Breviscapine suppresses the growth of non-small cell lung cancer by enhancing microRNA-7 expression

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Breviscapine (BVP) has previously been shown to inhibit the proliferation of hepatocellular carcinoma cells. However, little is known about the effects of BVP on non-small cell lung cancer (NSCLC) growth. Here, we aimed to study the effects of BVP on human NSCLC growth. We employed A549, NCL-H460 and A549 cells transfected with microRNA-7 (miR-7) mimic and inhibitor to investigate the effect of BVP on cell proliferation, apoptosis and apoptosis-associated molecules. The results showed that BVP significantly reduced the growth of A549 and NCL-H460 cells in a concentration-dependent and time-dependent manner, accompanied by a significant elevation of apoptosis. Additionally, the present study also confirmed that BVP-treated A549 cells showed increased levels of Bax and microRNA-7 (miR-7) and a decreased level of Bcl-2. The up-regulation of miR-7 enhanced the BVP sensitivity of NSCLC cells by suppressing cell proliferation and promoting cell apoptosis, while the inhibition of miR-7 reversed the anti-proliferative pro-apoptotic effects of BVP. Pre-treatment with miR-7 mimics enhanced the BVP-mediated down-regulation of Bax/Bcl-2 in NSCLC cells, while pre-treatment with the miR-7 inhibitor blocked the BVP-mediated down-regulation of Bax/Bcl. Taken together, these results confirm that BVP effectively inhibits NSCLC proliferation and that miR-7, as a novel target, is likely involved in BVP-induced growth suppression and the apoptosis of NSCLC cells.

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1. Introduction

Lung cancer is the second most common cancer worldwide (Siegel *et al.* 2012). Among lung cancer cases, approximately 85% develop into non-small cell lung cancer (NSCLC). It is estimated that lung cancer accounts for 1.6 million new cases of cancer and 1.37 million cancer deaths annually (Furrukh *et al.* 2013). In the United States, 215020 new cases were expected and 161840 persons were projected to die from the disease in 2008 (Molina *et al.* 2008). In China, lung cancer has an incidence rate of 19.59% and approximately 600000 people in China are diagnosed with lung cancer annually (Wu *et al.* 2015). Surgery, radiation therapy, chemotherapy and immunotherapy are all available for treating the disease, based on the clinical stage. However, due to limitations in early diagnostic techniques and the sustainable development of multiple drug resistance in NSCLC, the 5-year survival rate remains less than 15% (Hotta *et al.* 2011; Hayashi *et al.* 2012). Therefore, it is necessary to identify effective and safe drugs to treat NSCLC.

A better understanding of the molecular pathogenesis in cancer progression is useful to discover therapeutic targets and develop novel anti-carcinoma medicines. MicroRNA (miRNA) is a class of endogenous small non-coding RNA molecules with lengths of ~21–25 bases, which are widely distributed in eukaryotes (McManus 2003). As regulators, miRNAs can bind with imperfect complementarity to the 3′-untranslated regions of target mRNAs, causing translational repression or target mRNA degradation (McManus

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2003). In carcinogenesis, a wide variety of miRNAs, such as miR-155 (Hou *et al.* 2016), miR-21 (Zhao *et al.* 2015b), miR-34a (Zhao *et al.* 2015c) and miR-7 (He *et al.* 2015), act as oncogenes or tumour suppressors that affect cancer cell proliferation. MiR-7 is a tumour regulator that participates in the progression of lung cancer. As previously reported, miR-7 deficiency significantly promoted the growth of A549 and H1299 cells, and miR-7 overexpression decreased the proliferation of human lung cancer cells (Xiong *et al.* 2014). Interestingly, miR-7 is also involved in the prevention of small cell lung cancer via the repression of MRP1/ABCC1 (Liu *et al.* 2015). Notably, several medicines (Ma *et al.* 2014; He *et al.* 2015), such as docetaxel and curcumin, have been confirmed to suppress tumour growth via the regulation of miR-7 expression. Therefore, it has been proposed that miR-7 plays a negative role in NSCLC growth and could serve as a potential therapeutic target for NSCLC.

In recent years, with the development of phytochemistry, a variety of effective and safe ingredients from plants have been separated, providing a vast and alternative repository for the development of new antitumour agents. Breviscapine (BVP) is a natural flavonoid primarily extracted from *Erigeron breviscapus* (Wang *et al.* 2015a). In recent decades, several studies (Qi *et al.* 2006; Jia *et al.* 2008; Yiming *et al.* 2008; Wang *et al.* 2013) have confirmed that BVP possesses various pharmacologic effects, such as anti-inflammatory, neuroprotective, renoprotective and cardiovascular protective properties. Notably, recent studies have demonstrated the potential antitumour activity of BVP. For example, in 2010, Wu *et al.* (2010) reported that BVP treatment induced HepG2 apoptosis via the regulation of several apoptosis-associated proteins, including Bcl-2, Bax and caspase-3. In 2012, Wei *et al.* (2012) concluded that BVP repressed the metabolic transformation of DMBA-induced carcinogenesis. However, the activity of BVP against NSCLC and the underlying mechanism remain elusive. Therefore, in this paper, we first investigate the anti-proliferative action of BVP in NSCLC and then study whether miR-7 is involved in BVP-induced growth suppression and apoptosis in NSCLC cells.

2. Materials and methods

2.1 *Cell lines*

A549 and NCL-H460 cells were obtained from the Institute of Biochemistry and Cell Biology of Chinese Academy of Science (Shanghai, China). The normal lung fibroblast cell line MRC-5 was obtained from the American Type Culture Collection (Manassas, VA, USA). This study was approved by the ethical committee of Zhejiang Cancer Hospital.

2.2 *Cell culture*

All cells were grown in DMEM supplemented with 10% foetal bovine serum at 37°C and placed in an environment containing 5% CO_2.

2.3 *MTT assay*

To examine the cytotoxicity of BVP (purity>98%; Shanghai Rong He Bioengineering Company, China), the cells (96-well plates at a density of 1×10^4 cells/well) were pre-treated with BVP (dissolved in 0.9% NaCl) and incubated for 24, 48 and 96 h. Four hours before the end of the study period, 20 μL of MTT (5 mg/L, Sigma-Aldrich, St. Louis, MO, USA) was added to each well. Finally, the supernatant was removed, and the residual crystals were dissolved in 100 μL of DMSO, followed by measurement at 570 nm using a microplate reader (ELx808; BioTek Instruments, Winooski, VT, USA). Dose–response curves were generated using GraphPad Prism (version 5.0, GraphPad Software Inc., La Jolla, CA), and the half maximal inhibitory concentration (IC 50) was calculated.

2.4 *Apoptosis assay*

A549 cells (5×10^5/well) pre-treated with BVP (0, 25, 50, and 100 μM) were plated onto 6-well plates. After cultivation for 48 h, the apoptotic rate was analysed using a commercial kit (BD Pharmingen, San Diego, CA, USA). The apoptotic cells were analysed using an FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

2.5 *RNA extraction*

Extraction of total RNA was performed according to the manufacturer's instructions (TRIzol reagent, Invitrogen, CA, USA). The RNA was quantitated and then stored at −80°C prior to RT-PCR analysis.

2.6 *Quantitative RT-PCR (qRT-PCR) for miRNA*

Approximately 10 ng of total RNA was converted to cDNA using the PrimerScript RT reagent kit (Takara, Dalian, China) with miR-7-specific and u6 primers (RiboBio, Guangzhou, China). When the reverse transcription was finished, qRT-PCR was then performed using the All-in-One^{TM} miRNA qRT-PCR Detection Kit

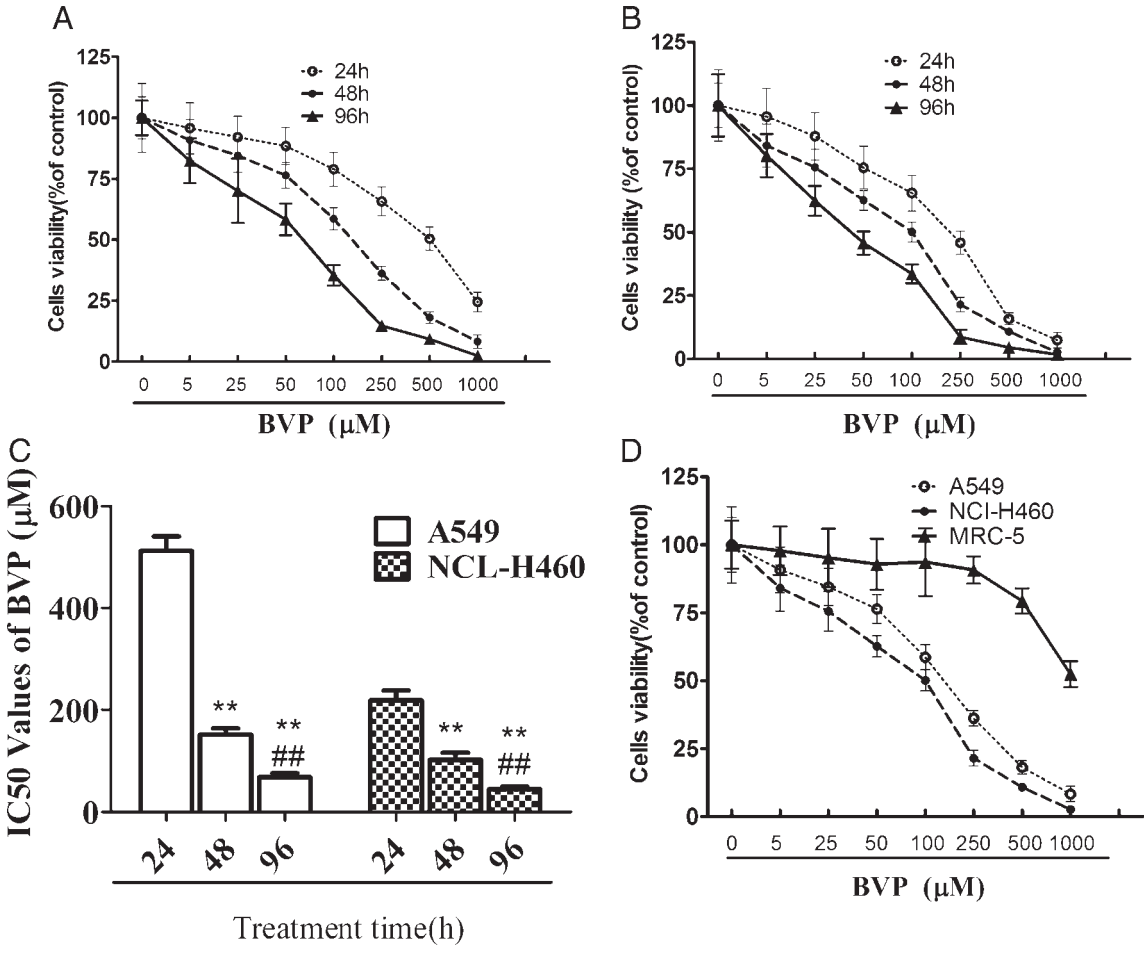

Figure 1. Inhibitory effects of BVP on growth of A549, NCL-H460 and MRC-5 cells as determined by MTT assay. (**A**) A549 was treated with BVP for 24, 48 and 96 h. (**B**) NCL-H460 cells were treated with BVP for 24, 48 and 96 h. (**C**) Increasing BVP treatment times resulted in a time-dependent reduction in IC50 values. (**D**) A549, NCL-H460 and MRC-5 (normal) cells were treated with BVP for 48 h. Cell viabilities were examined by MTT assay. Data are shown as averages with indicated standard deviations (n=3). $^{**}P<0.01$ compared with the 24 h group, and $^{\#\#}P<0.01$ compared with the 48 h group.

(GeneCopoeia, Rockville, MD, USA) on an Applied Biosystems 7500 RealTime PCR system (Applied Biosystems, White Plains, NY, USA). The U6 gene was used as a normalization control for all samples. The following primers were used: 5′-AGTCGCTAGCCACAAACCAG GAAGGGGAA-3′ and 5′-ATCGGAATTCAAAT GATAAAGCCTGAAGTC-3′, for miR-7, and 5′-CGCT TCGGCAGCACATATAC-3′ and 5′ -TTCACGAA TTTGCGTGTCAT-3′ for U6.

2.7 *Western blot analysis*

A549 cells were seeded in culture flasks and incubated with BVP for 48 h. Subsequently, the intracellular protein was extracted. Western blot analysis was performed according to a previously described method (Wu *et al.* 2015). The following primary antibodies were used: anti-Bcl-2 (1:1500 dilutions) and anti-Bax (1:1500 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA). All data were normalized to the β-actin loading controls.

2.8 *Plasmids and transfection*

Human miR-7 mimics (dsRNA oligonucleotides), miR-7 inhibitor (single-stranded chemically modified oligonucleotides), negative control mimic (miR-NC) and negative control inhibitor (Ctrl inhibitor) were purchased from RiboBio (Guangzhou RiboBio Co., Ltd) and were transfected into the cells using Lipofectamine®2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's

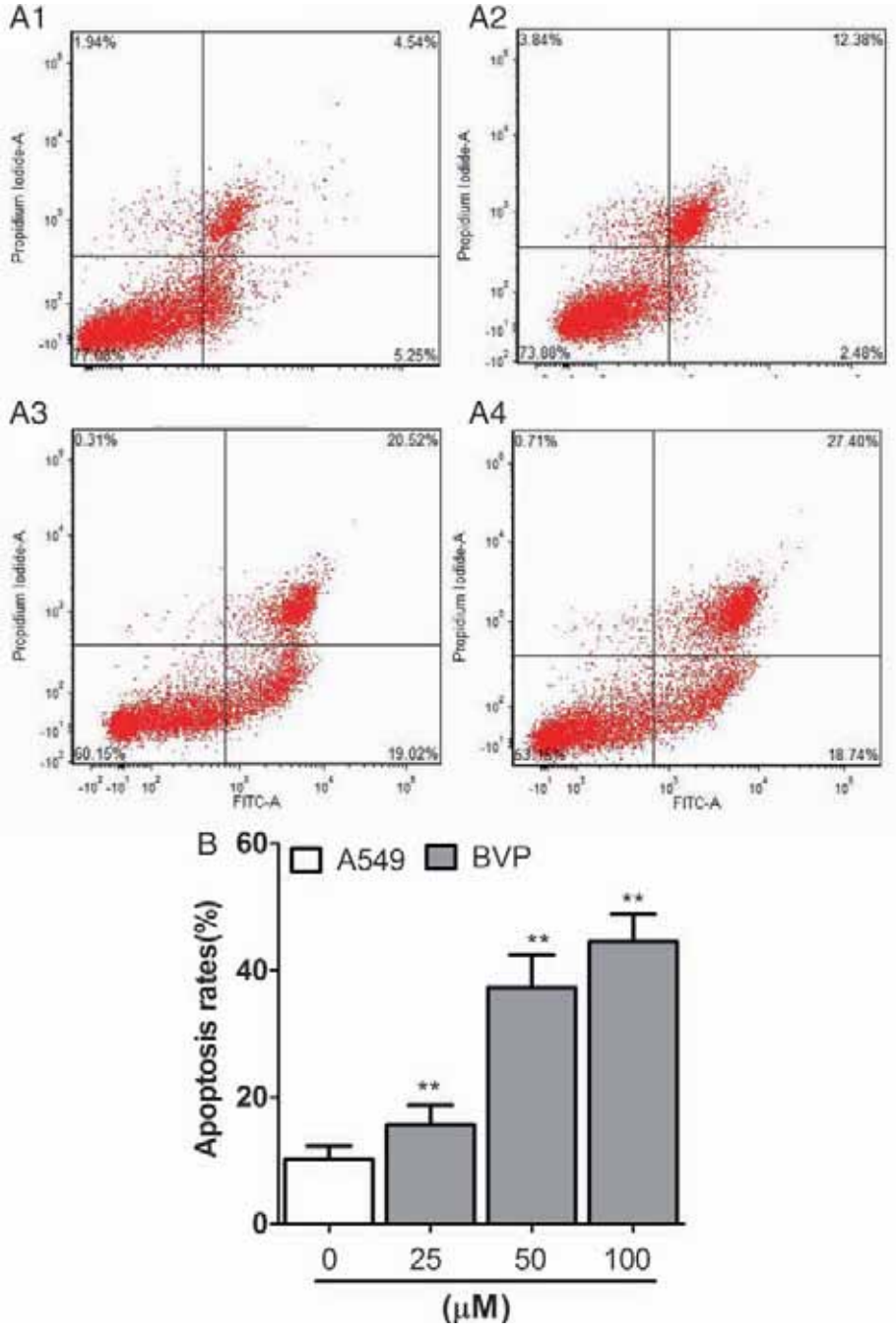

Figure 2. BVP increased apoptosis in A549 cells. The cells were treated with BVP at 0 μM (**A1**), 25 μM (**A2**), 50 μM (**A3**) and 100 μM (**A4**) concentrations for 48 h. (**B**) Histogram presenting the total percentages of apoptotic cells. Data are shown as averages with indicated standard deviations (n=3). $^{**}P<0.01$ compared with the group without BVP treatment.

instructions. Total RNA was extracted, and the miR-7 levels were measured at 24 h after transfection. Total cell protein was extracted at 72 h after transfection. Moreover, the cells were treated with BVP at 24 h after transfection, and then (48 h later) the proliferation levels and Bax/Bcl-2 expression were measured by the methods mentioned above. Moreover, A549 cells were transfected with the BCL-2 3′UTR plasmid using the Renilla luciferase pRL-TK vector (Promega USA), and a luminescence assay was performed after 24 h using the Dual-Luciferase Reporter Assay System (Promega USA) according to the manufacturer's instructions.

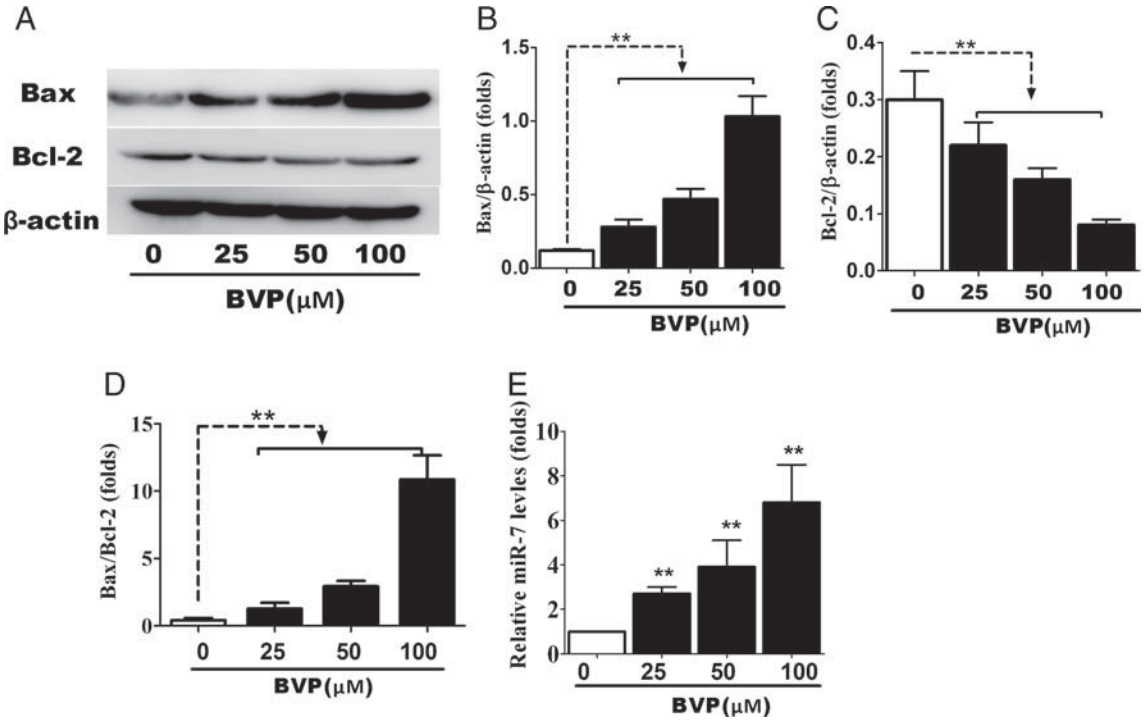

Figure 3. BVP increased the ratio of Bax/Bcl-2 and up-regulated miR-7 level in A549 cells. (**A**) Histogram showing the protein expression levels of Bax and Bcl-2. (**B**) Treatment with BVP significantly increased Bax expression. (**C**) Treatment with BVP significantly reduced Bcl-2 expression. (**D**) Treatment with BVP significantly increased the Bax/Bcl-2 ratio. (**E**) miR-7 expression by BVP. Data are shown as averages with indicated standard deviations (n=3). $^{**}P<0.01$ compared with the group without BVP treatment.

2.9 *Statistical analysis*

All experiments were performed in triplicate. The data are expressed as the means ± SD. ANOVA analysis based on the SPSS 16.0 software was used for the multigroup comparison, while Student's *t*-test was applied for comparisons between two groups. P-values <0.05 were considered to indicate statistically significant differences.

3. Results

3.1 *Breviscapine inhibited A549 and NCL-H460 cell proliferation*

A549 and NCL-H460 cells were treated with increasing concentrations of BVP, and the cell survival rates were assayed by MTT after 24, 48 and 96 h of treatment. As shown in figure 1, BVP shows dose-dependent (figure 1A–B) and time-dependent (figure 1C) inhibitory effects on the growth of A549 and NCL-H460 cells. Moreover, we tested if BVP had cytotoxicity to normal cells. The results in figure 1D show that MRC-5 (human lung fibroblast cells) is insensitive to BVP at a dose below 100 μM. Therefore, these data indicate BVP could inhibit the survival of NSCLC without significant cytotoxicity to normal cells.

3.2 *Breviscapine induced apoptosis in A549*

To further reveal the cytotoxicity of BVP, the effects of BVP on the apoptosis of A549 cells were examined. As shown in figure 2, BVP markedly increased the proportion of apoptotic cells in A549 cells ($P<0.01$).

3.3 *Breviscapine regulated the expression of Bax and Bcl-2 in A549 cells*

The results shown in figure 3A indicate that expression of pro-apoptotic protein Bax increased in response to BVP (figure 3B, $P<0.01$), whereas Bcl-2 was decreased by BVP treatment (figure 3C, $P<0.01$). Moreover, BVP caused an increase in the ratio of Bax/Bcl-2 (figure 3D, $P<0.01$).

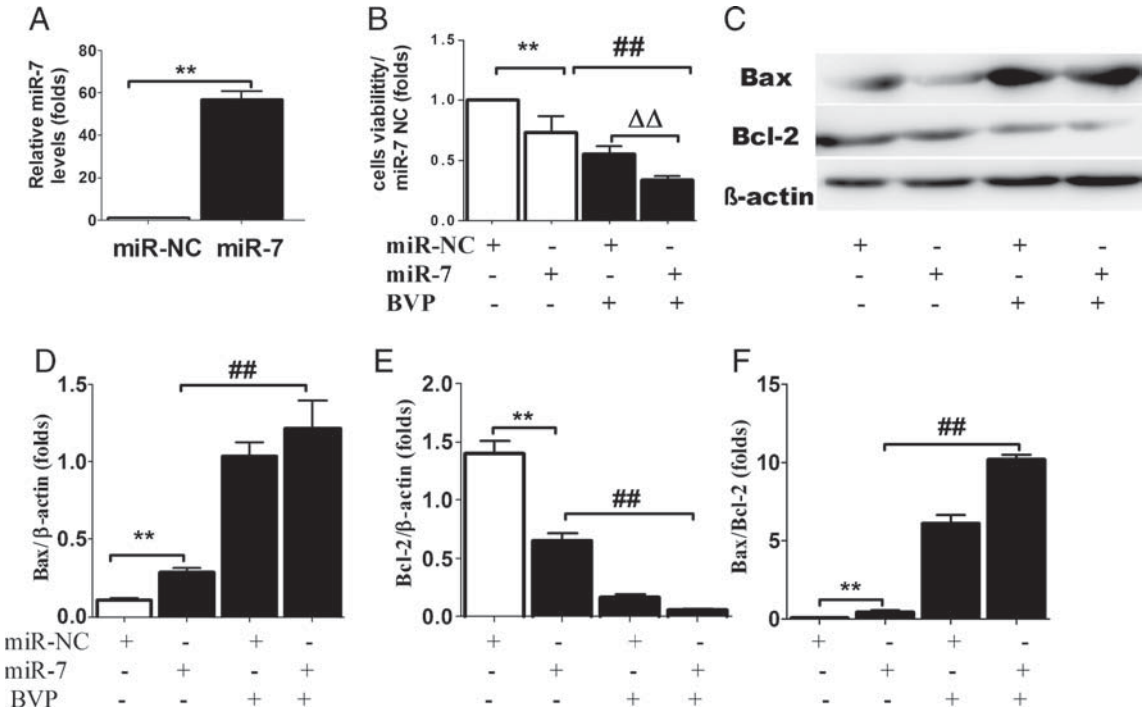

Figure 4. Overexpression of miR-7 enhanced A549 cell sensitivity to BVP (100 μM). (**A**) miR-7 expression of A549 cells following transfection with miR-7 or miR-NC. (**B**) Cell viability was evaluated by MTT after treatment with BVP for 48 h. (**C**) Histogram showing the protein expression levels of Bax and Bcl-2. (**D**) Bax expression was increased in A549 cells transfected with miR-7. (**E**) Bcl-2 expression was reduced in A549 cells transfected with miR-7. (**F**) The ratio of Bax/Bcl-2. Data are shown as averages with standard deviations (n=3). $^{**}P<0.01$ compared with miR-NC group without BVP treatment, $^{\#\#}P<0.01$ compared with miR-7 group without BVP treatment, and $^{\Delta\Delta}P<0.01$ compared with miR-7 group treated with BVP. miR-NC, microRNA-negative control mimic.

3.4 *Breviscapine up-regulated the expression of miR-7 in A549 cells*

Previous studies have demonstrated that miR-7 plays a negative role in NSCLC cell proliferation. As shown in figure 3E, qRT-PCR assays have confirmed that miR-7 expression levels are enhanced after treatment with BVP ($P<0.01$).

3.5 *Over-expression of miR-7 enhanced cytotoxicity of breviscapine to A549 cells*

As previously discussed (Xiong *et al.* 2014), miR-7 inhibits A549 cell growth by targeting Bcl-2. To further study the role of miR-7 in BVP-induced growth inhibitory effects and apoptosis in NSCLC cells, we transiently transfected A549 cells with miR-7 mimics and the negative control mimic (miR-NC). As shown in figure 4A, miR-7 overexpression in A549 cells transfected with miR-7 mimics results in the growth inhibition and increased apoptosis of A549 cells (figure 4B, $P<0.01$; supplementary figure 1, $P<0.01$). MiR-7 overexpression in A549 cells also increased the levels of Bax expression (figure 4C–D, $P<0.01$), reduced the levels of Bcl-2 (figure 4E, $P<0.01$), and increased the Bax/Bcl-2 ratio (figure 4F, $P<0.01$). Importantly, we also observed that pre-treatment with miR-7 mimics not only enhances BVP-induced growth inhibition and apoptosis in A549 cells (figure 4B, $P<0.01$; supplementary figure 1, $P<0.01$, Supporting information) but also reinforces its ability to restore the normalized expression of Bax and Bcl-2 (figure 4D–F, $P<0.01$).

3.6 *Inhibition of miR-7 reduced cytotoxicity of breviscapine to A549 cells*

As shown figure 5A, miR-7 was successfully knocked down in A549 cells (figure 5A, $P<0.01$). Transfection with miR-7 inhibitor strongly increased the luciferase activity of the WT BCL-2 3′UTR (figure 5B, $P<0.01$) but not the activity of the MT BCL-2 3′UTR. These results indicate that miR-7 suppresses BCL-2 expression by targeting the 3′-UTR of BCL-2 mRNA. Further analysis revealed that the decrease of miR-7 increased the survival and decreased the apoptosis of A549 (figure 5C, $P<0.01$; supplementary figure 2, $P<0.01$, Supporting information). Knockdown of miR-7 by miR-7

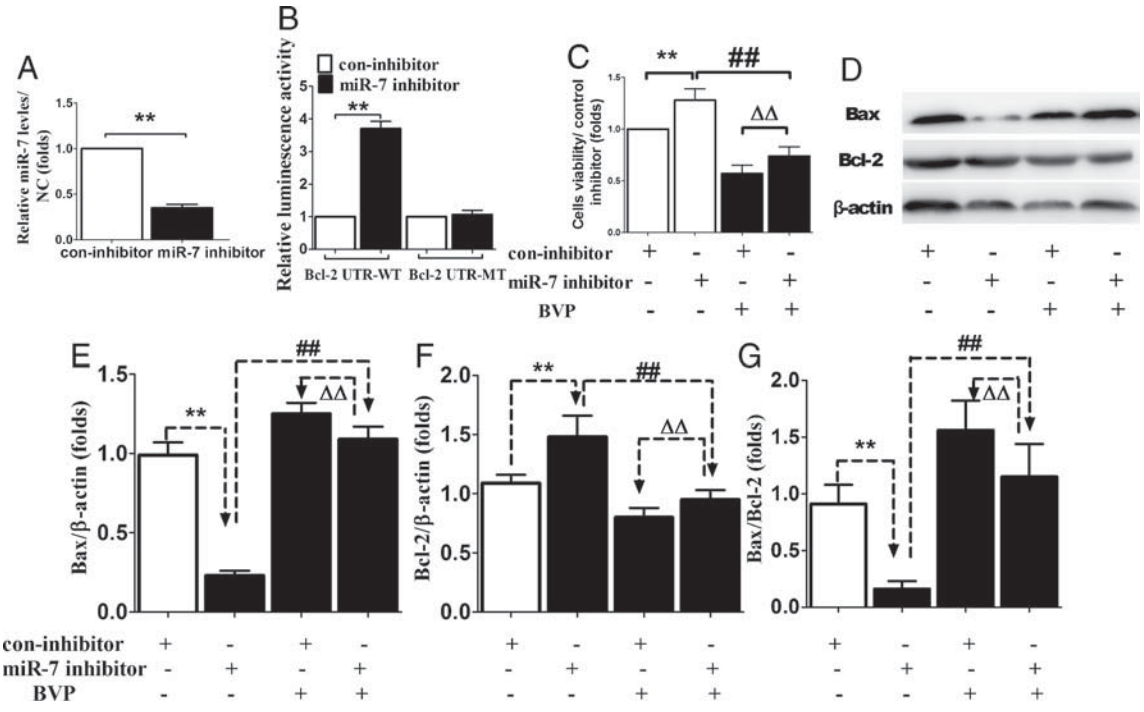

Figure 5. Knockdown of miR-7 reduced A549 cell sensitivity to BVP (100 μM). (**A**) miR-7 expression of A549 cells, following transfection with miR-7 inhibitor or Con-inhibitor. (**B**) A luciferase assay was conducted after co-transfection of Con-inhibitor or miR-7 inhibitor with reporter plasmids along with an endogenous control Renilla luciferase pRL-TK vector in A549 cells. (**C**) Cell viability was evaluated by MTT, after treatment with BVP for 48 h. (**D**) Histogram showing the protein expression levels of Bax and Bcl-2. (**E**) Bax expression was reduced in A549 cells transfected with miR-7 inhibitor. (**F**) Bcl-2 expression was increased in A549 cells transfected with miR-7 inhibitor. (**G**) The ratio of Bax/Bcl-2. Data are shown as averages with standard deviations (n=3). $^{**}P<0.01$ compared with group of Con-inhibitor without treatment of BVP, $^{\#\#}P<0.01$ compared with group of miR-7 inhibitor without treatment of BVP, and $^{\triangle\triangle}P<0.01$ compared with group of Con-inhibitor with treatment of BVP. Control inhibitor, negative control inhibitor.

inhibitor also reduced the level of Bax (figure 5D–E, $P<0.01$), increased the level of Bcl-2 (figure 5F, $P<0.01$) and decreased the Bax/Bcl-2 ratio (figure 5G, $P<0.01$). Following treatment with BVP, we found that the cells pre-treated with miR-7 inhibitor exhibited higher survival and lower apoptosis compared with those cells pre-treated with the control inhibitor (figure 5C, $P<0.01$; supplementary figure 2, $P<0.01$). As expected, the level of Bax/Bcl-2 in cells pre-treated with miR-7 inhibitor was lower than in those cells pre-treated with control inhibitor (figure 5D–G, $P<0.01$). Therefore, these findings indicate that the inhibition of miR-7 could reduce A549 sensitivity in response to BVP.

4. Discussion

A few recent studies have focused on the antitumour activity of BVP. For example, Wu *et al.* (2010) reported that BVP reduces the viability and enhances the apoptosis of HepG2 cells in a dose-dependent manner via the regulation of several apoptosis-associated proteins, and Wei *et al.* (2012) confirmed that BVP represses the metabolic transformation of DMBA-induced carcinogenesis. Since then, no further details have been disclosed. In the present study, we first showed that BVP reduced the proliferation of A549 and NCL-H460 cells in dose-dependent and time-dependent manners. Moreover, the increased apoptosis of NSCLC in response to BVP may be caused by the normalization of the expression of Bax and Bcl-2. These findings suggest that BVP is an effective drug for preventing NSCLC.

MiR-7 is a tumour suppressor widely distributed in solid tumours and is usually down-regulated. Previous studies have shown that miR-7 expression was significantly higher in high-grade ovarian cancer than it was in low-grade (Swiercz *et al.* 2015) and that levels of miR-7-5p in glioblastoma microvessels were significantly reduced compared with levels in normal brain capillaries (Liu *et al.* 2014b). Additionally, the antitumour activity of miR-7 has also been confirmed by the fact that overexpression of miR-7 in tumour cell lines inhibited growth and survival, whereas the knockdown of miR-7 produced the opposite phenotype (Ma *et al.* 2013; Xiong *et al.* 2014). Similarly, a recent paper revealed that systemic miRNA-7 delivery inhibits tumour angiogenesis and growth in murine glioblastoma (Babae

et al. 2014). In NSCLC, pro-apoptotic mechanisms of miR-7 have been widely studied. Several molecules (EGFR, IRS, RAF1, paired box 6 and PAK1) and signalling pathways (PI3K/AKT, Raf/MEK/ERK) have been reported to be involved in the tumour inhibitory activity of miR-7 (Reddy *et al.* 2008; Liu *et al.* 2014a, b; Luo *et al.* 2015; Zhao *et al.* 2015a; Zhou *et al.* 2014). Interestingly, Bcl-2 is down-regulated by miR-7 at both transcriptional and translational levels through direct 3′ UTR interactions (Xiong *et al.* 2014). Therefore, miR-7-mediated growth suppression and apoptosis of A549 cells may be via the modulation of Bcl-2. These findings imply that miR-7 affects NSCLC progression and could serve as a potential diagnostic marker and therapeutic target.

Currently, the pro-apoptotic activity of BVP has been well established in several cell models, such as cardiomyocytes, neurons and the hepatocellular carcinoma cell line. In 2004, the first report (Li *et al.* 2004) showed that BVP treatment at a dose of 5–20 mg/mL significantly reduced LDH leakage and intracellular free $Ca2^{+}$ levels and promoted apoptosis in cardiomyocytes. Similarly, Yiming *et al.* (2008) further reported BVP attenuated neuroapoptosis and regulated the apoptotic protein expression after transient focal cerebral ischaemia. Furthermore, mitogen-activated protein kinase (MAPK) and PI3K/Akt/eNOS signalling pathways have been confirmed to be involved in the apoptosis induction of BVP (Wang *et al.* 2015a, b, c). In this paper, we first reported that BVP remarkably up-regulated miR-7 expression in A549 cells. In addition, to further study the role of miR-7 in the growth inhibition effect of BVP-and apoptosis in NSCLC cells, we transiently transfected A549 with miR-7 mimics and miR-7 inhibitor. These results indicate that the antineoplastic activity of BVP was affected by endogenous miR-7 expression in A549 cells. Thus, the overexpression of miR-7 enhanced NSCLC cells reactivity to the BVP, while knockdown of miR-7 neutralized the cytotoxicity of BVP.

In summary, our data first confirm that BVP is effective in preventing NSCLC cell proliferation. Moreover, BVP-induced growth inhibition and apoptosis of A549 cells via the up-regulation of miR-7.

Acknowledgements

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