

C-Phycocyanin Suppresses Cell Proliferation and Promotes Apoptosis by Regulating the AMPK Pathway in NCL-H292 Non-Small Cell Lung Cancer Cells

(AMPK / C-phycocyanin / mTOR / NSCLC / NBR2)

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Abstract. Non-small cell lung cancer (NSCLC) results in high mortality and has gained increasing attention. C-Phycocyanin (C-PC) has been identified as a potential therapeutic inhibitor for NSCLC, but its underlying mechanism remains obscure. The gene expression of the long noncoding RNA neighbour of BRCA1 RNA 2 (NBR2) in NSCLC cells was evaluated by quantitative reverse transcription-PCR. The cell capacity for proliferation and migration was examined by EdU and wound-healing assays. Furthermore, the viability and apoptosis of cells was measured with CCK-8 and annexin V/PI, respectively. Next, the protein level of activation of adenosine monophosphate-activated protein kinase and the rapamycin kinase (mTOR) signalling pathway-associated molecules was evaluated by western blotting. H292 cells were pre-treated with C-PC or transfected with plasmids encoding *NBR2* or the sh*NBR2* plasmid, to over-express or knock down *NBR2* expression, respectively. *NBR2* expression was robustly down-regulated in NSCLC cell lines compared with a normal cell line (BEAS-2B). *NBR2* over-expression inhibited migration and promoted apoptosis of H292 cells. Treatment of H292 cells with C-PC enhanced *NBR2* levels in a dose- and time-dependent manner. Down-regulation of *NBR2* in H292 cells inhibited the activity of C-PC on cell proliferation, viability and clone

formation. Further mechanistic investigation showed that the down-regulation of *NBR2* abolished the modulatory effects of C-PC on the AMPK/mTOR signalling pathway. In conclusion, C-PC inhibits H292 cell growth by enhancing the *NBR2*/AMPK signalling pathway.

Introduction

Lung cancer (LC) remains the most common cancer in the world, affecting up to 1.4 million persons every year (Brozos-Vazquez et al., 2020). Non-small cell lung cancer (NSCLC) accounts for about 80 % of LC (Liu et al., 2019). Although chemotherapy, surgery, radiotherapy and molecular targeted drugs, such as gefitinib and erlotinib, have inhibitory effects on the development of NSCLC, the overall 5-year survival rate of advanced patients with NSCLC is still only 20 % (Miller et al., 2019; Zhang et al., 2020a), and traditional treatments have severe side effects and increase treatment costs (Schutt et al., 2010). Thus, it is urgent to develop new drugs for treating patients with NSCLC and to identify the exact molecular mechanisms involved in cancer progression.

Long noncoding RNAs (lncRNAs) are more than 200 nucleotides in length and have been attributed with diverse molecular functions, ranging from negative regulation of miRNAs by serving as miRNA ‘sponges’ to marking mRNAs for degradation and transcriptional regulation of genes (Ulitsky and Bartel, 2013). Previous studies have revealed that lncRNAs can act as tumour suppressors or oncogenes in malignant tumours, demonstrating that abnormal expression of lncRNA is associated with tumour development in various cancers (Pandya et al., 2020; Zhang et al., 2020b). Schmidt et al. (2014) indicated that MALAT-1 lncRNA influences expression of prognostic markers in NSCLC, which could improve the risk prediction in resectable patients with NSCLC. In addition, another lncRNA molecule (ABHD11-AS1) has been observed to promote NSCLC proliferation and the Warburg effect (Xue et al., 2020). Furthermore,

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Abbreviations: AMPK – adenosine monophosphate-activated protein kinase, C-PC – C-phycocyanin, lncRNA – long noncoding RNA, mTOR – mechanistic target of rapamycin kinase, *NBR2* – neighbour of BRCA1 RNA 2, NSCLC – non-small cell lung cancer, qRT-PCR – quantitative reverse transcription-PCR.

lncRNA00673 facilitates cell proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) of NSCLC through sponging miR-150-5p (Lu et al., 2017). However, the function of lncRNA neighbour of BRCA1 RNA 2 (NBR2) in the progression of NSCLC remains unclear.

The sequence locus of NBR2 is adjacent to tumour suppressor gene *BRCA1* (Xu et al., 1997). NBR2 has been reported to be decreased in tissue specimens from NSCLC patients, and these NSCLC patients with low expression of NBR2 show a poor prognosis. NBR2 over-expression suppresses the viability and migration of NSCLC cells (Gao et al., 2019). However, the functional target of NBR2 and the underlying mechanisms in the regulation of NSCLC remain obscure. Furthermore, NBR2 may also interact with intracellular energy sensor adenosine monophosphate-activated protein kinase (AMPK), which modulates the activation of AMPK. Depletion of NBR2 suppressed deprivation-induced activation of the AMPK signalling pathway, including anabolic processes and catabolism of adenosine triphosphate (ATP) production (Liu et al., 2016). This suggests that NBR2 can enhance AMPK expression, which may be involved in the development of NSCLC.

C-Phycocyanin (phycocyanin, C-PC) is a natural antineoplastic marine protein derived from *Spirulina platensis* with multiple biological functions (Mitra et al., 2015), including anti-inflammatory (Mitra et al., 2015), antioxidant (Sonani et al., 2017), immunomodulatory (Cian et al., 2012), and antibacterial activities (Basha et al., 2008; Abd El-Baky and El-Baroty, 2012; Jiang et al., 2018). Furthermore, many tumours, including NSCLC, can be treated with C-PC. The anticancer activities of C-PC are well defined (Wang et al., 2007; Hao et al., 2019), but the exact function and molecular mechanism that C-PC plays in NSCLC remain poorly understood.

To investigate the molecular mechanism of C-PC activity in NSCLC, we performed an *in vitro* assay in which H292 cells were treated with C-PC. This study demonstrated that C-PC inhibited tumour proliferation and migration by promoting NBR2 expression, which activated AMPK and suppressed the mechanistic target of rapamycin kinase (mTOR) signalling pathway that ultimately triggered cancer cell death.

Material and Methods

Materials, cell lines and transfection

Phosphate-buffered solution (PBS) was used to prepare phycocyanin treatments (C-PC, MedChemExpress, Monmouth Junction, NJ, USA). Human NSCLC H1299 (ATCC® CRL-5803), H292 (ATCC® CRL-1848), PC-9 (ATCC® HTB-177), and Beas-2B (ATCC® CRL-9609) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA). Cell lines were cultured in DMEM media including 10 % foetal bovine serum (FBS) (Gibco, Australia) and a 37 °C hu-

midified incubator with 5 % CO₂. Cells were passaged every two days.

For cell transfection experiments, vectors including empty control (pcDNA3.1, pc-NC) and lncRNA NBR2 plasmid (pc-NBR2) were transfected into H292 cells using Lipofectamine™ 2000 (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. Briefly, cells were seeded in 6-well plates and transfected at 90% confluence. Initially, 500 µl of Opti-MEM medium was added to two 1.5 ml centrifuge tubes each. Then, Lipofectamine™ 2000 and NBR2 expression plasmids were individually added to the two tubes and mixed with OPTI-MEM medium. After incubation for 5 min at room temperature, the reagents were mixed and incubated for an additional 20 min. The transfection mixture was added to wells containing 1 ml OPTI-MEM medium and gently shaken. After 7 h incubation at 37 °C, the OPTI-MEM medium was replaced by normal cell culture medium and the plasmid-transfected cells were maintained in culture for the duration of the experiments (Deng et al., 2018).

Cell counting kit-8 assay

A total of 5000 H292 cells per well were seeded in 96-well plates (Corning Inc., Corning, NY, USA) and were then subjected to different treatments for the indicated times. The OD value of 450 nm was detected after incubation with 10 µM of the cell counting kit-8 (CCK-8) (Beyotime, Shanghai, China) solution for another 2 h.

Colony formation assay

A total of 200 cells were seeded into each well of a 6-well plate. After the cells were incubated for 14 days with the different treatments for the indicated times, a 0.05 % (w/v) crystal violet solution was added to each well of the 6-well plates and then incubated for an additional 1 hour at room temperature. The number of colonies of each well could be observed by light-field microscopy. Three replicates were performed for each experiment.

EdU assay

Each well of any 6-well plate was seeded with 1×10^5 H292 cells. The cells were then subjected to the indicated treatments, and then co-cultured with 10 µM EdU for 2 h. The cells were then fixed in 4 % paraformaldehyde. Finally, these cells were stained with Alexa Fluor 488 probes and DAPI (nuclear stain; Sigma, USA). The number of EdU+ cells in five random fields was calculated with an IX71 microscope and DP80 camera (200×, Olympus GmbH, Hamburg, Germany).

Quantitative RT-PCR (qRT-PCR)

According to the manufacturer's protocols, TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) was used to separate total RNA from each group of cells. Briefly, 1 ml of TRIzol was added to each centrifuge tube containing 1×10^7 cells. After 5 min of in-

cubation, 0.3 ml of chloroform was added to each tube. All tubes were shaken vigorously by hand for 15 s and centrifuged for 15 min at $12,000 \times g$ at 4°C . The upper aqueous phase was transferred to a clean 1.5 ml centrifuge tube and mixed with 0.5 ml isopropyl alcohol to precipitate RNA. After centrifugation for 10 min at $12,000 \times g$ at 4°C , the RNA pellet was washed with 1 ml 75% ethanol. Then, the RNA pellet was dissolved with 30 μl RNase-free water for further experiments. The cDNA was obtained by reverse transcription using a reverse transcription kit (TaKaRa, Tokyo, Japan) from 500 ng total RNA (Rio et al., 2010; Yang et al., 2022). The qRT-PCR assay was performed in a Bio-Rad CFX96 system using SYBR Master Mix (TaKaRa, Tokyo, Japan). The gene expression ratio of *NBR2* to *GAPDH* was calculated as relative fold changes against control values using the $2^{-\Delta\Delta\text{Ct}}$ method (Rao et al., 2013). The specific primers used in this study were as follows: *NBR2* (Forward: 5'-GGAGGTCTCCAGTTTCGGTA-3'; reverse: 5'-TTGATGTGTGCTTCCTGGG-3'). *GAPDH* (forward: 5'-CCATGGGAAGGTGAAGGTC-3'; reverse: 5'-GAAGGGTCATTGATGGCAAC-3').

Western blotting

According to manufacturer's protocols, RIPA lysis buffer (Beyotime, Shanghai, China) was used to separate total proteins from each group of cells. Cell lysates were centrifuged at $12,000 \times g$ for 10 min at 4°C and the supernatant was transferred to new 1.5 ml centrifuge tubes. The BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to assay the protein concentration. After that, these protein samples were electrophoresed by 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted on polyvinylidene fluoride membranes. The membranes were immersed in TBST (TBS + 0.5 % Tween 20) containing 5 % non-fat dry milk (Fdbio, Shanghai, China) for 1 h, and then primary antibodies were added to bind to the proteins under study. These primary antibodies were anti-phospho-acetyl-CoA carboxylase (Ser79) antibody (CST, #3661S, 1 : 1000, USA), acetyl-CoA carboxylase antibody (CST, 3662S, 1 : 1000, USA), phospho-AMPK α (Thr172) (40H9) rabbit mAb (CST, 2535S, 1 : 1000, USA), and AMPK α (D63G4) Rabbit mAb (CST, 5832S, 1 : 1000, USA). Following incubation, the membranes were washed five times for 5 min in TBST and incubated for 1 h in secondary antibody (1 : 1000 dilution in TBST containing 5 % bovine serum albumin). Following five washes of 5 min each in TBST, the membranes were visualized with chemiluminescence solution (Fdbio-Dura ECL kit, Hanzhou, China) at room temperature using the ChemiDoc/XPS+ gel imaging system (Bio-Rad Laboratories, Hercules, CA).

Annexin V-FITC/PI analysis

To compare the effects on cells under different indicated treatments, cells were harvested and stained with the Annexin V-FITC-PI test kit (Solarbio, Beijing, China). A FACS flow cytometer (CytoflexLX, Beckman, USA)

was used to analyse the results. Three replicates were established for each experiment (Yang et al., 2021).

Wound-healing assay

For each type of experimental conditions, cells were grown in 6-well culture plates. Once the cells of each well reached a confluence of 90 %, a 200 μl pipette tip was used to produce a scratch on the cell layer. The cells were washed with PBS and cultured in low-serum DMEM medium. A DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) was used to observe the wounds and photographed at multiple time points. ImageJ software (US National Institutes of Health, Bethesda, MD, USA) was used to analyse the wound areas.

Statistical analysis

All experimental data were analysed by the SPSS software (version 20.0, IBM, USA). The mean \pm standard deviation (mean \pm SD) was used to present all data. To compare two groups of experimental data, the Student's *t*-test method was used. Comparing multiple groups of experimental data, one-way analysis of variance was used. P values less than 0.05 were considered statistically significant.

Results

NBR2 was down-regulated in cancer cell lines, and its over-expression inhibited migration and promoted apoptosis of H292 cells

The gene expression of *NBR2* was down-regulated in H1299, H292, H1650 and PC-9 cells, and especially in H292 cells, compared to BEAS-2B cells ($P < 0.01$, Fig. 1A). Therefore, H292 cells were chosen for further experiments.

To investigate the role of *NBR2* in H292 cells, *NBR2* was over-expressed by plasmid transfection. *NBR2* gene levels were up-regulated more than 15-fold in the pc-*NBR2* group compared to the negative control group ($P < 0.05$, Fig. 1B), which revealed that effective transfection had been achieved. Functionally, *NBR2* over-expression inhibited migration of H292 cells *in vitro* (Fig. 1C and D). Furthermore, *NBR2* over-expression reduced the cell viability (Fig. 1E) and promoted H292 apoptosis (4.52 ± 0.92 % in the pc-NC group vs 22.31 ± 2.13 % in the pc-*NBR2* group, $P < 0.01$) (Fig. 1F and G). These results suggested that *NBR2* over-expression could inhibit migration and induce apoptosis in H292 cells.

NBR2 was up-regulated by C-PC in a time- and concentration-dependent manner in H292 cells

Studies demonstrating AMPK activation by C-PC (Ren et al., 2018) and *NBR2* (Yang et al., 2020) have raised interest in the potential relationship between C-PC and *NBR2* in H292 cells. The results of RT-qPCR revealed that *NBR2* gene levels were significantly ele-

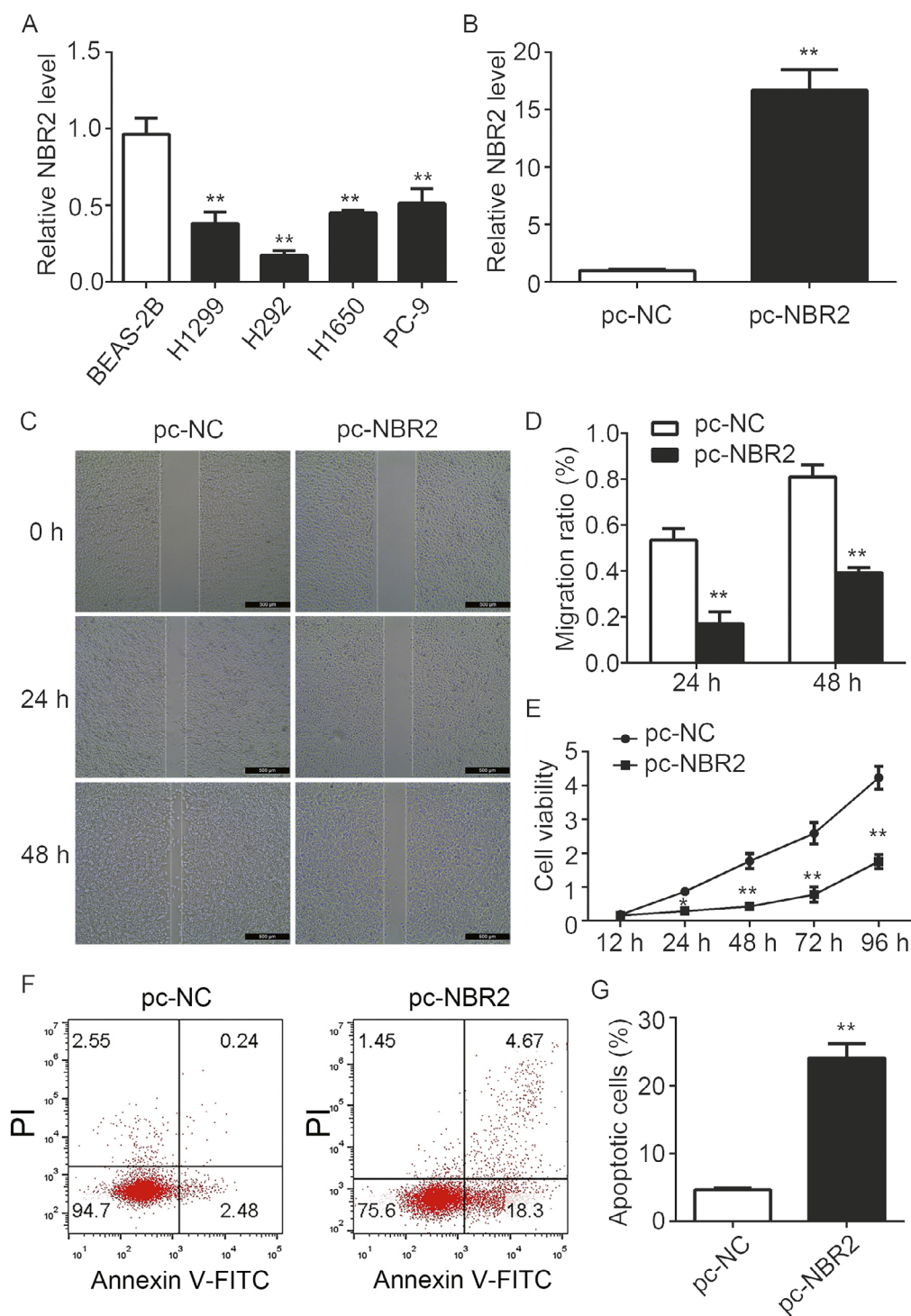


Fig. 1. Over-expression of NBR2 inhibited cell migration and induced apoptosis of H292 cells. **(A)** qRT-PCR detection of NBR2 down-regulation in NSCLC lines (H1299, H292, H1650 and PC-9) compared to BEAS-2B cell lines. **(B)** NBR2 was up-regulated after treatment with pc-NBR2 (pcDNA-NBR2) compared to the empty vector pc-NC. **(C, D)** Wound-healing analysis was used to evaluate H292 cell migration. **(E)** CCK-8 assay showing the cell viability after transfection with the empty vector or pc-NBR2. **(F, G)** Flow cytometry analysis of apoptotic cells. Data are shown as mean \pm SD, * $P < 0.05$; ** $P < 0.01$. Three replicates were performed for each experiment.

vated by C-PC at 10 μ M after 12 h. The *NBR2* gene levels were further up-regulated over a 48-h period (Fig. 2A). After transfection of shNBR2 into H292 cells, the

NBR2 gene levels decreased either in the presence or absence of C-PC ($P < 0.01$, Fig. 2B). Thus, C-PC influenced H292 cells.

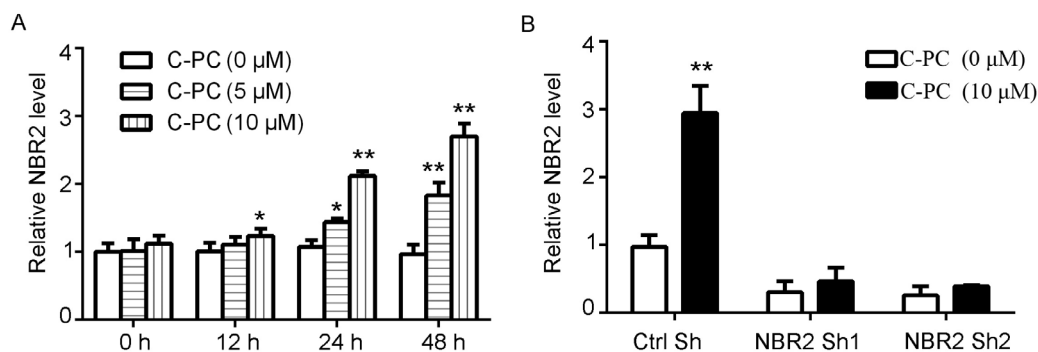


Fig. 2. C-PC treatment increases the gene expression of *NBR2*. (A) RT-qPCR showing the changes in gene expression of *NBR2* in H292 cells treated with 0, 5, 10 μM C-PC for 12, 24 and 48 h using two primer sets. (B) RT-qPCR histogram showing the efficiency of *NBR2* shRNA-mediated knock-down in H292 cells. Data are shown as mean \pm SD, * $P < 0.05$; ** $P < 0.01$. Three replicates were performed for each experiment.

Knock-down of *NBR2* attenuated the inhibitory effects of C-PC in H292 cells

To further examine whether the inhibitory effects of C-PC were dependent on *NBR2*, EdU staining, colony formation and wound-healing assays were carried out. The results of EdU assay demonstrated that C-PC could suppress proliferation of H292 cells (Fig. 3A and B, $P < 0.05$). However, *NBR2* depletion could reverse the inhibitory effect of C-PC (Fig. 3A and B, $P < 0.01$). Furthermore, sh*NBR2* could reverse the C-PC inhibitory effect on colony formation in H292 cells (Fig. 3C and D, $P < 0.05$). We also found that sh*NBR2* attenuated the effect of C-PC on the inhibition of H292 cell migration (Fig. 3E and F, $P < 0.01$).

NBR2 knock-down influenced C-PC activity on AMPK/mTOR signalling

Finally, the expression of p-AMPK, p-ACC, p-S6K, and p-S6 was examined in H292 cells treated with C-PC. C-PC not only clearly declined phosphorylation of p-AMPK and p-ACC (Fig. 4A and C), but also reduced the protein levels of total S6K. In contrast, the C-PC treatment up-regulated phosphorylation of p-S6K (Fig. 4B and C). The depletion of *NBR2* abolished the C-PC down-regulated p-AMPK and p-ACC, but the expression of p-S6K and p-S6 were not affected by the exposure to C-PC (Fig. 4C). These findings indicated that *NBR2* was a bridging gene between C-PC and the AMPK/mTOR signalling pathway.

Discussion

Lung cancer accounts for almost one-third of all cancer-related deaths. NSCLC is the leading subtype of lung cancer and accounts for around 80 % of all cancers (Nussinov et al., 2021). Distant metastasis and rapid proliferation are the main causes of mortality in patients with NSCLC (Hamouri et al., 2022). Despite the suc-

cessful application of radiation and chemotherapy for the treatment of NSCLC since the 1990s, accumulating evidence has shown that NSCLC cells could acquire resistance to therapy agents and the additional dose results in unaffordable toxicity (Huang et al., 2017). It is necessary to identify new agents to inhibit the proliferation and metastasis abilities of NSCLC cells and to reveal the molecular mechanisms. C-PC is a natural substance having anti-cancer activity, which is obtained from aquatic species *Spirulina platensis* (Hao et al., 2019; Ji et al., 2020; Kaur et al., 2020). The role and mechanism of *NBR2* in the C-PC inhibition of cancer cells has not been fully elucidated. In this study, we found that C-PC could inhibit proliferation and migration of H292 cells. Next, *NBR2* was found to be highly up-regulated in NSCLC cells treated with C-PC based on qRT-PCR. These results indicated that *NBR2* was associated with the inhibitory effects of C-PC on the proliferative and invasive ability of NSCLC cells. Furthermore, C-PC could not further activate AMPK and inhibited mTOR-associated pathway molecules in H292 cells with *NBR2* knock-down. In brief, C-PC exerted significant antitumour effects on NSCLC cells, and *NBR2* was an important factor in the C-PC-mediated suppression of NSCLC cell migration and proliferation.

Some studies have found that *NBR2* suppresses migration, invasion and EMT of colorectal cancer cells (Dimitrov et al., 2001; Bai et al., 2020). Our study showed that *NBR2* was down-regulated in NSCLCs *in vitro*, which is consistent with previous reports that low expression of *NBR2* correlated with poor clinical outcomes in NSCLC patients (Gao et al., 2019). Furthermore, we found that *NBR2* over-expression inhibited NSCLC cell malignancy by reducing NSCLC cell migration and promoting apoptosis. Therefore, *NBR2* has an inhibitory effect on the progression of NSCLC, which suggests that increasing *NBR2* transcripts may represent a therapeutic target and interventional strategy in tumour cells.

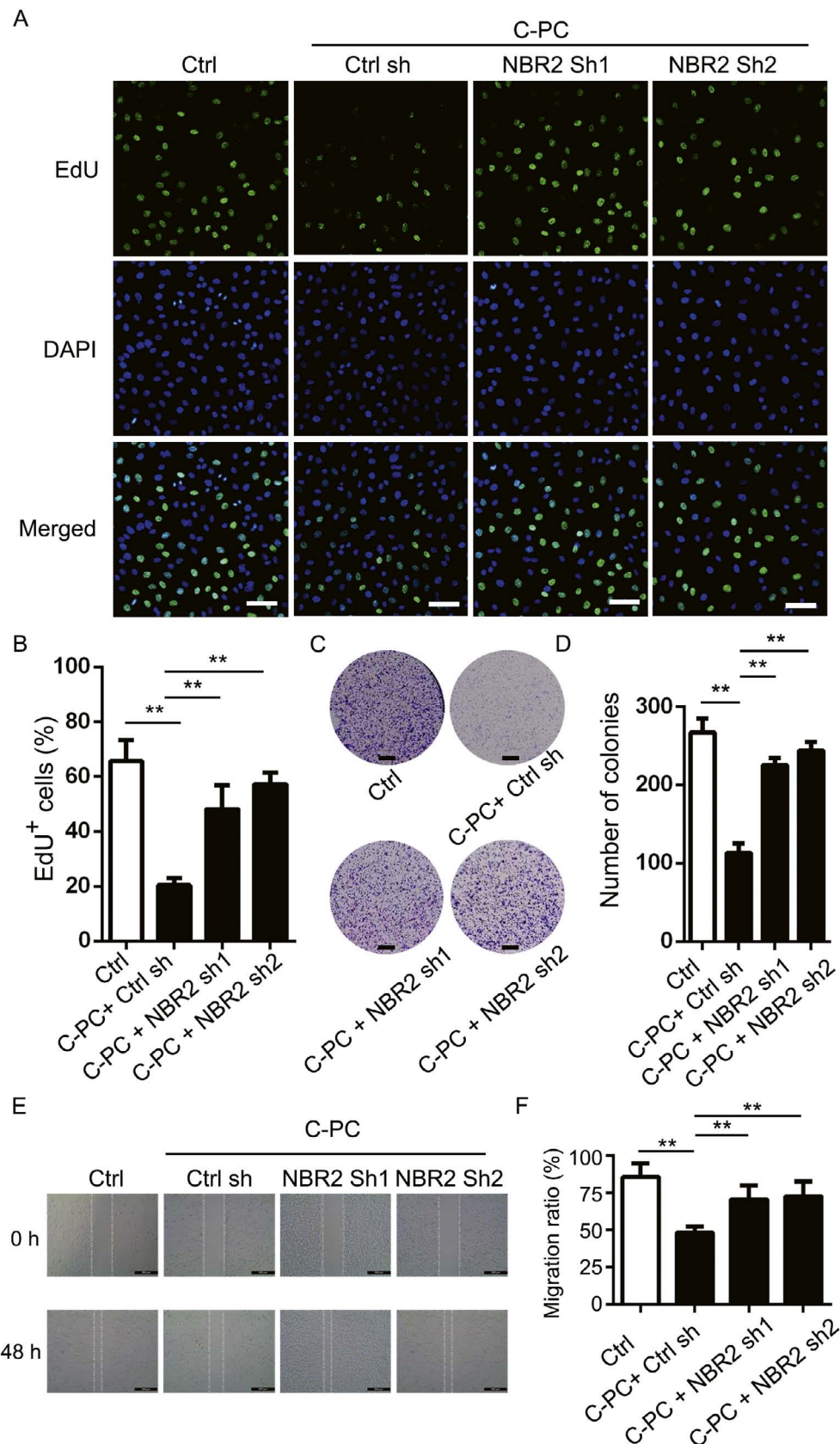


Fig. 3. C-PC mediates the inhibitory effects on the growth of H292 cells via NBR2. (**A, B**) EdU assay showing that the proliferation of H292 cells was enhanced following treatment with 0 or 10 μ M C-PC for 24 h. (**C, D**) The clone formation observed after H292 shNBR2-transfected cells were treated with 0 or 10 μ M C-PC for 24 h. (**E, F**) Wound-healing assay demonstrated migration of H292 cells following transfection with shNBR2 followed by 0 or 10 μ M C-PC for 24 h. Three replicates were performed for each experiment. Data are shown as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$.

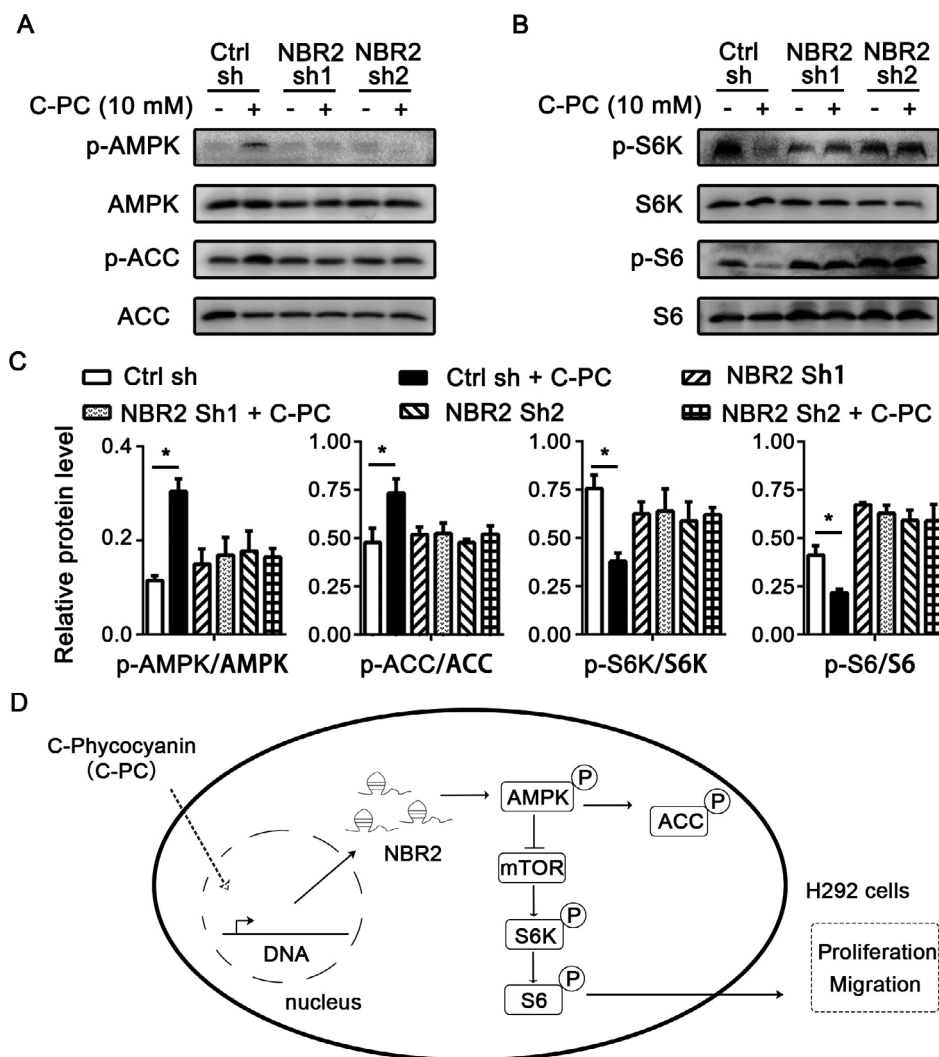


Fig. 4. Knock-down of *NBR2* in H292 cells compromised the activity of C-PC in activation of the AMPK signalling pathway. **(A, B)** Western blotting revealed that the protein phosphorylation of AMPK, ACC, S6K, and S6 was enhanced after H292 cells were transfected with the sh*NBR2* plasmid followed by 0 or 10 μ M C-PC for 24 h. **(C)** Quantitative analysis of the level of protein phosphorylation of AMPK, ACC, S6K, and S6 following transfection of H292 cells with sh*NBR2* was followed by exposure to 0 or 10 μ M C-PC for 24 h. Data are shown as the mean \pm SD. Three replicates were performed for each experiment.

C-PC has been definitively demonstrated to exert multiple anti-cancer functions in lung (Bingula et al., 2016), breast (Baudelet et al., 2013), liver (Basha et al., 2008), and cervical cancer (Yuan and Gurunathan, 2017; Hao et al., 2019) and in melanoma. Some studies have found that C-PC down-regulates EMT in cervical cancer cells (Ji et al., 2020). As a key sensor of cellular energy status, the AMPK pathway plays an important role in regulating tumour cell growth and proliferation (Inoki et al., 2012; Li et al., 2021). AMPK can be activated by C-PC in HepG2 liver cancer cells (Ren et al., 2018). Recently, AMPK activity has been shown to be regulated by interacting directly with *NBR2* (Liu et al., 2016). Our study demonstrated that C-PC is also an important negative factor in NSCLC cells. Furthermore, *NBR2*

expression could be significantly enhanced by C-PC in NSCLC cells, while *NBR2* depletion reversed the inhibitory effect of C-PC on NSCLC cells. These studies established that C-PC could have an important influence on the progression of NSCLC by increasing *NBR2* expression and triggering activation of AMPK signalling. In NSCLC, abnormal activation of the mTOR signalling pathway has been closely related to the resistance to apoptosis of tumour cells and to promote tumour metastasis (Deng et al., 2018; Wang et al., 2018). Recent studies have shown that mTOR inhibition promotes transmission of DNA damage signals to the apoptotic machinery and enhances antitumour effects in NSCLC (Piao et al., 2016). In this study, C-PC inhibited downstream mTOR signalling in NSCLC cells, a mechanism that can increase

the loss of DNA damage recognition and enhance the resistance of tumour cells.

Conclusion

In this study, we provide evidence that C-PC inhibits migration and promotes apoptosis of NSCLC cells. These effects are exerted by up-regulating NBR2 expression and regulating activity of the AMPK/mTOR signalling pathway. Thus, C-PC may represent a potential anti-cancer agent for the treatment of NSCLC.

Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

(I) study concepts and design: Chaowen He; (II) data acquisition: Chaowen He, Dongxuan Huang, Dongsheng Huang, Jianfeng Peng; (III) data analysis: Chaowen He, Fan Yang; (IV) manuscript preparation and editing: all authors.

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