reporter activity of the wild-type but not that of the mutant CARMA1 3'-UTR, suggesting that miR-539 directly binds to this site (**Figure 3A**). We next investigated the effect of miR-539 on endogenous CARMA1 expression. Overexpression of miR-539 dramatically decreased CARMA1 at the mRNA and protein level, whereas anti-miR-539 increased CARMA1 levels in A549 cells (**Figure 3B**). Taken together, these results suggest that miR-539 directly targets and modulates the expression of CARMA1 in NSCLC cells.

Decreased expression of mir-539 in NSCLC and its correlation with CARMA1 levels

We further compared expression levels of CARMA1 and miR-539 in NSCLC patient tissues and found that CARMA1 protein level was also upregulated in miR-539 downregulated NSCLC tissues confirming that endogenously expressed CARMA1 is regulated by miR-539 expression. Bivariate correlation analysis revealed a significant inverse correlation between expression of CARMA1 and miR-539 ($r=-0.320$; $P<0.05$; **Figure 4**). Thus, these data further support that downregulation of miR-539 was inversely correlated with upregulation of CARMA1 in NSCLC tissues. Taken together, these data demonstrate that CARMA1 is a direct target of miR-539 and further suggest that miR-539 may exert its apoptosis promoting effect through inhibition of CARMA1 expression.

Discussion

In this study, we firstly revealed the expression of miR-539 in NSCLC and its mechanism for the regulation of NSCLC cell proliferation and invasion. We found that miR-539 evidently deregulated in NSCLC tissues and it can repress the proliferation and invasion of NSCLC cells by directly targeting the 3'-UTR of CARMA1. In addition, we found that miR-539 levels are inversely correlated with of CARMA1 mRNA expression in NSCLC tissues and cancer cell lines.

Accumulating studies demonstrated that microRNAs play critical roles in tumorigenesis by involving the regulation of cell differentiation, proliferation, apoptosis and invasion [20].

However, to our knowledge, few reports have described the function of miR-539. miR-539 has been reported to play an important role in the failing heart by suppress O-GlcNAcase expression. Recently, miR-539 as tumor suppressors, shows a global decrease in expression in two human cancers, including osteosarcoma and thyroid cancer [18, 19]. In this study, we demonstrated that miR-34c-3p is downregulated in NSCLC tissues as well as in NSCLC cell lines. Therefore, miR-539 was suggested to function as a tumor suppressor gene in NSCLC, which is consistent with its roles in osteosarcoma and thyroid cancer [18, 19]. To verify this hypothesis, we validated the tumor suppressive roles of miR-539 in NSCLC cell line A549 cells. Restoration of miR-539 expression could dramatically inhibit growth and invasion ability of NSCLC cell lines in vitro.

CARMA1 (CARD domain and MAGUK domain-containing protein-1), also known as CARD11 and Bimp3, is a CARD-containing membrane-associated guanylate kinase family protein that plays an essential role in antigen receptor-induced nuclear factor κB activation in T and B cells [21, 22]. In the previous study, Gu et al. observed that CARMA1 exerts a pro-metastasis function in thyroid cancer by involving in cell migration and invasion [18]. However, the expression of CARMA1 and the relationship of CARMA1 with miR-539 in NSCLC remain unclear. In this study, we found that CARMA1 was evidently upregulated in NSCLC tissues. The expression level of CARMA1 is negative correlation with miR-539 expression level in NSCLC. Furthermore, luciferase activity assays showed that CARMA1 was a direct target gene of miR-539. Exogeneticover expression of miR-539 resulted in a remarkable decrease of CARMA1.

In conclusion, our data offer convincing evidence that miR-539 may function as a tumor suppresssor miRNA in NSCLC, partly by regulating CARMA1 expression. Modulating miR-539 expression represents a potential strategy for the treatment of NSCLC patients.

Disclosure of conflict of interest

None.

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References

[1] Zhou YL, Xu YJ and Qiao CW. MiR-34c-3p suppresses the proliferation and invasion of non-small cell lung cancer (NSCLC) by inhibiting PAC1/MAPK pathway. Int J Clin Exp Pathol 2015; 8: 6312-6322.

[2] Dong W, Yao C, Teng X, Chai J, Yang X and Li B. MiR-140-3p suppressed cell growth and invasion by downregulating the expression of ATP8A1 in non-small cell lung cancer. Tumour Biol 2015; [Epub ahead of print].

[3] Song Y, Dou H, Wang P, Zhao S, Wang T, Gong W, Zhao J, Li E, Tan R and Hou Y. A novel small-molecule compound diaporine A inhibits non-small cell lung cancer growth by regulating miR-99a/mTOR signaling. Cancer Biol Ther 2014; 15: 1423-1430.

[4] Li Z, Lei H, Luo M, Wang Y, Dong L, Ma Y, Liu C, Song W, Wang F, Zhang J, Shen J and Yu J. DNA methylation downregulated mir-10b acts as a tumor suppressor in gastric cancer. Gastric Cancer 2015; 18: 43-54.

[5] Xiao X, Tang C, Xiao S, Fu C and Yu P. Enhancement of proliferation and invasion by MicroRNA-590-5p via targeting PBRM1 in clear cell renal carcinoma cells. Oncol Res 2013; 20: 537-544.

[6] Yin WZ, Li F, Zhang L, Ren XP, Zhang N and Wen JF. Down-regulation of microRNA-205 promotes gastric cancer cell proliferation. Eur Rev Med Pharmacol Sci 2014; 18: 1027-1032.

[7] Yang X, Ni W and Lei K. miR-200b suppresses cell growth, migration and invasion by targeting Notch1 in nasopharyngeal carcinoma. Cell Physiol Biochem 2013; 32: 1288-1298.

[8] Yang H, Luo J, Liu Z, Zhou R and Luo H. MicroRNA-138 Regulates DNA Damage Response in Small Cell Lung Cancer Cells by Directly Targeting H2AX. Cancer Invest 2015; 33: 126-136.

[9] Zhang Y, Yang Q and Wang S. MicroRNAs: a new key in lung cancer. Cancer Chemother Pharmacol 2014; 74: 1105-1111.

[10] Wang M, Zhang Q, Wang J and Zhai Y. MicroRNA-498 is downregulated in non-small cell lung cancer and correlates with tumor progression. J Cancer Res Ther 2015; 11 Suppl: c107-111.

[11] Jiang YW and Chen LA. microRNAs as tumor inhibitors, oncogenes, biomarkers for drug efficacy and outcome predictors in lung cancer (review). Mol Med Rep 2012; 5: 890-894.

[12] Gao W, Xu J and Shu YQ. miRNA expression and its clinical implications for the prevention and diagnosis of non-small-cell lung cancer. Expert Rev Respir Med 2011; 5: 699-709.

[13] Yang M, Shen H, Qiu C, Ni Y, Wang L, Dong W, Liao Y and Du J. High expression of miR-21 and miR-155 predicts recurrence and unfavourable survival in non-small cell lung cancer. Eur J Cancer 2013; 49: 604-615.
[14] Chen Y, Min L, Zhang X, Hu S, Wang B, Liu W, Wang R, Gu X, Shen W, Lv H, Zou J, Chen Y, Xu X and Chen L. Decreased miRNA-148a is associated with lymph node metastasis and poor clinical outcomes and functions as a suppressor of tumor metastasis in non-small cell lung cancer. Oncol Rep 2013; 30: 1832-1840.
[15] Zhu WY, Luo B, An JY, He JY, Chen DD, Xu LY, Huang YY, Liu XG, Le HB and Zhang YK. Differential expression of miR-125a-5p and let-7e predicts the progression and prognosis of non-small cell lung cancer. Cancer Invest 2014; 32: 394-401.
[16] Cortinovis D, Monica V, Pietrantonio F, Ceresoli GL, La Spina CM and Wannesson L. MicroRNAs in non-small cell lung cancer: current status and future therapeutic promises. Curr Pharm Des 2014; 20: 3982-3990.
[17] Muthusamy S, DeMartino AM, Watson LJ, Brittian KR, Zafir A, Dassanayaka S, Hong KU and Jones SP. MicroRNA-539 is up-regulated in failing heart, and suppresses O-GlcNAcase expression. J Biol Chem 2014; 289: 29665-29676.
[18] Gu L and Sun W. MiR-539 inhibits thyroid cancer cell migration and invasion by directly targeting CARMA1. Biochem Biophys Res Commun 2015; 464: 1128-1133.
[19] Jin H and Wang W. MicroRNA-539 suppresses osteosarcoma cell invasion and migration in vitro and targeting Matrix metallopeptidase-8. Int J Clin Exp Pathol 2015; 8: 8075-8082.
[20] Zhao W, Cao L, Zeng S, Qin H and Men T. Up-regulation of miR-556-5p promoted prostate cancer cell proliferation by suppressing PPP2R2A expression. Biomed Pharmacother 2015; 75: 142-7.
[21] Blonska M and Lin X. NF-kappaB signaling pathways regulated by CARMA family of scaffold proteins. Cell Res 2011; 21: 55-70.
[22] Blonska M, Joo D, Zweidler-McKay PA, Zhao Q and Lin X. CARMA1 controls Th2 cell-specific cytokine expression through regulating JunB and GATA3 transcription factors. J Immunol 2012; 188: 3160-3168.