MicroRNA-101 downregulation increases C-Fos expression and contributes to the pathogenesis of non-small cell lung cancer

MikroRNA-101’in aşağı düzenlenmesi C-Fos ekspresyonunu artırır ve küçük hücreli dışı akciğer kanserinin patogenezine katkıda bulunur

In addition, knockdown of C-Fos induced similar effects as overexpression of miR-101 in NSCLC cells.

**Conclusion:** These findings indicate that miR-101 regulate NSCLC cells growth through targeting C-Fos. Thus, strategies on C-Fos and/or miR-101 may be a potential molecular therapy for NSCLC.

**Keywords:** MiR-101; C-Fos; Non-small cell lung cancer; Pathogenesis; Cell proliferation; Cell apoptosis.

**Amaç:** Akciğer kanserinde tümöre neden olan moleküler mekanizmalar aydınlatılmasını devam etmektedir. MikroRNA (miRNA), karsinogenez ile ilişkili kodlamayan RNA’ların sınıfındandır. Bu çalışma akciğer kanserinde miRNA-101’in (miR-101) rolünü araştırmayı amaçlamaktadır.

**Yöntem:** MiR-101 insan küçük hücreli dışı akciğer kanseri (KHDAK) dokularında geliştirilmiş bitişik dokulara kıyaslamanak olabilirdi. MiR-101 KHDAK A549 hücrelerine nakledildi. Biyoinformatik öngörüler, C-Fos’un 3’UTR’sinde miR-101’in potansiyel bir bağımlığı yerde olduğunu ortaya koydu; bu durum lusihefrez testi ile de tayit edildi. C-Fos’un ekspresyonunu ve fonksiyonunu teşpit etmek için Western blot analizi ve knockdown yöntemi kullanıldı.

**Bulgular:** KHDAK’də miR-101’in ekspresyonu aşağı düzenlenmiştir. MiR-101’in aşıri ekspresyonu, KHDAK A549 hücrelerinin hücre proliferasyonunu baskılamış ve hücre apoptozunu indüksiyon etmiştir. C-Fos, miR-101 tarafından protein seviyesinde aşıri düzenlenmiştir. Buna ek olarak, C-Fos’un iptal edilmiş, KHDAK hücrelerinde miR-101’in aşıri ekspresyonu ile benzer etkilere neden olmuştur.
Sonuç: Bu bulgular, miR-101’in C-Fos’u hedefleyerek KHDAK hücrelerinin büyümesini düzenlediğini göstermektedir. Bu nedenle C-Fos ve/veya miR-101 üzerindeki stratejiler, KHD için potansiyel bir moleküler terapi olabilir.

Anahtar Kelimeler: MiR-101; C-Fos; Küçük hücreli dış akciğer kanseri; Patogenez; Hücre çoğalması; Hücre apoptozisi.

Introduction

Lung cancer has been the most common cancer and lethal malignancies all over the world for decades. In 2012, it was estimated that there were 1.8 million new cases and 1.5 million deaths globally. With the increasing of aging population and environmental pollution, the upward trend in lung cancer incidence will continue. Non-small cell lung cancer (NSCLC) represents around most of all lung cancers, with around 80% [1]. Unfortunately, the prognosis for NSCLC is still dismal and outcome of the NSCLC is poor. A major population of NSCLC patients are already in an advanced or metastatic stage of the disease at the time of diagnosis [2, 3]. Thus, development of new early diagnosis method and treatment against NSCLC is still an unmet medical need. Accordingly, further knowledge and understanding the mechanisms that control NSCLC development is of significance.

The molecular network of protein-coding genes and pathways has been demonstrated to include functional regulators, such as p53, EGFR, p21, p65, ALT and AKT signaling [4, 5]. The discovery of these proteins especially EGFR and ALT, has led to new therapy and a marked change in the outcome of patients. Besides protein coding genes, non-coding genes are also important in tumorigenesis. However, knowledge of non-coding genes, for example miRNAs, and their roles in NSCLC, are relatively limited [1, 6]. MiRNAs are a class of non-coding small RNAs with 20–25 nucleotides in length, processed by Drosha to form 70–80-nt precursor miRNAs, which are later processed by Dicer into mature miRNAs [7]. The work model of mature miRNAs is to bind to the 3′ untranslated region (UTR) of their target genes through partial sequence complementarity. And it results in mRNA degradation and/or translational repression [8]. Through silencing multiple genes involved in diverse biological processes, such as cell differentiation, proliferation and apoptosis, miRNAs are emerging as key regulators in tumors. They can function as oncogenes or tumor suppressors [9]. Some previous studies have released the critical roles of miRNAs in NSCLC pathogenesis. A group of miRNAs, such as Let-7, miR-34a, miR-7, miR-155 and miR-21, have been found to be associated with carcinogenesis and control tumor cell proliferation and metastasis [7, 10–13]. This may provide rationale and basis for the development of targeted diagnosis and therapies for cancer. Recently, miR-101 was identified as a tumor suppressor in several kinds of human cancers, such as osteosarcoma, bladder cancer, hepatocellular carcinoma and endometrial cancer [14–17]. However, the biological expression, function and mechanisms of miR-101 in lung cancer, especially, in NSCLC, remain largely unknown.

In the present study, we examined the expression of miR-101 in NSCLC clinical tissues and explored its function in NSCLC.

Materials and methods

Tissue samples and cell lines

Eleven pairs of primary NSCLC tissues and matched adjacent tissues were collected from Department of Thoracic Surgery, the Third People’s Hospital of Shenzhen, China from 2009 to 2010 with informed consent and approval. Our studies have been reviewed and approved by the Ethics Committee of the Third People’s Hospital of Shenzhen and all the experiments were carried out in accordance with relevant guidelines and regulations.

NSCLC cell line A549 was obtained from the Institute of Biochemistry and Cell Biology of Chinese Academy of Science (China) and originated from the ATCC (Manassas, VA, USA). All cells were cultured in DMEM with 10% fetal bovine serum, 2 μM glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin sulfate.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using TRzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNAs were then stored at −80°C before analysis.

For miRNA expression, around 10 ng of RNA were converted to cDNA using the ABI miRNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA). U6 gene was used as a normalization control for analysis.

For C-Fos expression, synthesis of cDNA was performed with 1 μg of total RNA per sample with the ABI reverse transcription kit (Applied Biosystems, USA)
according to the manufacturer’s manual. QRT-PCR was performed in triplicate for each sample using ABI SYBR Green Master kit (Applied Biosystems, USA) according to the manufacturer’s instructions. GAPDH was used as a housekeeping gene for normalization. The primer sequences used are: (1) C-Fos: 5′-CGTGCCAGACATGGAC-CTAT-3′ (forward) and 5′-CGGGGTAGGTGAAGACGAAG-3′ (reverse); (2) GAPDH: 5′-TGCACCACCAACTGCTTAGC-3′ (forward) and 5′-GCATGGACTGTGGTCATGAG-3′ (reverse).

The expression ratio calculation is based on the following calculation strategy.
- MiR-101 expression = 2^(-ΔCT value(miR-101)-CT value(U6)).
- C-Fos expression = 2^(-ΔCT value(C-Fos)-CT value(GAPDH)).

### MiRNA mimics, C-Fos siRNA and transfection

The human miR-101 duplex mimics (miR-101) and negative control oligonucleotide duplex mimics (miR-NC) were designed and obtained from Ribobio (Guangzhou, China). C-Fos siRNA and control siRNA oligonucleotides were purchased from Santa Cruz (Santa Cruz, USA). MiRNA or siRNA transfection was performed using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer’s protocol.

### MiRNA target prediction

Putative miRNA target genes were searched by the website program, www.microRNA.org.

### Luciferase assay

A549 cells were transfected with a C-Fos 3′UTR plasmid, Renilla luciferase pRL-TK vector (Promega, Madison, WI, USA) and miR-101 mimics/miR-NC mimics. The transfection is by Lipofectamine 2000 reagent (Invitrogen, USA) following the instruction. Luminescence was then examined 48 h later on the Dual-Luciferase Reporter Assay System (Promega, USA).

### Cell viability analysis

Cell viability was determined by the Cell Counting Kit-8 assay (Dojindo, Japan) following the manufacturer’s protocol.

### Caspase 3/7 activity

Caspase 3/7 activity was analyzed following the manufacturer’s instructions (Pierce, USA). Briefly, A549 cells were cultured in a white-walled 96-well plate. Before testing, cells were washed with PBS and culture with Caspase-Glo® 3/7 Reagent at room temperature for 1–2 h. The luminescence of each sample was measured in a plate-reading luminometer (Tecan Infinite M200, Switzerland).

### Western blot analysis

For total protein isolation, cells were washed with 1× PBS, lysed with RIPA buffer for 15 min on ice. After centrifugation at 12,000 rpm for 15 min, the supernatants were collected. Proteins extracted from cells were immunoblotted with different antibodies following the protocol. The primary antibodies used were C-Fos (dilution 1:2000) and GAPDH (1:10,000; Cell Signaling Technology, USA).

### Statistical analysis

Data were expressed as mean±SEM of three independent experiments. For all statistical tests, PRISM 5.0 (San Diego, CA, USA) was used. Analysis of intergroup differences between two groups was carried out using t-test, while analysis of more than two groups was by ANOVA. p-Values < 0.05 were considered statistically significant.

### Results

#### Expression of miR-101 is downregulated in human NSCLC tissues

To investigate the miR-101 expression in NSCLC, 11 cases of human NSCLC tissues along with matched adjacent tissues were recruited and examined. As shown in Figure 1, miR-101 expression was significantly decreased in NSCLC tissues compared to their matched adjacent tissues (p = 0.036).

#### MiR-101 suppresses cell proliferation of A549 cells and induces apoptosis

The biological function of miR-101 in NSCLC A549 cells was next under investigation. Mature miR-101 mimics or negative
control mimics were transiently transfected into A549 cells by Lipofectamine 2000. The expression of miR-101 exhibited around 18-times of increase of transfection of miR-101 at 24 h (p < 0.01) (Supplemental Figure 1). Cell viability was analyzed by the Cell Counting Kit-8 proliferation assay after 48 h. As a result, A549 cells showed decreased cell proliferation after transfection with miR-101 compared with negative control (Figure 2A). These results suggest that miR-101 has an inhibitory effect on the proliferation of A549 cells.

To further investigate the role of apoptosis in miR-101 function, caspase 3/7 activity in A549 cells was analyzed. Caspase 3/7 activity significantly increased in miR-101-transfected cells (Figure 2B). It indicated the increase of cell apoptosis. Together, these results indicated that the inhibition of cell growth by miR-101 was associated with increased cell apoptosis.

MiR-101 targets C-Fos

To further explore the mechanisms through which miR-101 suppresses A549 cell growth, we analyzed probable downstream genes related to cell proliferation or apoptosis. Computational prediction was used to search for miR-101 likely target genes. Among them, C-Fos was found to have a putative miR-101 binding site in its 3′UTR (Figure 3A). The 3′-UTRs of C-Fos bear binding sites of miR-101 with highly conservative sequences in mammals.

To determine whether C-Fos is directly regulated by miR-101 through 3′UTR binding, we performed a dual-luciferase assay to verify the relationship. The 3′UTR fragment with the predicted binding site, was cloned and inserted into the downstream region of a luciferase reporter gene in a pGL3-con vector. A549 cells were co-transfected with C-Fos 3′UTR-reporter plasmid and miR-101 or miR-NC mimic or miR-NC for luciferase activity analysis. As shown in Figure 3B, miR-101 reduced the activity of luciferase containing C-Fos 3′UTR compared to control ones. These results indicate that miR-101 may suppress C-Fos expression by targeting its 3′UTR.

To further investigate the effects of miR-101 on C-Fos gene expression, protein level of C-Fos was analyzed by Western blotting. A549 cells were transfected with miR-101 mimics for 48 h. In A549 cells transfected with miR-101 mimics, the protein expressions of C-Fos were significantly less than that of the negative control of cells (Figure 3C).

Taken together, these results suggest that miR-101 decreased the expression of C-Fos through direct 3′UTR interactions.

Inhibition of C-Fos reduces cell proliferation of A549 cells through apoptosis

Since miR-101 decreased C-Fos expression, we further investigated the function of such alteration. A549 cells were transfected with C-Fos siRNA for 72 h, and then cell viability was examined. As shown in Figure 4A, cell proliferation was inhibited after transfection with C-Fos siRNA. In addition, cell apoptosis was analyzed through caspase 3/7 activity. After knockdown of C-Fos by siRNA, caspase 3/7 activity significantly increased compared with the control cells. It suggests the increase of cell apoptosis.

These results are similar to the effect of miR-101 overexpression (Figure 2A and B). It supports the involvement of C-Fos in miR-101-mediated cell growth inhibition and cell apoptosis induction of A549 cells.
To further explore the functional role of C-Fos in NSCLC, we examined its expression in clinical samples. A total of 11 cases of human NSCLC tissues along with matched adjacent tissues were examined. As shown in Figure 5, C-Fos expression was significantly \( p = 0.0086 \) increased in NSCLC tissues compared to the matched adjacent tissues.

These results indicate the opposite expression pattern of C-Fos to that seen for miR-101.

**Discussion**

Emerging evidence has indicated miRNAs to be critical regulators in tumorigenic processes by targeting a variety...
of oncogenes or tumor suppressors [15–22]. Alteration in miRNA expressions occur in NSCLC. In the present study, we identified interaction and function of miR-101 and C-Fos in NSCLC. The expression of miR-101 is downregulated in human NSCLC tissues, which is consistent with a previous study [23]. Overexpression of miR-101 suppressed cell growth of NSCLC A549 cells, suggesting that miR-101 is a tumor suppressor in NSCLC. These results together with previous reports [14–17, 24], support overexpression of miR-101 to be a potential gene therapy, which may provide therapeutic benefits for treating human NSCLC.

The regulatory function of miRNAs is through direct targets and interactions with their 3′UTRs. Reported targets of miR-101 include mRNAs of signaling proteins such as vascular endothelial growth factor C, stathmin 1, RAF proto-oncogene serine/threonine-protein kinase, zester homolog-2, Ras-related C3 botulinum toxin substrate 1, and other functional genes related to cell growth and migration [25]. In this study, we identified C-Fos as a target of miR-101 in human NSCLC A549 cells. Overexpression of miR-101 significantly reduced the relative luciferase activity of a reporter containing its potential binding sites on the 3′UTR of C-Fos, as well as the endogenous C-Fos protein level.

C-Fos is a 380-amino-acid protein containing a basic leucine zipper region for dimerization and DNA-binding, as well as a transactivation domain at the C-terminus [26]. Thus, it regulates gene expression and is involved in various biological processes. Activation of the C-Fos transgene in mice results in increased production of proteins, such as cyclin D1, A and E in osteoblasts and chondrocytes, which may contribute to the uncontrolled growth leading to tumorigenesis [27]. C-Fos expression was observed in human osteosarcomas in more than half of the cases, and is associated with higher frequency of relapse and poor response to chemotherapy [28]. In lung cancer, transcriptome network analysis identified C-Fos as a candidate gene for squamous lung cancer. Considering the importance of C-Fos in activation of several genes related to tumor growth and tumor metastasis, we investigated the role of C-Fos in miR-101-mediated antitumor activity. Knockdown of C-Fos elicited a similar effect to miR-101 overexpression. Thus, these results suggest that the biological function of miR-101 on NSCLC A549 cells was at least partially through targeting C-Fos. The interaction between miR-101 and C-Fos has been previously reported in other cancer types, such as osteosarcoma, bladder cancer, hepatocellular carcinoma and endometrial cancer [14–17]. Subsequently, C-Fos was identified as a direct target of miR-101 in these cancers and was negatively regulated by miR-101 at the post-transcriptional level, which is consistent with the present study. Furthermore, miR-101 inhibits the proliferation, migration and invasion of cancer cells [14–17]. These results together with us released that miR-101 acts as a tumor suppressor in multiple types of human cancer to a certain extent, via direct suppression of the protein expression levels of C-Fos.

In this study, we found an inverse correlation between miR-101 and C-Fos expression in human NSCLC tissues. MiR-101 was downregulated, while C-Fos was upregulated in NSCLC. When it is analyzed in the same tissue, the trend of the reverse pattern could be seen but with large variation. However, the present study only recruited 11 cases of the samples (Supplemental Figure 2). Thus, a larger sample needs to be further analyzed and confirmed.

These findings indicate that downregulation of miR-101 in NSCLC may contribute to the malignant phenotype by maintaining a high level of C-Fos. Thus, miR-101 and its target C-Fos are two potential functional therapeutic targets for NSCLC treatment.

Conflict of interest statement: The authors declare that they have no conflict of interest.

References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
2. Kalathiya U, Padariya M, Baginski M, Padariya C. SiMiSnoRNA: collection of siRNA, miRNA, and snoRNA database for RNA interference. Turk J Biochem 2015;40:524–32.
3. Barger JF, Nana-Sinkam SP. MicroRNA as tools and therapeutics in the treatment of lymphoid malignancies. Semin Hematol 2014;51:219–27.
4. Calin GA, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. Cancer Res 2006;66:7390–4.
5. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–8.
6. Deng Y, Deng H, Bi F, Liu J, Bemis LT, Norris D, et al. MicroRNA-137 targets carboxyl-terminal binding protein 1 in melanoma cell lines. Int J Biol Sci 2011;7:133–7.
7. Mishra S, Lin CL, Huang TH, Bouamar H, Sun LZ. MicroRNA-21 inhibits p57Kip2 expression in prostate cancer. Mol Cancer 2014;13:212.
11. Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, et al. MicroRNA-21 (miR-21) post-transcriptionally down-regulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 2008;27:2128–36.

12. Hiroki E, Akahira J, Suzuki F, Nagase S, Ito K, Suzuki H, et al. Changes in microRNA expression levels correlate with clinicopathological features and prognoses in endometrial serous adenocarcinomas. Cancer Sci 2010;101:241–9.

13. Bhatt K, Zhou L, Mi QS, Huang S, She JX, Dong Z. MicroRNA-34a is induced via p53 during cisplatin nephrotoxicity and contributes to cell survival. Mol Med 2010;16:409–16.

14. Wang Z, He R, Xia H, Wei YU, Wu S. MicroRNA-101 has a suppressive role in osteosarcoma cells through the targeting of c-FOS. Exp Ther Med 2016;11:1293–9.

15. Long Y, Wu Z, Yang X, Chen L, Han Z, Zhang Y, et al. MicroRNA-101 regulates expression of the v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) oncogene in human hepatocellular carcinoma. Hepatology 2009;49:1194–202.

16. Li S, Fu H, Wang Y, Tie Y, Xing R, Zhu J, et al. MicroRNA-101 inhibits the proliferation and invasion of bladder cancer cells via targeting c-FOS. Mol Med Rep 2016;14:2651–6.

17. Konno Y, Dong P, Xiong Y, Suzuki F, Lu J, Cai M, et al. MicroRNA-101 targets EZH2, MCL-1 and FOS to suppress proliferation, invasion and stem cell-like phenotype of aggressive endometrial cancer cells. Oncotarget 2014;5:6049–62.

18. 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.

19. Chen D, Guo W, Qiu Z, Wang Q, Li Y, Liang L, et al. MicroRNA-30d-5p inhibits tumour cell proliferation and motility by directly targeting CCNE2 in non-small cell lung cancer. Cancer Lett 2015;362:208–17.

20. Duncavage E, Goodgame B, Sezhiyan A, Govindan R, Pfeifer J. Use of microRNA expression levels to predict outcomes in resected stage I non-small cell lung cancer. J Thorac Oncol 2010;5:1755–63.

21. Santos AO, Pereira JP, Pedroso de Lima MC, Simoes S, Moreira JN. In vitro modulation of Bcl-2 levels in small cell lung cancer cells: effects on cell viability. Braz J Med Biol Res 2010;43:1001–9.

22. Huang Z. Bcl-2 family proteins as targets for anticancer drug design. Oncogene 2000;19:6627–31.

23. Yan F, Shen N, Pang J, Xie D, Deng B, Molina JR, et al. Restoration of miR-101 suppresses lung tumorigenesis through inhibition of DNMT3a-dependent DNA methylation. Cell Death Dis 2014;5:e1413.

24. Wang L, Zhang LF, Wu J, Xu SJ, Xu YY, Li D, et al. IL-1beta-mediated repression of microRNA-101 is crucial for inflammation-promoted lung tumorigenesis. Cancer Res 2014;74:4720–30.

25. Xu C, Zheng Y, Lian D, Ye S, Yang J, Zeng Z. Analysis of microRNA expression profile identifies novel biomarkers for non-small cell lung cancer. Tumori 2015;101:104–10.

26. Caputto BL, Cardozo Gizzi AM, Gil GA. c-Fos: an AP-1 transcription factor with an additional cytoplasmic, non-genomic lipid synthesis activation capacity. Biochim Biophys Acta 2014;1841:1241–6.

27. He L, Lee J, Jang JH, Lee SH, Nan MH, Oh BC, et al. Ginsenoside Rh2 inhibits osteoclastogenesis through down-regulation of NF-kappaB, NFATc1 and c-Fos. Bone 2012;50:1207–13.

28. Weissstein JS, Majeska RJ, Klein MJ, Einhorn TA. Detection of c-Fos expression in benign and malignant musculoskeletal lesions. J Orthop Res 2001;19:339–45.

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