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Original Article

MiR-21 expression significance in non-small cell lung cancer tissue and plasma

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Abstract: Lung cancer has the leading cause of morbidity and mortality among malignant tumor worldwide. Non-small cell lung cancer (NSCLC) accounts for 85% in all patients with lung cancer. MiR-21 is a kind of evolutionary conservative noncoding RNA molecule that participates in regulating cell behavior. This study detected miR-21 expression difference in tumor tissue and plasma from NSCLC patients and explored its clinical significance to analyze its impact on cells. Tumor tissue and plasma were collected from 118 NSCLC patients received surgery. MiR-21 expression in tumor tissue was tested by in situ hybridization. MiR-21 level in plasma was determined by RT-PCR. MiR-21 mimics was used to overexpress miR-21 in A549 cell line. MTT assay was applied to detect cell proliferation. Transwell assay was adopted to evaluate cell invasion. Western blot was performed to analyze phosphatase and tensin homolog (PTEN) and protein kinase B (AKT) expressions. In situ hybridization showed that miR-21 positive rate in NSCLC patients with higher clinical stage was significantly higher than that in patients with lower clinical stage ($P < 0.05$). The rate of miR-21 overexpression in metastatic NSCLC patients plasma was obviously higher than that without metastasis ($P < 0.05$). Cell transfection revealed that PTEN expression reduced, p-AKT level enhanced, cell proliferation increased, and cell invasion improved in A549 after miR-21 transfection. MiR-21 expression in NSCLC tumor tissue and plasma might be correlated to cancer clinical staging and metastasis. Cell transfection showed that miR-21 overexpression can downregulate PTEN and enhance cell proliferation and invasion.

Keywords: NSCLC, miR-21, plasma, PTEN, cell proliferation, metastasis

Introduction

Lung cancer has the leading cause of morbidity and mortality among malignant tumor worldwide. Non-small cell lung cancer (NSCLC) accounts for 85% in all patients with lung cancer [1]. Although the treatment of NSCLC patients has gained great progress, and the patient's survival rate and quality of life have been greatly improved, the diagnosis and therapeutic effect of advanced NSCLC patients is still poor [2, 3]. Thus, screening molecular markers closely associated with NSCLC diffusion, metastasis, and disease progression, and easy to detect and monitor is of great significance to improve the diagnosis and therapeutic effect of patients with advanced NSCLC.

MicroRNAs are small non-coding RNA molecules with hairpin structure [4]. It was showed that miRNAs can affect multiple cell behaviors via RNA interference [5]. Through miRNA expression spectrum analysis on a variety of human malignant tumor cells, it was found that miR-21 expression was significantly abnormal in various malignant tumor cells, including lung cancer, liver cancer, and esophageal cancer. It suggested that miR-21 expression may be related to cancer occurrence of development [6-8]. Meanwhile, it was also reported that miR-21 positive rate in the plasma form metastatic colorectal cancer patients was significantly higher than that in non-metastatic patients [9], indicating that we could monitor NSCLC progress through detecting miR-21 level in the plasma of NSCLC patients. This study detected miR-21 expression difference in tumor tissue and plasma from NSCLC patients and explored its clinical significance to analyze its impact on cells.

Materials and methods

Object selection

A total of 118 NSCLC patients between Jan 2014 and Dec 2015 in Jiangsu Cancer Hospital & Institute Affiliated to Nanjing Medical

Table 1. Primer sequences used for RT-PCR

Name	Sequence	Tm (°C)
MiR-21-F	5'-GTGTAGCTTATCAGACTGATG-3'	53.9
MiR-21-R	5'-TGTCAACATCAGTCTGATAAG-3'	54.9
U6-F	5'CTCGCTTCGGCAGCACA3'	55.3
U6-R	5'AACGCTTCACGAATTTGCGT3'	55.4

University were enrolled, including 74 males and 44 females with mean age at 54.1 ± 6.7 (48-72) years old. All the patients were primary without chemoradiotherapy or surgery. The patients were diagnosed and staged according to the NSCLC guideline 2015 published by NCCN. There were 18 cases in stage I, 26 cases in stage II, and 32 cases in stage III, and 42 cases in stage IV. Tumor tissue and para-carcinoma tissue were obtained from the surgery. The tissue was used for RNA extraction or 10% formalin fixation. The study was approved by Jiangsu Cancer Hospital & Institute Affiliated to Nanjing Medical University Ethics Committee and all the subjects had signed informed consent.

In situ hybridization

Antisense nucleic acid modified miR-21 oligonucleotide probe was applied for in situ molecular hybridization (Boster, Wuhan, China). The paraffin section received routine dewaxing, washing, peroxidase blocking, and proteinase K digestion. After rinsed by 0.5 M PBS (pH7.4), the section was washed by distilled water. Next, the section was pre-hybridized under 63~65°C for 3~4 h. Then the section was added with hybrid liquid containing miR-21 probe (Exiqon) and hybridized under 55°C for 24 h. After washed by SSC solution, the section was blocked at room temperature. Next, the section was treated by rabbit anti digoxin antibody and incubated at 37°C for 60 min. After washed by PBS and alkaline phosphatase buffer, the section was treated by TMB substance for development. After development, the section was washed by PBS and fixed by 4% paraformaldehyde for 2 h for storage [10].

The section was observed under the BX61 microscope (Olympus). MiR-21 expression in the cells would be stained as blue, and the intensity was followed by expression level. Tumor cells were found and the region with uniform staining was used for score. 0 point means miR-21 negative, 1 point represents miR-21 weak positive, 2 points refers to miR-21 moderate positive, and 3 points shows miR-21 strong positive [10].

RT-PCR

A total of 10 ml venous blood was extracted using vacuum EDTA anticoagulation tube and centrifuged at 2000 g for 10 min. The plasma on the superstratum was moved to a new tube and centrifuged at 8000 g for 10 min. Total RNA was extracted from the plasma using the kit (QIAGEN). The primers were designed based on the sequence of miR-21 (**Table 1**). RT-PCR was performed on Real-Time PCR amplifier (Bio-Rad) using the mirVanatqRT-PCR miRNA detection kit (Ambion). The RT-PCR reaction was composed of 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. U6 RNA was selected as internal reference. The results were analyzed by supporting analysis software V2.02 and presented as $2^{-\Delta\Delta Ct}$ [11].

Cell transfection and miR-21 overexpression

MiR-21 overexpression was designed based on the sequence of miR-21. Corresponding oligonucleotide sequence was selected as negative control. The sequences were synthetized by SunShineBio (Nanjing, China). It was expressed by pMIR-REPORT system (Ambion). NSCLC cell line A549 was purchased from the cell bank, Chinese academy of sciences. A549 cells were maintained in DMEM medium containing 10% FBS and gentamycin. The cells in logarithmic phase were digested and seeded in 96-well plate at 3×10^5/well for transfection according to the manual [12]. The cells were divided into three groups, including miR-21 group, negative group, and blank group. A total of 1 μl lipofectamine 2000 (Invitrogen) was diluted in 50 μl serum free DMEM medium and incubated for 5 min. MiR-21 expression vector, negative control, and PBS were added to prepare the transfection liquid, respectively. A total of 100 μl transfection liquid was added to each well and incubated for 6 h. Then the cells were further changed to antibiotics and serum free medium and incubated for 48-72 h.

MTT assay

A549 cells were seeded in 96-well plate and incubated at 37°C and 5% CO_2 for 44 h. After added with 70 μl MTT solution, the plate was further incubated for 4 h. At last, the plate was treated by DMSO and tested at λ=490 nm.

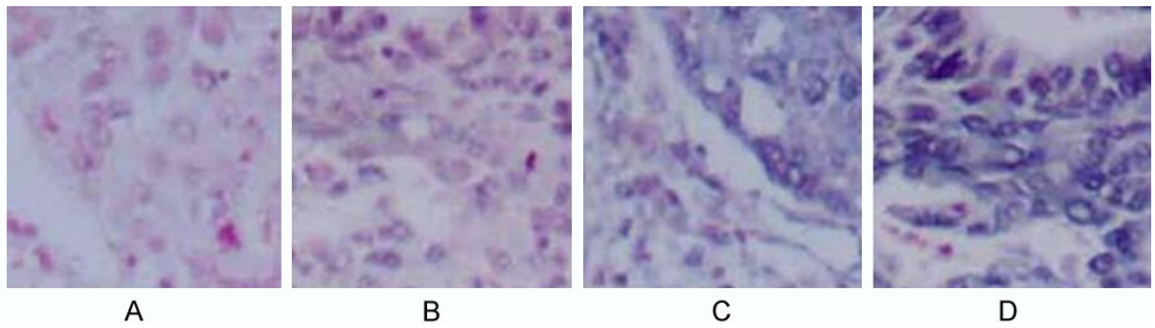

Figure 1. In situ hybridization. A. miR-21 negative expression (score 0). B. miR-21 low expression (score 1). C. miR-21 moderate expression (score 2). D. miR-21 strong expression (score 3).

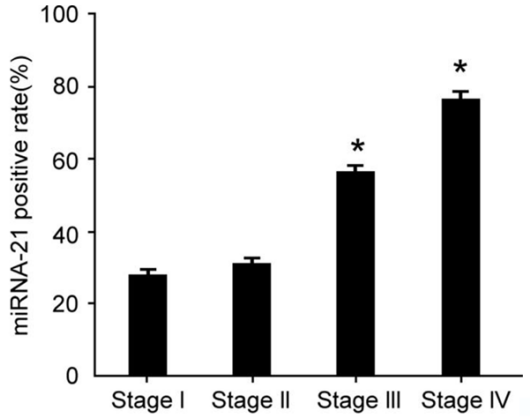

Figure 2. MiR-21 positive rate in NSCLC patients at different clinical stages. *P < 0.05, compared with stage I.

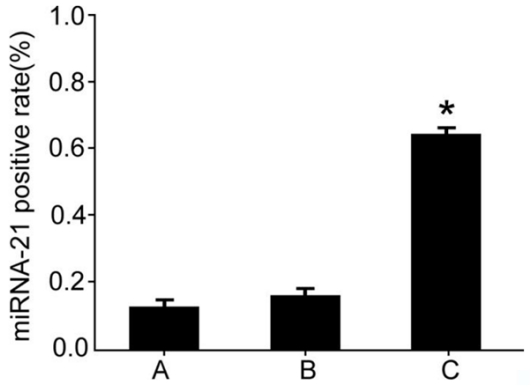

Figure 3. MiR-21 expression in A549 cells detected by RT-PCR. A. untransfected A549. B. negative control. C. miR-21 transfected A549. *P < 0.05, compared with group A.

Table 2. The relationship between plasma miR-21 and clinicopathological parameter

	miR-21 high expression	miR-21 low expression	*P* value
Age (year)			
≥ 60	32	31	> 0.05
< 60	20	35	
Gender			
Male	40	34	> 0.05
Female	24	20	
Clinical stage			
I+II	4	30	< 0.05
III+IV	60	14	
Metastasis			
Yes	52	12	< 0.05
No	12	42	

Transwelll assay

A total of 60 μl matrigel (BD) at 5 mg/ml was added to the upper chamber at 4°C for air dry. A549 cells in logarithmic phase were digested by trypsin and resuspended at 1×10^6/mL. A total of 200 μl cell suspension was seeded into the upper chamber, while 600 μl medium containing 10% FBS was added to the lower chamber. After incubated for 24 h, the matrigel and cells in the upper chamber were removed. The penetrated cells were stained by crystal violet at room temperature for 30 min and washed by 10% acetic acid. At last, the cells were tested on microplate reader at 570 nm. Each group was repeated for three times.

Western blot

A549 cells were collected in 100 μl lysis and centrifuged at 13000 g for 10 min. The protein was separated by 15% SDS-PAGE and transferred to PVDF membrane. After blocked by 5% skim milk at 37°C for 1 h, the membrane was incubated in primary antibody (1:2000) at 4°C overnight. After washed by TBST, the membrane was further incubated in HRP-labeled

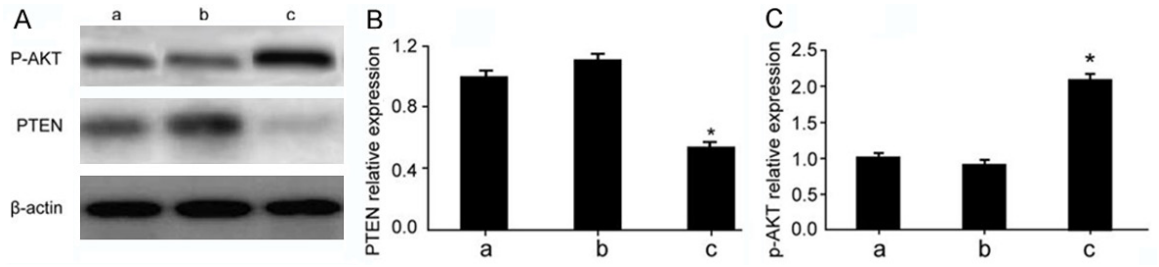

Figure 4. PTEN and p-AKT expression detected by Western blot. A. Western blot. B. PTEN expression level. C. p-AKT expression level. a, untransfected A549. b, negative control. c, miR-21 transfected A549. *P < 0.05, compared with group a.

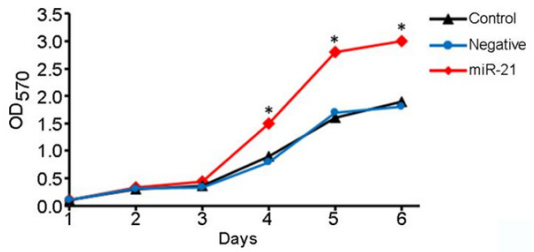

Figure 5. Cell proliferation detected by MTT assay. *P < 0.05, compared with control.

goat anti mouse IgG secondary antibody (1:1000) at room temperature for 1 h. At last, the membrane was added with DAB developing solution avoid of light for 10 min and scanned to obtain the result. β-actin was selected as internal reference.

Statistical analysis

All data analysis was performed on SPSS 17.0 software. The data was depicted as mean ± standard deviation. The results in each group were compared by t test, ANOVA, or q test when necessary. P < 0.05 was treated as statistical significance.

Results

MiR-21 expression in NSCLC tissue

In situ hybridization was applied to test miR-21 expression in NSCLC tumor tissue in different clinical stages. **Figure 1** showed the staining intensity upon different score. MiR-21 positive expression was evaluated as score ≥ 2, while negative expression was treated as score ≤ 1. As shown in **Figure 2**, miR-21 positive rate in patients at stage III and IV was 56.3% and 76.2%, which was significantly higher than that at stage I as 27.8%.

The relationship between miR-21 and NSCLC in plasma

RT-PCR was performed to test miR-21 expression in the plasma from NSCLC patients. U6 RNA was selected as internal reference to calculate the relative expression level of miR-21. The follow-up results were listed in **Table 2**. There were 18 patients appeared bone metastasis (15.2%), 19 patients occurred pulmonary metastasis (14.4%), 14 cases showed pleural metastasis (11.9%), 14 cases appeared brain metastasis (11.9%), 8 patients occurred liver metastasis (6.8%), and 10 cases with other types of metastasis (8.5%). MiR-21 level in the plasma from patients in stage III and IV was obviously higher than that in stage I and II (P < 0.05), which was in accordance with the result of in situ hybridization. It was demonstrated that miR-21 high expression rate in metastatic patients was 81.3%, which was markedly higher than that in patients without metastasis as 22.2%.

Cell transfection and miR-21 overexpression

pMIR-REPORT system was adopted to establish miR-21 overexpressed A549 cell line. Total RNA was extracted to detect miR-21 relative expression. It was showed that miR-21 expression was markedly higher in A549 cells after transfection compared with blank control (P < 0.05), suggesting successful transfection (**Figure 3**).

PTEN expression detected by Western blot

Western blot was applied to test PTEN and p-AKT expression in A549 cells. As shown in **Figure 4**, PTEN expression significantly decreased, while p-AKT level markedly elevated in A549 cells after miR-21 transfection (P < 0.05).

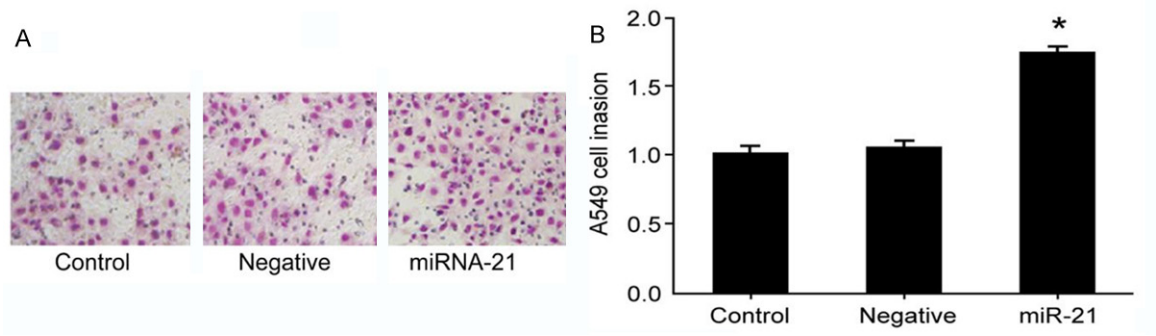

Figure 6. Cell invasion detected by Transwell assay. *P < 0.05, compared with control.

A549 cell proliferation

MTT assay was performed to determine A549 cell proliferation after transfection. Untransfected cell line was selected as blank control. As shown in **Figure 5**, A539 cell proliferative ability obviously enhanced after miR-21 transfection compared with control (P < 0.05).

A549 cell invasion

Transwell assay was adopted to evaluate A549 cell invasion after transfection for 24 h. As shown in **Figure 6**, A549 cell invasion was markedly elevated after miR-21 transfection (P < 0.05). OD570 measurement revealed that A549 cell invasion enhanced by 0.73 times compared with untransfected cells.

Discussion

MiRNAs are a type of noncoding RNA molecules at the length of about 21 to 25 bp. It is highly conserved in evolution and closely associated with growth and development by participating in the regulation of cell proliferation, differentiation, and apoptosis [2]. Recent studies found that miRNAs were closely related to the occurrence and development of a variety of malignant tumors and associated with cell proliferation and invasion [7, 13]. This study discovered that miR-21 positive rate in the tumor tissue from NSCLC patients in advanced stage was significantly higher than that in early stage, speculating that miR-21 expression may be related to NSCLC occurrence and progress. Next, it was demonstrated that miR-21 high expression rate in metastatic patients was markedly higher than that in patients without metastasis, suggesting that miR-21 level in plasma may be closely related to NSCLC metastasis.

Through miRNA analysis in various human malignant tumors, it was found that many miRNAs exhibited abnormal expression in malignant tumor cells compared with normal cells. For instance, brain glioma demonstrated miR-21 overexpression, while miR-124 and miR-137 downregulation [12, 14]. It was also revealed that miR-340 and miR-200 declined in NSCLC cells [15, 16]. Malignant tumor cells can promote proliferation, invasion, and metastasis, and inhibit apoptosis via changing the expression pattern of miRNAs [17]. It is generally considered that miRNA regulated protein expression at posttranscriptional level through binding with targeted mRNA [18]. There are a lot of studies demonstrated that miR-21 was correlated with multiple cancer cells proliferation, invasion, and metastasis, including glioma, prostate cancer, and esophageal cancer [12, 19].

Liu found that miR-21 showed high homology with the 3'UTR of PTEN mRNA through sequence alignment. Further experimental result proved that miR-21 regulated PTEN expression by targeting the 3'UTR of PTEN, thus to mediate PTEN/PI3K/Akt signaling pathway to regulate cell proliferation and division [20]. It was consistent with our results.

Lung cancer is a common human malignant tumor characterized as high malignancy, rapid progress, and high mortality. Its diagnosis and treatment have become the hot issue concerned by medical staff and patients [21]. Our results confirmed the relationship between miR-21 expression in NSCLC tumor tissue and plasma and disease progression and clinical stage. It was further proved that miR-21 can regulate PTEN expression in cancer cells, which provided theoretical basis to improve the diagnosis accuracy in NSCLC progress.

Conclusion

MiR-21 positive rate in tumor tissue and plasma from NSCLC patients in advanced stage was higher than that in early stage. Plasma miR-21 expression was correlated with NSCLC metastasis. Upregulating miR-21 in NSCLC cells can suppress PTEN expression and increase p-AKT level to facilitate cell proliferation and invasion.

Disclosure of conflict of interest

None.

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References

[1] Malvezzi M, Bosetti C, Rosso T, Bertuccio P, Chatenoud L, Levi F, Romano C, Negri E and La Vecchia C. Lung cancer mortality in European men: trends and predictions. Lung Cancer 2013; 80: 138-145.

[2] Vansteenkiste J, De Ruysscher D, Eberhardt WE, Lim E, Senan S, Felip E, Peters S; ESMO Guidelines Working Group. Early and locally advanced non-small-cell lung cancer (NSCLC): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2013; 24 Suppl 6: vi89-98.

[3] Peters S, Adjei AA, Gridelli C, Reck M, Kerr K, Felip E; ESMO Guidelines Working Group. Metastatic non-small-cell lung cancer (NSCLC): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2012; 23 Suppl 7: vii56-64.

[4] Iorio MV and Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med 2012; 4: 143-159.

[5] Ameres SL and Zamore PD. Diversifying microRNA sequence and function. Nat Rev Mol Cell Biol 2013; 14: 475-488.

[6] Li B, Ren S, Li X, Wang Y, Garfield D, Zhou S, Chen X, Su C, Chen M, Kuang P, Gao G, He Y, Fan L, Fei K, Zhou C and Schmit-Bindert G. MiR-21 overexpression is associated with acquired resistance of EGFR-TKI in non-small cell lung cancer. Lung Cancer 2014; 83: 146-153.

[7] Wagenaar TR, Zabludoff S, Ahn SM, Allerson C, Arlt H, Baffa R, Cao H, Davis S, Garcia-Echeverria C, Gaur R, Huang SM, Jiang L, Kim D, Metz-Weidmann C, Pavlicek A, Pollard J, Reeves J, Rocnik JL, Scheidler S, Shi C, Sun F, Tolstykh T, Weber W, Winter C, Yu E, Yu Q, Zheng G and Wiederschain D. Anti-miR-21 suppresses hepatocellular carcinoma growth via broad transcriptional network deregulation. Mol Cancer Res 2015; 13: 1009-1021.

[8] Nouraee N, Van Roosbroeck K, Vasei M, Semnani S, Samaei NM, Naghshvar F, Omidi AA, Calin GA and Mowla SJ. Expression, tissue distribution and function of miR-21 in esophageal squamous cell carcinoma. PLoS One 2013; 8: e73009.

[9] Toiyama Y, Takahashi M, Hur K, Nagasaka T, Tanaka K, Inoue Y, Kusunoki M, Boland CR and Goel A. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. J Natl Cancer Inst 2013; 105: 849-859.

[10] Xu L, Huang Y, Chen D, He J, Zhu W, Zhang Y and Liu X. Downregulation of miR-21 increases cisplatin sensitivity of non-small-cell lung cancer. Cancer Genet 2014; 207: 214-220.

[11] Shen H, Zhu F, Liu J, Xu T, Pei D, Wang R, Qian Y, Li Q, Wang L, Shi Z, Zheng J, Chen Q, Jiang B and Shu Y. Alteration in Mir-21/PTEN expression modulates gefitinib resistance in non-small cell lung cancer. PLoS One 2014; 9: e103305.

[12] Quintavalle C, Donnarumma E, Iaboni M, Roscigno G, Garofalo M, Romano G, Fiore D, De Marinis P, Croce CM and Condorelli G. Effect of miR-21 and miR-30b/c on TRAIL-induced apoptosis in glioma cells. Oncogene 2013; 32: 4001-4008.

[13] Lujambio A, Calin GA, Villanueva A, Ropero S, Sanchez-Cespedes M, Blanco D, Montuenga LM, Rossi S, Nicoloso MS, Faller WJ, Gallagher WM, Eccles SA, Croce CM and Esteller M. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci U S A 2008; 105: 13556-13561.

[14] Zhu X, Li Y, Shen H, Li H, Long L, Hui L and Xu W. miR-137 inhibits the proliferation of lung cancer cells by targeting Cdc42 and Cdk6. FEBS Lett 2013; 587: 73-81.

[15] Pacurari M, Addison JB, Bondalapati N, Wan YW, Luo D, Qian Y, Castranova V, Ivanov AV and Guo NL. The microRNA-200 family targets multiple non-small cell lung cancer prognostic markers in H1299 cells and BEAS-2B cells. Int J Oncol 2013; 43: 548-560.

[16] Fernandez S, Risolino M, Mandia N, Talotta F, Soini Y, Incoronato M, Condorelli G, Banfi S and Verde P. miR-340 inhibits tumor cell proliferation and induces apoptosis by targeting multiple negative regulators of p27 in non-small cell lung cancer. Oncogene 2015; 34: 3240-3250.

[17] Lin S and Gregory RI. MicroRNA biogenesis pathways in cancer. Nat Rev Cancer 2015; 15: 321-333.

[18] Fu X, Tian J, Zhang L, Chen Y and Hao Q. Involvement of microRNA-93, a new regulator of PTEN/Akt signaling pathway, in regulation of chemotherapeutic drug cisplatin chemosensitivity in ovarian cancer cells. FEBS Lett 2012; 586: 1279-1286.

[19] Ribas J and Lupold SE. The transcriptional regulation of miR-21, its multiple transcripts, and their implication in prostate cancer. Cell Cycle 2010; 9: 923-929.

[20] Liu ZL, Wang H, Liu J and Wang ZX. MicroRNA-21 (miR-21) expression promotes growth, metastasis, and chemo- or radioresistance in non-small cell lung cancer cells by targeting PTEN. Mol Cell Biochem 2013; 372: 35-45.

[21] Grunnet M and Sorensen JB. Carcinoembryonic antigen (CEA) as tumor marker in lung cancer. Lung Cancer 2012; 76: 138-143.