Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

miR-675 promotes disease progression of non-small cell lung cancer via activating NF-κB signaling pathway

Y. Feng[1#*], C. Yang[2#], D. Hu[3], X. Wang[2], X. Liu[2]

[1] Department of Pathology, The First Hospital of Wuhan, Wuhan, Hubei, China
[2] Department of Respiratory, The third people's hospital of Hubei Province, Wuhan, Hubei, China
[3] Department of hepatopathy of Hubei Zhongshan Hospital, Wuhan, Hubei, China

Correspondence to: gukenliangnai@sohu.com

Received October 14, 2017; **Accepted** May 15, 2017; **Published** May 20, 2017

[#] These authors contributed equally to this work

Doi: http://dx.doi.org/10.14715/cmb/2017.63.5.2

Abstract: Non-small cell lung cancer (NSCLC) is a major type of lung cancer significantly affected human life. Current studies have revealed potential role of miR-675 in the etiology of lung cancer. Therefore, in this study, we aimed to investigate the role of miR-675 in NSCLC cell line. We recruited 92 cases of NSCLC patients from July 2009 to February 2011 in the oncology department. Tumor and peripheral tissue was collected for miR-675 expression. NSCLC cell lines with either miR-675 over-expression or low-expression were established. Western blotting and immunofluorescence staining were used for detecting P65 and pP65 expression and translocation in the nuclei. MiR-675 expression was significantly higher in the lung cancer tissue than the peripheral normal tissue ($P < 0.001$). The gene expression of pP65 was also increased and decreased in cells with over-expression or low-expression of miR-675, respectively (both $P < 0.05$). Whereas the trend of P65 expression was on the opposite (both $P < 0.05$), indicating the NF-κB signaling pathway was activated when miR-675 was expressed and vice versa. Immunofluorescence staining showed that more pP65 expression was clustered in the nuclei in miR-675 over-expressed cells, which further demonstrated NF-κB signaling pathway activation. Increased miR-675 expression is associated with NSCLC progression through activation of NF-κB signaling pathway.

Key words: miR-675; NF-κB; Non-small cell lung cancer.

Introduction

Lung cancer has been reported to be the leading type of cancer and be responsible for a great proportion of mortality in China as well as worldwide (1-3). The etiology of non-small cell lung cancer (NSCLC), a major type of lung cancer, has not been understood completely. Genetic studies have shown that NSCLC involved a complex process with a series of gene mutations and abnormal expression regulations (4,5). Recent studies have suggested potential roles of microRNAs (miRNA) in the progression of lung cancer (6) based on their important physiological roles in modulating cell proliferation, differentiation and apoptosis (7,8). Micro RNAs were demonstrated to modulate cancer progression by either promoting or inhibiting cancer. A previous miRNA profiling study revealed that miR-675, located on chromosome 11p15.5 cluster, was the most significantly dysregulated micro RNA in lung cancer tissue, indicating its specific role in NSCLC (9). However, scarce evidence was published later as if miR-675 has direct role in the progression of NSCLC.

NF-κB, the central transcription factor in inflammation, has been reported to be involved in the occurrence and development of tumors (10), which was acknowledged to be evolved from inflammation (11). NF-B activation involved its phosphorylation of P65 subunit and translocation into the nuclei (12), which further affect the occurrence and development of tumors. However, to date no study has address the correlation between miR-675 and NF-κB signaling pathway. Therefore, in this study, we aimed to investigate the role of miR-675 in NSCLC cell line and its signaling pathway.

Materials and Methods

Clinical data

This study recruited 92 patients diagnosed with non-small cell lung cancer from July 2009 to February 2011 in the oncology department of our hospital. The tumor tissues and peripheral tissues were regarded as the object of study. All patients had complete clinical data, including the general situation, pathological diagnosis, staging, treatment, and survival status. All patient data including follow-up data were well preserved.

Gene expression assay

Reverse transcription kit was purchased from TaKaRa company. The same amount of tissue and Trizol was mixed and centrifuged at 12000 r/min for 10 min under 4ºC. The supernatant was transferred to a new EP tube followed by standing for 5 min for fully lysis. Chloroform was added followed by oscillator shock for 15 s, 3 min of standing and centrifuge at 12000 r/min for 15 min under 4ºC. The upper layer (the middle layer was protein) was again transferred to a new EP tube. The same volume of isopropyl alcohol was added to the EP tube under 4ºC and the EP tube was

reversed for several times followed by precipitation of 10 min at room temperature, and centrifuge at 12000 r/min for 10 min under 4ºC. Once the supernatant was discarded, 75% ethanol (DEPC dissolved in water) was added followed by centrifuge at 7500 r/min for 5 minutes under 4ºC.The supernatant was removed carefully (meanwhile the white precipitation is RNA). When the RNA was slightly dry, 20 μL of DEPC water was added. Nucleic acid dye method was used for real time fluorescence quantitative PCR in the study of miR-675 in non-small cell lung cancer. Primer sequences were: miR-675:F- GCCGGUCAUGUCCAAAGUATT; R-UACUUUGGACAUGACCGGCTTGAPDH:F-GCCCTGAGGGCCCGAACTGTTACT;R- CAGACG-CACGGCTTTGACCTTCTT,

The reaction process was40 reaction cycles of 95ºC for 10 min for pre-denaturation, Cycle C; degeneration under 95ºC for 30sec, 55ºC for 30sec for annealing, and extension under 72ºCfor 30 sec followed by melting curve analysis.

In vitro transfection

A549 cells (purchased from ATCC) in the logarithmic phase was seeded into six-well plates with a cell concentration of about 3×105in each well. Lipo2000 was added to 100ul serum free medium and stand for five minutes. Plasmids (12 μL) with high expression of miR-675 and low expression (purchased from RiboBio Co., Guangzhou, China)was added into 100 μL of serum-free culture media. After five minutes, plasmids and Lipo2000 were mixed for 20 minutes and then added into 1800ul of serum-free media. RNA extraction was performed after 48 hours. The miR-675 expression plasmids was synthesized by RiboBio Co., Guangzhou and the corresponding control group was plasmids with missense replacement. MiR-675 and U6 primer sequences have been mentioned above.

Western blot

Extraction of serum protein was performed using protein extraction kit and Quantitated by BCA method. BCA solution is made by combination of Solution A and Solution B with a ratio of 50:1. Two μL of the supernatant was collected and 18μL of PBS and 200μL of AB mixture were added. All the protein samples were set to the same concentration, while 5× bromophenol blue was added (20% of the total volume). The bubbles were eliminated and all the protein samples were set to the same concentration before loading. Six μL of protein marker was loaded together with other protein samples. The initial voltage was 80V is for the concentrated gel, and 120V for the separation gel. When the target protein electrophoresis moved to the 1cmabove the lower edge of the gel, the electrophoresis was over. PVDF film was soaked methanol for 5 minutes with constant current of 250mA for 90 minutes. It was then transferred from the electrophoresis tank and rinsed by TBST slightly. Antibody blocking was performed by 5% skim milk solution while swaying for one hour. TBST was then used for rinse and the incubation with the primary antibody was performed overnight. On the second days, the film was placed at room temperature for 40 minutes, and rinsed by TBST for three times with each time for 5 minutes. The secondary antibody was selected based on the source of the primary antibody. Incubation with the secondary antibody was for an hour under room temperature. TBST was used for rinse for three times with each time for 5 minutes. After the washing of the PVDF film, luminescence and development was performed using ECL light-emitting liquid, which is made by combining A liquid 1:1 with liquid B.

Immunofluorescence assay

Tissue was fixed, dehydrated by gradients of sucrose, frozen sectioned, and sliced into a thickness of 6μm. Tissue section was then incubated under high temperature for 5 minutes and washed by PBS after cooling (three times, five minutes each). 10% of BSA was used for blocking for 50 minutes and primary anti-rabbit pP65 antibody (Cell Signaling Technology, Inc., United States) and secondary rabbit-anti-rat antibody (Cell Signaling Technology, Inc., United States) were added. DAPI staining was then performed under 1:100 (Santa Cruz Biotech Co., United States).

Statistical analysis

All data was analyzed using SPSS11.0 statistical software package. The statistical methods included t test, Pearson correlation analysis, and $\chi 2$ test. $P<0.05$ was considered as statistically significant.

Results

Expression of miR-675 in non-small cell lung cancer

Gene expression assay of tumor and peripheral tissue specimens from92 patients with NSCLC were performed and the results demonstrated a higher expression of miR-675 in cancer tissues, which was statistically significant than the expression in the peripheral tissue (P=0.000871, as shown in Figure 1). Our results suggest that miR-675may play an important role in the occurrence and development of NSCLC.

Expression of miR-675 activated NF-κB signaling pathway

First, we successfully constructed two NSCLC cell line A549 with either high or low miR-675 expression. As shown in Figure 2A and B, expressions of miR-675 were as expected after transfection. As shown in Figure 2C, compared to the control group, pP65 expression and translocation into nuclei were both increased whereas the P65 expression was decreased in cells with high ex-

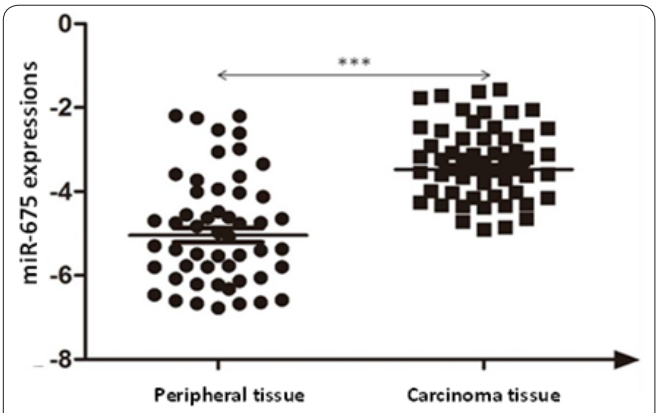

Figure 1. miR-675 expressions in the carcinoma and peripheral tissue by Q-PCR.

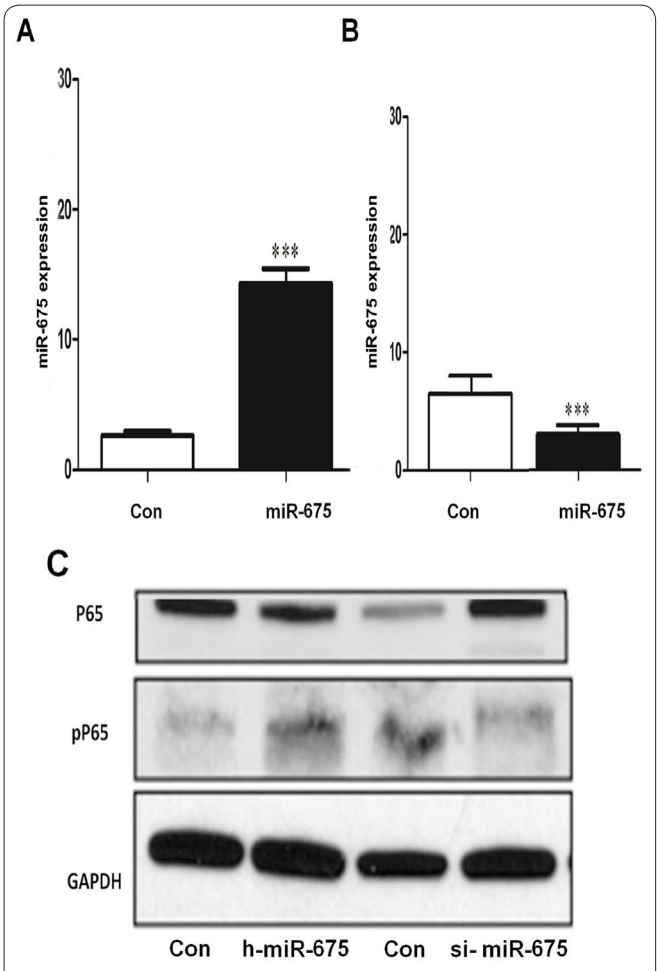

Figure 2. miR-675 activated NF-κB signaling pathway. A) successfully constructed TE-1 cells transfected with miR-675 high expression plasmid; B) successfully constructed A549 cells transfected with miR-675 low expression plasmid; C) expressions of pP65 and P65 in A549 cells with either miR-675 high or low expression plasmids.

pression of miR-675. In theA549 cells with low expression of miR-675, pP65 expression and translocation into nuclei were decreased. Therefore we have demonstrated in both ways that the NF-κB signaling pathway is activated.

pP65 nuclear translocation

We also detected NF-κB nuclear translocation in A549 cell lines after transfecting miR-675. DMSO and miR-675 plasmid was transfected into A549 cells and we observed a significant increase of pP65 translocation into the nuclei, indicating that the NF-κB signaling pathway is activated. On the opposite, when only DMSO was transfected, few pP65entered the nucleus in A549 cell line, indicating that NF-κB signaling pathway was not activated (as shown in Figure 3).

Discussion

In this current study we demonstrated the potential role of miR-675 in the progression of human non-small cell lung cancer. In human cancer patients, miR-675 was highly expressed in cancer tissue rather than peripheral tissue. In a human NSCLC cell line, higher expression of miR-675 increased the NF-κB signaling by increasing more pP65 expressions and nuclear translocation.

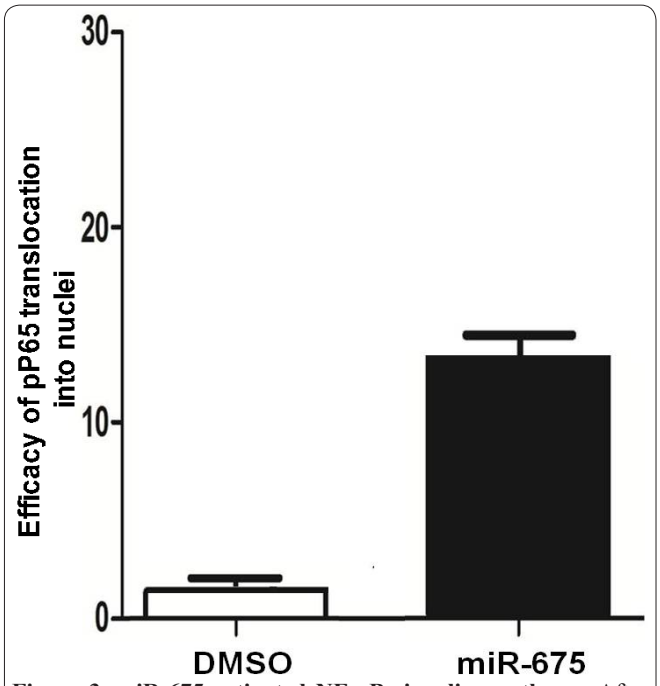

Figure 3. miR-675 activated NF-κB signaling pathway. After A549 cells were transfected with miR-675, pP65 translocation into nuclei was significantly increased indicating an activation of NF-κB signaling pathway; When A549 cells were treated by DMSO, almost no pP65 entered the nuclei indicating NF-κB signaling pathway was not activated.

To date there have not been extensive cellular or molecular studies on the role of miR-675 on lung cancer cells. Although our data was in accordance with previous reports showing that miR-675 promotes carcinogenesis, it was contradictory to a study by He D. et al. (13) They showed that the expression of miR-675 precursor hampered the NSCLC cell line proliferation and migration therefore it inhibited tumor progression (13). However, more studies have shown the potential of miR-675 in increasing tumor progression. It was demonstrated to significantly increased cell proliferation in human colorectal cancer through binding to tumor suppressor retinoblastoma (RB) (14). Zhuang M et al. also showed that miR-675 enhanced human gastric cancer cell proliferation by targeting tumor suppressor RUNX1 (15). More recently, it was also demonstrated that the overexpression of miR-675 promoted bladder cancer cell proliferation by inhibiting P53 activation (16). More importantly, all these studies have provided evidence on higher expression of miR-675 in tumor tissue than peripheral tissues, which were also consistent with our results (14-16). More importantly, we demonstrated a new signaling pathway of miR-675/NF-κB in promoting NSCLC cell proliferation. Taken together, these findings indicated that abnormally enhanced miR-675 expression had an important role in tumor progression.

NF- κB is a classic inflammatory signaling pathway involving in regulation of cell division, proliferation and apoptosis, and modulation of inflammatory or immune response and it has central role in lung cancer cell carcinogenesis (17). The NF- κB signaling pathway has been studied extensively and NF- κB pP65 protein transfers from cytoplasm to nuclei is the key point of NF- κB signaling pathway activation(18). In this study, we found that pP65 expression and translocation into the nuclei were increased significantly when miR-675

was highly expressed, suggesting that NF- κB signaling pathway was activated. The transfection of miR-675 led to increase in nuclear translocation of pP65 confirmed that NF-κB signaling pathway was activated by the high expression of miR-675. We speculate that the low expression of miR-675 may alleviate the disease of patients with non-small cell lung cancer. Further research is warrant to confirm the exact role of miR-675 in non-small cell lung cancer.

In conclusion, miR-675 expression was higher in human non-small cell lung cancer tissue than non-tumor peripheral tissue. Cell line model showed that the enhanced miR-675 expression promoted cell proliferation by targeting pP65 nuclear translocation and expression level. Future studies in animal model can further elucidate the role of miR-675 in lung cancer and it can be a candidate for cancer therapy.

References

1. Hong QY, Wu GM, Qian GS, Hu CP, Zhou JY, Chen LA, et al. Prevention and management of lung cancer in China. Cancer. 2015;121:3080-8.

2. Islami F, Torre LA, Jemal A. Global trends of lung cancer mortality and smoking prevalence. Transl Lung Cancer Res. 2015;4:327-38.

3. Torre LA, Siegel RL, Jemal A. Lung Cancer Statistics. Adv Exp Med Biol. 2016;893:1-19.

4. Saintigny P, Burger JA. Recent advances in non-small cell lung cancer biology and clinical management. Discov Med. 2012;13:287-97.

5. Califano R, Abidin A, Tariq NU, Economopoulou P, Metro G, Mountzios G. Beyond EGFR and ALK inhibition: unravelling and exploiting novel genetic alterations in advanced non small-cell lung cancer. Cancer Treat Rev. 2015;41:401-11.

6. Ortholan C, Puissegur MP, Ilie M, Barbry P, Mari B, Hofman P. MicroRNAs and lung cancer: new oncogenes and tumor suppressors, new prognostic factors and potential therapeutic targets. Curr Med Chem. 2009;16:1047-61.

7. Bueno MJ, Malumbres M. MicroRNAs and the cell cycle. Biochim Biophys Acta. 2011;1812:592-601.

8. Su Z, Yang Z, Xu Y, Chen Y, Yu Q. MicroRNAs in apoptosis, autophagy and necroptosis. Oncotarget. 2015;6:8474-90.

9. Wang J, Zhao YC, Lu YD, Ma CP. Integrated bioinformatics analyses identify dysregulated miRNAs in lung cancer. Eur Rev Med Pharmacol Sci. 2014;18:2270-4.

10. Del Prete A, Allavena P, Santoro G, Fumarulo R, Corsi MM, Mantovani A. Molecular pathways in cancer-related inflammation. Biochem Med (Zagreb). 2011;21:264-75.

11. Crusz SM, Balkwill FR. Inflammation and cancer: advances and new agents. Nat Rev Clin Oncol. 2015;12:584-96.

12. Karin M. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene. 1999;18:6867-74.

13. He D, Wang J, Zhang C, Shan B, Deng X, Li B, et al. Down-regulation of miR-675-5p contributes to tumor progression and development by targeting pro-tumorigenic GPR55 in non-small cell lung cancer. Mol Cancer. 2015;14:73.

14. Tsang WP, Ng EK, Ng SS, Jin H, Yu J, Sung JJ, et al. Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer. Carcinogenesis. 2010;31:350-8.

15. Zhuang M, Gao W, Xu J, Wang P, Shu Y. The long non-coding RNA H19-derived miR-675 modulates human gastric cancer cell proliferation by targeting tumor suppressor RUNX1. Biochem Biophys Res Commun. 2014;448:315-22.

16. Liu C, Chen Z, Fang J, Xu A, Zhang W, Wang Z. H19-derived miR-675 contributes to bladder cancer cell proliferation by regulating p53 activation. Tumour Biol. 2016;37:263-70.

17. Chen SJ, Huang SS, Chang NS. Role of WWOX and NF-κB in lung cancer progression. Transl Respir Med. 2013;1:15.

18. Lee JI, Burckart GJ. Nuclear factor kappa B: important transcription factor and therapeutic target. J Clin Pharmacol. 1998;38:981-93.