MiR-222-3p Promotes Cell Proliferation and Inhibits Apoptosis by Targeting PUMA (BBC3) in Non-Small Cell Lung Cancer

Weijun Chen, MM¹ and Xiaobo Li, MM²

Abstract
MicroRNAs have been demonstrated to be critical regulators in tumor progression, including non-small cell lung cancer. MicroRNA-222-3p has been reported to function as a tumor suppressor or oncogene in several types of cancer, but its function role in non-small cell lung cancer has not been uncovered. In this study, we first found the expression of microRNA-222-3p was significantly increased in non-small cell lung cancer tissues and cell lines. MicroRNA-222-3p inhibitor decreased the activity of non-small cell lung cancer cells to proliferate and increased cell apoptosis using cell counting kit-8, flow cytometry, and caspase-3 activity analysis. Overexpressed microRNA-222-3p in non-small cell lung cancer cells promoted cell proliferation, but decreased cell apoptosis. Moreover, Bcl-2-binding component 3 was the target gene of microRNA-222-3p, and its knockdown weakened the regulatory effect of microRNA-222-3p inhibitor on cell proliferation and apoptosis in non-small cell lung cancer cells. In conclusion, microRNA-222-3p plays a significant role in the regulation of Bcl-2-binding component 3 expression and might be a promising target for clinical non-small cell lung cancer therapy.

Keywords
non-small cell lung cancer, miR-222-3p, BBC3, apoptosis

Abbreviations
BBC3, Bcl-2-binding component 3; CCK, cell counting kit; FBS, fetal bovine serum; miR-222-3p, microRNA-222-3p; mRNA, messenger RNA; MUT, mutant; NSCLC, non-small cell lung cancer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; PUMA, p53 upregulated modulator apoptosis; UTR, untranslated region; WT, wild type

Introduction
Lung cancer is the most common cause of cancer-related deaths among males and females in both developed and developing countries.¹ In 2019, about 228,150 people are diagnosed as lung cancer in the United States, and 142,670 people die of this disease.² Non-small cell lung cancer (NSCLC) as the most commonly type of lung cancer³ has a dismal 5-year survival of just 15% and unexpectedly high recurrence rate of 30% to 50%.⁴ The current standard treatment for advanced NSCLC is platinum-containing chemotherapy, followed by selective use of maintenance therapy, and combined with second-line cytotoxic chemotherapy, but the patients’ survival remains unsatisfactory.⁵ Therefore, exploration of the molecular mechanisms and regulation of NSCLC progression might well provide therapeutic opportunities for NSCLC.

MicroRNAs (miRNAs) constitute of a large family of 21-24 nt noncoding, endogenous, single-stranded RNAs that play important modulatory roles by base-pairing to messenger RNAs (mRNAs) and triggering either RNA degradation or posttranscriptional depression.⁶ Nearly 20 years, a
considerable of research spanning has documented evidence that abnormal expression of miRNAs are associated with cell growth and tumorigenesis of NSCLC.7 Many miRNAs are involved in the initiation and progression of NSCLC, including proliferation, invasion, migration, and apoptosis.8 Moreover, miRNAs have been established as key regulators of diverse cellular procedures, playing tumor suppressor or oncogenic roles in cancers.9 MicroRNA-222-3p is one member of the miRNA family that enrichment occurs in exosomes when released from epithelial ovarian cancer cells and can induce a polarization of the M2 phenotype.10 Liu et al11 revealed that miR-222-3p contribute to growth and invasion of endometrial carcinoma by downregulating ERα. Cell studies have shown that miR-222-3p overexpression in papillary thyroid carcinoma cells depresses cell proliferation.12 Interestingly, a higher level of exosomic miR-222-3p has been reported to predict worse prognosis in NSCLC.13 Ulivi et al14 further showed that miR-222-3p was significantly associated with disease-free survival in early-stage NSCLC. Nevertheless, the biological functions and the underlying mechanisms of miR-222-3p in NSCLC remain largely undefined.

The BCL2 family member BBC3, encoded by Bcl-2-binding component 3, also known as p53 upregulated modulator apoptosis (PUMA), is a proapoptotic BH3-only protein.15 Bel-2-binding component 3 mRNA production is dramatically induced under conditions of various apoptotic stimuli, such as DNA damage, p53 tumor suppressor, glucocorticoid treatment, and growth factor deprivation.16 A recent study have shown that BBC3 is frequently downregulated in head and neck cancer tissues compared with adjacent normal tissue, and can be used to tumor size-classify.17 Altered expression of BBC3 is concerned with cell proliferation, migration, and apoptosis.10,18 Moreover, BBC3 has been demonstrated to be the target gene of miR-222 in liver cancer.19 However, whether BBC3 was a functional regulator involved in miR-222-3p in NSCLC has not been reported yet.

In the current study, we analyzed the function of miR-222-3p in NSCLC in vitro by gain- and loss-of-function experiments. Considering the potential crosstalk between miR-222-3p and BBC3, we sought to investigate the miR-222-3p/BBC3 axis as a complementary tool to enhance our understanding the development and progression of NSCLC.

### Materials and Methods

#### Tissue Samples

Total 60 pairs of tumor tissues and matched adjacent tissues were collected from patients with NSCLC between June 2017 and March 2018 at Taizhou Center Hospital (Zhejiang, China), which were immediately snap frozen in liquid nitrogen. Before surgery, all the patients were confirmed to not receive any treatments, including chemotherapy or radiotherapy and signed the informed consent. The basic clinicopathological features of each patient were listed in Table 1. This study obtained the Ethical approval from the Ethics Technology in Cancer Research & Treatment

| Table 1. Association Between miR-222-3p Expression and Clinicopathological Characteristics in Patients With Non-Small Cell Lung Cancer. |
|---|---|---|---|
| **Clinicopathological Characteristics** | **Cases (n = 60)** | **Expression of miR-222-3p** | **P Value** |
| | | **High (n = 39)** | **Low (n = 21)** | (χ² test) |
| Sex | | | | |
| Male | 37 | 26 | 11 | .278 |
| Female | 23 | 13 | 10 | |
| Age | | | | |
| < 60 | 48 | 30 | 18 | .417 |
| ≥ 60 | 12 | 9 | 3 | |
| Tumor size (cm) | | | | |
| < 4 | 35 | 19 | 16 | .005b |
| ≥ 4 | 25 | 20 | 5 | |
| Histological type | | | | |
| Squamous cell carcinoma | 25 | 14 | 11 | .217 |
| Adenocarcinoma | 35 | 25 | 10 | |
| Differentiation | | | | |
| Well/moderate | 32 | 18 | 14 | .129 |
| Poor/not stated | 28 | 21 | 7 | |
| TNM stage | | | | |
| I + II | 46 | 30 | 16 | .949 |
| III + IV | 14 | 9 | 5 | |
| Lymph node metastasis | | | | |
| Negative | 38 | 19 | 19 | .001b |
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*a* n = 60.  
*b* P < .05.
Committee of Taizhou Center Hospital and conducted in accordance to the Declaration of Helsinki.

**Cell Culture**

Four human NSCLC cell lines (AH1299, SPC-A1, A549, and 95D), normal human bronchial epithelial cell line (BEAS-2B), and human embryonic kidney-derived cell line (HEK293T) were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). SPC-A1 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco). The other cell lines were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS. All the cell lines were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

**Cell Transfection**

MicroRNA-222-3p mimic, miR-222-3p inhibitor, and their negative control (mimic NC and inhibitor NC, respectively) were synthesized by the GenePharma Biotech (Shanghai, China). Small interfering RNA against human BBC3 mRNA and the control siNC were synthesized by Guangzhou Ribobio Co, Ltd. (Guangzhou, China). SPC-A1 and 95D cells were seeded in 6-well plates at a density of 4 × 10⁴ cells per well and cultured overnight until reached 70% to 90% confluence. Cell transfection was performed using Lipofectamine™ 2000 reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s instructions.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was extracted from tissues or cell lines using TRIzol Isolation Reagents (Invitrogen, Carlsbad, California) and complementary DNA was synthesized using a TaqMan miRNA reverse transcription kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer’s instructions. Quantitative real-time polymerase chain reaction (PCR) was conducted on CFX96™ Real-Time PCR detection System (Bio-Rad Laboratories, Inc) using miRNA-specific TaqMan miRNA assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the following primers: miR-222-3p forward 5'-AGCTACATCTGGCTACTGGGT-3' and reverse 5'-GCGGACACAGAATTAATACGAC-3', U6 forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCAGAATTGCGT-3'. The relative expression of miR-222-3p was calculated using the 2⁻ΔΔCq method and normalized to U6 as an internal control.

**Cell Counting Kit-8 Assay**

SPC-A1 and 95D cells were seeded into 96-well plates at a density of 3000 cells per well after transfection. At the indicated time points (24 hours, 48 hours, and 72 hours, respectively), cell counting kit-8 (CCK-8) solution (Beyotime, Shanghai, China) was added into each well and cells were incubated for 2 hours. Then, the optical density values at 450 nm were determined using a microplate reader (Bio-Tek, Winooski, Vermont).

**Western Blot Analysis**

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Solarbio, Beijing, China). Protein concentration was measured using BCA assay kit (Beyotime). Equal amounts of...
protein were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred to polyvinylidene fluoride membrane (EMD Millipore, Billerica, Massachusetts). After blocking with 5% nonfat milk for 1 hour at room temperature, the membranes were incubated with primary antibodies against BBC3 (1:1000, Abcam, Cambridge, Massachusetts) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)(1:5000, Santa Cruz Biotechnology, Inc, Dallas, Texas) overnight at 4°C. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature, followed by signals detection using enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Statistical Analysis
All experiments were performed at least 3 times and data were expressed as mean ± standard deviation. All statistical analyses were performed using SPSS version 21.0 software (IBM, Chicago, Illinois). Significant differences were evaluated using Student t test between 2 groups and 1-way analysis of variance followed by post hoc Dunnett test among groups. The values of P less than .05 were considered as statistically significant.

Results
MicroRNA-222-3p Was Significantly Up-Regulated in NSCLC Tissues and Cell Lines
To identify the potential role of miR-222-3p in NSCLC, the expression levels of miR-222-3p were determined in tumor tissues and matched adjacent tissues derived from 60 patients with NSCLC. The expression levels of miR-222-3p were significantly upregulated in 60 pairs of tumor tissues compared with matched adjacent tissues (Figure 1B, P < .001). Next, we investigated whether miR-222-3p expression levels were associated with...
clinical features. The results showed that miR-222-3p expression levels were significantly correlated with tumor size \( (P = .005) \) and lymph node metastasis \( (P = .001) \), but not sex \( (P = .278) \), age \( (P = .417) \), histological type \( (P = .217) \), differentiation \( (P = .129) \), and TNM stage \( (P = .949) \), as shown in Table 1. In addition, we analyzed the expression levels of miR-222-3p in NSCLC cell lines. As shown in Figure 1C, miR-222-3p expression levels in 4 NSCLC cell lines (AH1299, SPC-A1, A549, and 95D) were markedly increased compared with the normal human bronchial epithelial cell line (BEAS-2B). Considering that 95D and SPC-A1 cells exhibited higher and lower expression of miR-222-3p, they were selected to perform the following in vitro experiments.

### MicroRNA-222-3p Promoted Cell Proliferation and Colony Formation Ability in NSCLC Cells

95D and SPC-A1 cells were transfected with miR-222-3p inhibitor and miR-222-3p mimic, respectively, to investigate the biological function of miR-222-3p in NSCLC cells. First, quantitative real-time PCR demonstrated that the expression of miR-222-3p was significantly decreased in 95D cells after miR-222-3p inhibitor transfection but increased in SPC-A1 cells after miR-222-3p mimic transfection (Figure 2A, \( *P < .05 \), \( **P < .01 \), \( ***P < .001 \) versus inhibitor NC or mimic NC). NC indicates negative control; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction.

**Figure 2.** MicroRNA-222-3p promoted cell proliferation and colony formation in NSCLC cells. (A) 95D and SPC-A1 cells were transfected with miR-222-3p inhibitor and miR-222-3p mimic, respectively, and miR-222-3p expression was measured using quantitative real-time PCR. (B) Cell proliferation was assayed in 95D and SPC-A1 cells. Colony formation was assayed in (C) 95D and (D) SPC-A1 cells. \( *P < .05 \), \( **P < .01 \), \( ***P < .001 \) versus inhibitor NC or mimic NC. NC indicates negative control; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction.

MicroRNA-222-3p Decreased Cell Apoptosis in NSCLC Cells

Furthermore, the effects of miR-222-3p on cell apoptosis were evaluated by flow cytometry. As shown in Figure 3A, the average apoptosis rate was significantly elevated from 16.8% ± 0.4% in inhibitor NC group to 29.8% ± 0.4% in miR-222-3p
inhibitor group in SPC-A1 cells ($P < .001$). On contrary, miR-222-3p mimic transfection remarkably decreased the average apoptosis rate from 23.9% ± 0.6% to 12.9% ± 0.7% in 95D cells (Figure 3B, $P < .001$). To further confirm the apoptosis status, caspase-3 activity assay was performed in 95D and SPC-A1 cells. The cells treated with 1 μM cisplatin (DDP) for 2 hours were used as positive control. ***$P < .001$ versus inhibitor NC or mimic NC. NC indicates negative control; NSCLC, non-small cell lung cancer.

**Bcl-2-Binding Component 3 Is a Target of miR-222-3p**

Potential targets of miR-222-3p were predicted by TargetScanHuman7.1. Among these predicted target genes, BBC3 as an essential apoptosis inducer was selected as a potential target gene of miR-222-3p in NSCLC. As shown in Figure 4A,
the potential miR-222-3p binding sites in the BBC3 mRNA 3′-UTR were presented. The WT and MUT of miR-222-3p binding sites in the BBC3 3′-UTR of mRNA luciferase reporter were constructed. Subsequently, luciferase reporter assay was performed to validate the interaction between miR-222-3p and BBC3. The results showed miR-222-3p mimic significantly reduced the relative luciferase activity of the WT BBC3 but did not affect the MUT BBC3 compared with the mimic NC (Figure 4B, \( P < .01 \)). In addition, the protein levels of BBC3 were elevated following transfection with miR-222-3p inhibitor in 95D cells (Figure 4C), while the miR-222-3p mimic group in SPC-A1 cells showed the opposite effect (Figure 4D). This data revealed that miR-222-3p may negatively regulate BBC3 by directly binding to the 3′-UTR in NSCLC cells.

**Bcl-2-Binding Component 3 Knockdown Abolished the Downregulation of miR-222-3p-Mediated Cell Proliferation and Apoptosis of NSCLC Cells**

To further explore the function of BBC3 in miR-222-3p-mediated proliferation and apoptosis of NSCLC cells, we conducted rescue experiment in 95D cells after transfection with miR-222-3p inhibitor alone or together with siBBC3. Western blot analysis confirmed the protein expression of BBC3 was upregulated after miR-222-3p inhibitor transfection alone, but downregulated after cotransfection with miR-222-3p inhibitor and siBBC3 (Figure 5A). Then, the proliferation, apoptosis rate, and caspase-3 activity were investigated. Our data showed BBC3 knockdown could rescue the proliferation suppression (Figure 5B) and apoptosis promotion (Figure 5C and D) of 95D cells by the downregulation of miR-222-3p. These results suggest that miR-222-3p-mediated regulation of NSCLC cell might be dependent on regulating BBC3.

**Discussion**

Despite abnormal expression of miRNAs has been reported in diverse human tumor types, the potential role of miRNAs in NSCLC remains largely unclear. MicroRNA-222-3p has emerged as an inducer of tumor-associated macrophages polarization in epithelial ovarian cancer. In addition, miR-222-3p plays oncogenic roles in regulating cellular growth of human endometrial carcinoma and papillary thyroid carcinoma by suppressing target genes ERα and Gas5, respectively. However, the expression profile and biological function of miR-222-3p in NSCLC has not been characterized and understood.

Here, we observed that miR-222-3p was dramatically upregulated in NSCLC tissues compared to adjacent tissues. Compared with bronchial epithelial cell line (BEAS-2B), increased miR-222-3p expression levels were detected in NSCLC cell lines. Evidence of high level of miR-222-3p suggest that it may also exerts oncogenic properties in...
NSCLC. As expected, knockdown of miR-222-3p by transfection significantly decreased NSCLC cell proliferation and increased apoptosis, while overexpression of miR-222-3p could promote the malignant biological behaviors of NSCLC cells. In line with our data, miR-222 promoted colorectal cell function reported by Luo et al.\textsuperscript{20} Pan et al\textsuperscript{21} also showed that miR-222 exerts an oncogene in cervical cancer. Moreover, significantly elevated miR-222 expression was identified to be involved in key processes associated with NSCLC initiation and development.\textsuperscript{13}

What’s more, we found that miR-222-3p can directly and negatively modulate BBC3 mRNA expression by base-pairing with the latter’s 3’ end. The antitumorigenicity of NSCLC cells transfected by miR-223-3p inhibitor could be reversed by knockdown of BBC3, indicating that miR-222-3p promoted proliferation and colony formation and restricted apoptosis of NSCLC cells through directly targeting BBC3. As a member of the BH3-only Bcl-2 family, BBC3 was found to be a novel tumor suppressor in many types of human cancer.\textsuperscript{18} Bcl-2-binding component 3 is a powerful inducer of apoptosis which was induced by p53-dependent and -independent apoptotic signals.\textsuperscript{22} Two proteins (BBC-\( \alpha \) and BBC-\( \beta \)) containing a BH3 domain are encoded by BBC3, which have been shown to bind to members of the Bcl-2 family of pro-survival proteins, such as BCL2, BCL-XL, and MCL-1.\textsuperscript{23} The attractive interaction between BBC3 and Bcl-2 family members could relieve their inhibition of BAK and BAX, leading to mitochondrial membrane permeabilization and activates the caspase cascade.\textsuperscript{18} Several lines of evidence have implicated a potential role of BBC3 in inhibiting cell proliferation. For instance, EZH2-mediated upregulation of BBC3 makes an important contribution to the decreased proliferation of NSCLC cells.\textsuperscript{18} Wu et al\textsuperscript{24} indicated that miR-483-3p exhibited the in vitro proliferative activities in neuroblastoma cells through downregulating PUMA (also named as BBC3) expression. However, the molecular mechanisms underlying the action of BBC3 in cancer cells proliferation are largely unknown. In this study, our observations suggest a role for BBC3 as a tumor suppressor through inhibiting NSCLC proliferation. In addition, the downstream events of miR-222-3p/BBC3 axis are unknown and need further investigation. Considering these, our next work will further explore the effects of miR-222-3p on apoptosis-related factors, including Bcl-2 family members. Of course, there are some limitations in our study as follows: (1) Lack of the effects of miR-222-3p on apoptosis-related factors (caspase-3, Bax, Bcl-2, etc); (2) Lack of the role of miR-222-3p in tumor growth in vivo.

In conclusion, our data showed that miR-222-3p acts as an oncomiR through depressing tumor suppressor BBC3. The miR-222-3p/BBC3 pathway constitutes a novel controller of cellular growth and apoptosis in NSCLC. Despite further studies are necessary to thoroughly determine the biological function of miR-222-3p/BBC3 axis interplay, the identification and characterization of their functional crosstalk will help us understand the development and progression of NSCLC.

Authors’ Note
Patient samples were collected with written informed consent in accordance to the Declaration of Helsinki and under the Ethical Committee of Taizhou Center Hospital (Approval number: 20180342).

Authors Contributions
XL conceived and designed the research. WC performed experiments and performed statistical analysis. XL and WC drafted the manuscript. Both the authors read and approved the final manuscript.

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The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors Contributions
XL conceived and designed the research. WC performed experiments and performed statistical analysis. XL and WC drafted the manuscript. Both the authors read and approved the final manuscript.

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