

# Neochlorogenic acid enhances the antitumor effects of pingyangmycin via regulating TOP2A

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Received March 18, 2020; Accepted July 13, 2020

DOI: 10.3892/mmr.2020.11797

**Abstract.** Neochlorogenic acid (NCA), a natural compound found in honeysuckle, possesses prominent anti-inflammatory and antitumor effects. Pingyangmycin (PYM) induces DNA damage and has been used for the treatment of oral and maxillofacial tumors. Oral care serves an important role in promoting wound healing during chemotherapy in patients with oral squamous cell carcinoma (OSCC). Therefore, the present study aimed to analyze the effects of NCA and PYM on OSCC cells and to investigate the potential underlying mechanism. Reverse transcription-quantitative PCR and western blotting were conducted to analyze the expression levels of DNA topoisomerase II  $\alpha$  (TOP2A) in different OSCC cell lines. TOP2A-overexpression cells were constructed via transfection of TOP2A-overexpression plasmids. Following NCA or PYM treatment, cell proliferation was assessed using Cell Counting Kit-8 and colony formation assays, whereas cell apoptosis and the cell cycle distribution were assessed via TUNEL staining and flow cytometry, respectively. In addition, the expression levels of apoptosis- and cell cycle-related proteins were detected via western blotting. Moreover, co-immunoprecipitation (Co-IP) was conducted to determine whether TOP2A interacted with CDK1. The results of the present study indicated that NCA treatment significantly enhanced the suppressive effects of PYM on OSCC cell proliferation and apoptosis. The results also indicated that PYM arrested the cell cycle in the G<sub>0</sub>/1 by regulating cyclin dependent kinase 1 (CDK1)/cyclin B1, which was enhanced by the cotreatment of NCA and PYM. In addition, NCA and PYM treatment altered the expression levels of apoptosis-related

proteins. The Co-IP assay indicated that TOP2A interacted with CDK1. Moreover, TOP2A overexpression significantly reversed the effects of NCA and PYM treatment on OSCC cell proliferation and apoptosis. In addition, NCA significantly decreased PYM-induced toxicity in normal oral epithelial cells. In conclusion, the results of the present study suggested that NCA may promote the inhibitory effects of PYM in OSCC via TOP2A.

## Introduction

Pingyangmycin (PYM) has been widely used to treat head and neck squamous cell carcinoma, the cytotoxic effects of which depend on its ability to bind to iron and directly damage DNA, subsequently promoting cell death (1-4). In addition to inhibiting tumor growth, PYM affects cell apoptosis, chronic inflammation and angiogenesis (2,5,6). PYM also exerts a similar mechanism as bleomycin in inducing cell cycle arrest and cell apoptosis (2). A previous study suggested that PYM promoted cell apoptosis, potentially via activating the p53-dependent signaling pathway (7). PYM, a novel, hydrophilic, antitumor glycopeptide antibiotic, is produced by *Streptomyces* in the soil of Pingyang county (2). In addition, neochlorogenic acid (NCA), as one of main components of *Cyclocarya paliurus*, was suggested to be involved in suppressing cell apoptosis by regulating the mitogen-activated protein kinase/AKT signaling pathway (8). Moreover, NCA, as a phenolic in pectinase-treated *Prunus mume* fruit concentrate, exerts antitumor effects via promoting cell cycle arrest at the S phase and cell apoptosis via the mitochondrial-dependent apoptotic pathway, but does not demonstrate cytotoxicity in normal cells (9). A previous study has also indicated that NCA may be involved in the anticancer effects of *Leonurus sibiricus* extract, which are closely associated with DNA damage (10). The effects of phenolic compounds on DNA damage may be attributed to mutagenic activity, possibly by intercalating with DNA (11).

Topoisomerases function as important ribozymes that primarily regulate the topological structure of DNA by catalyzing the formation or disconnection of phosphodiester bonds (12). DNA topoisomerase II  $\alpha$  (TOP2A), which is a target

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**Key words:** neochlorogenic acid, pingyangmycin, DNA topoisomerase II  $\alpha$ , oral squamous cell carcinoma, cell cycle

of several anticancer drugs, can be damaged or suppressed, which hinders the replication and transcription of DNA, further promoting cell death (13). The abnormalities in TOP2A caused by anticancer drugs induce DNA double-strand breaks and lead to cell apoptosis by stabilizing the TOP2A cleavage complex (14). TOP2A is necessary for cell replication and is expressed at elevated levels during cell proliferation (14). In a previous study, compared with normal cells, cancer cells displayed upregulated expression levels of topoisomerase, independent of other factors (15,16). Oral squamous cell carcinoma (OSCC) is the most common type of malignant oral and maxillofacial tumor (17). Therefore, the present study aimed to analyze the effects of NCA and PYM on OSCC cells and to investigate the potential underlying mechanism.

## Materials and methods

**Cell lines and reagents.** HOK (human normal oral keratinocytes), A-253, HSC-4, CAL-27 and SCC-4 cells lines (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in DMEM (Hyclone; Cytiva) supplemented with 10% FBS (Biowest), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO<sub>2</sub>. PYM (Harbin Pharmaceutical Group Co., Ltd.) was dissolved in 1 mg/ml PBS to make a stock solution.

**Reverse transcription-quantitative PCR (RT-qPCR).** Cells were washed twice with PBS and total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration of RNA was detected using a spectrophotometer. Total RNA was reverse transcribed into cDNA using a ReverTra Aceq PCR RT kit at 37°C for 15 min (Toyobo Life Science). Subsequently, qPCR was performed using the FastStart Universal SYBR Green Master mix (Sigma-Aldrich; Merck KGaA). The following primer sequences were used for the qPCR: TOP2A forward, 5'-CATTGAAGACGCTTC GTTATGG-3' and reverse, 5'-CAGAAGAGAGGGCCAGTT GTG-3'; and β-actin forward, 5'-ATAGCACAGCCTGGA TAGCAACGTAC-3' and reverse, 5'-CACCTTCTACAATGA GCTGCGTGTG-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation for 1 min at 95°C; followed by 40 cycles at 95°C for 30 sec and 60°C for 40 sec; and the reaction was then maintained at 72°C for 5 min. TOP2A mRNA expression levels were quantified using the 2<sup>-ΔΔC<sub>q</sub></sup> method (18).

**Western blotting.** Cells were washed with precooled PBS and total protein was extracted from cells using RIPA lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.). Total protein was quantified using a BCA assay. Proteins (40 µg protein/lane) were separated via 12% SDS-PAGE and transferred onto PVDF membranes, which were blocked with 5% skim milk for 2 h at room temperature. The membranes were incubated with the following primary antibodies at 4°C overnight: Anti-TOP2A (1:10,000; cat. no. ab52934; Abcam), anti-CDK1 (1:2,000; cat. no. ab32094; Abcam), anti-Bax (1:1,000; cat. no. ab32503; Abcam), anti-caspase-3 (1:500; cat. no. ab13847; Abcam), anti-Bcl-2 (1:1,000; cat. no. ab32124; Abcam), anti-Cyclin B1 (1:1,000; sc-245; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (1:5,000; cat. no. ab8245; Abcam) Following the

primary antibody incubation, the membranes were rinsed three times with TBS-0.05% Tween-20 at 37°C and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; cat. no. ab6721; Abcam) or rabbit anti-mouse IgG (1:10,000; cat. no. ab6728; Abcam) secondary antibody at 37°C for 2 h. Protein bands were visualized using an ECL reagent (EMD Millipore). Protein expression was semi-quantified using ImageJ software version 1.46 (National Institutes of Health) with GAPDH as the loading control.

**Cell transfection.** TOP2A overexpression plasmids [pcDNA3.1(+)-TOP2A] were constructed and packaged by Shanghai GenePharma Co., Ltd. Cells (1.5x10<sup>6</sup> cells/well) were transfected with 0.5 µg/µl TOP2A overexpression plasmids or empty plasmids as the control group using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 24 h. Subsequently, the cells were used for subsequent experiments.

**Cell Counting Kit-8 (CCK-8) assay.** Cell viability was assessed using a CCK-8 kit (cat. no. CK04; Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. A total of 8x10<sup>3</sup> cells/well were seeded into 96-well plates at 37°C with 5% CO<sub>2</sub>. Cells were treated with 10 µg/ml PYM or 20 µM NCA at 37°C for 24 h. Subsequently, 10 µl CCK-8 solution was added to each well and incubated at 37°C for 4 h. The absorbance was detected at a wavelength of 450 nm using a microplate reader (EnSpire; PerkinElmer, Inc.).

**Colony formation assay.** Cells were quantified using a blood counting chamber. Briefly, cells were seeded into a 10 ml petri-dish (2x10<sup>2</sup> cells) containing 10 ml DMEM and 10% FBS, and were cultured for 14 days at 37°C with 5% CO<sub>2</sub>. When visible colonies (>50 cells) appeared in the petri-dishes, the supernatant was discarded, and cells were washed twice with PBS. Cells were fixed with 5 ml 10% methanol at 4°C for 10 min. Following 0.1% crystal violet staining (Sigma-Aldrich; Merck KGaA) at 37°C for 20 min, the number of cell colonies formed was counted under a light microscope (magnification, x20; CKX31SF; Olympus Corporation).

**TUNEL staining.** Cells were fixed with 4% paraformaldehyde at 37°C for 20 min and washed twice with PBS. Subsequently, cells were treated using 0.2% Triton X-100 at 37°C for 5 min and washed twice with PBS. Cells were incubated with the prepared TUNEL reaction liquid at 37°C for 1 h. Following incubation, cells were washed with PBS and stained with 80 µl 20% DAB at 37°C for 10 min. Cells were subsequently stained with 5 µg/ml hematoxylin at 37°C for 5 sec. Dehydrated transparent neutral gum was used to mount the sections. The cells were then observed under a fluorescent microscope (magnification, x200) in six randomly selected fields of view to observe TUNEL positive cells.

**Cell cycle analysis.** The cell cycle distribution was detected using a Cell Cycle kit (EZCell™ Cell Cycle Analysis kit; BioVision, Inc.). Cells were seeded (1x10<sup>5</sup> cells/ml) into 6-well plates. Following PYM or NCA treatment for 24 h at 37°C, cells were washed twice with precooled PBS and fixed with precooled 70% ethanol for 24 h at 4°C. Cells were washed twice with PBS and incubated with 400 µl PI (50 µg/ml) and

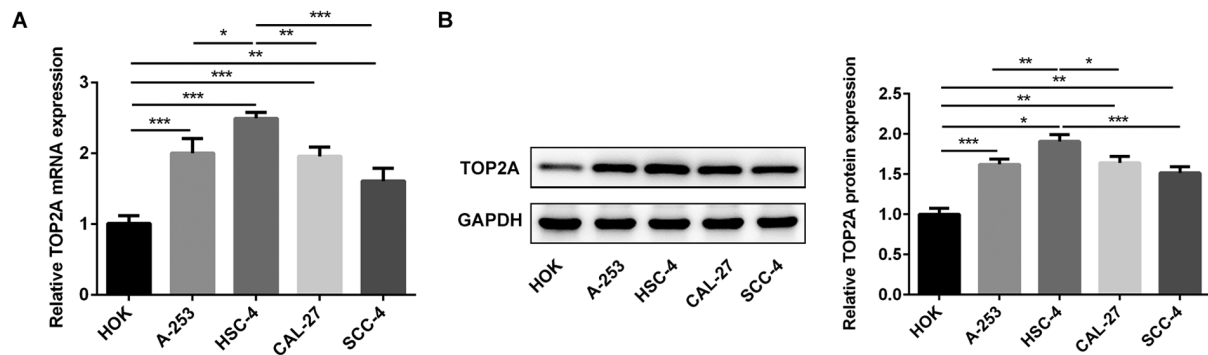


Figure 1. Expression levels of TOP2A in different OSCC cell lines. TOP2A (A) mRNA and (B) protein expression levels in OSCC cell lines. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . TOP2A, DNA topoisomerase II  $\alpha$ ; OSCC, oral squamous cell carcinoma.

100  $\mu$ l RNaseA (100  $\mu$ g/ml) for 1 h in the dark at 4°C (BD Biosciences). Cell cycle distribution was analyzed via flow cytometry (LSR-II; BD Biosciences) and analyzed using FlowJo version 10 software (FlowJo LLC).

**Co-immunoprecipitation (Co-IP).** Cells were collected and washed twice with PBS. Cell lysate was prepared using 500  $\mu$ l RIPA lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.), centrifuged at 24,148.8  $\times$  g for 10 min at 4°C and part of the supernatant was used to perform western blotting analysis as the input. Then, 1  $\mu$ g anti-TOP2A (1:10,000; cat. no. ab52934; Abcam) and anti-rabbit IgG (1:1,000; ab172730; Abcam; negative control) primary antibodies were incubated with the remaining supernatant at 4°C overnight. Following the incubation, 10  $\mu$ l Protein A agarose beads (Cell Signaling Technology, Inc.) were added to the remaining supernatant to capture antigen-antibody complexes at 4°C for 4 h. Then, the supernatant was centrifuged for 3 min at 1,509.3  $\times$  g at 4°C. Immunoprecipitation was performed by conducting western blotting according to the aforementioned protocol.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc.). Data are presented as the mean  $\pm$  SD of  $\geq 3$  experimental repeats. Comparisons among multiple groups were analyzed using one-way ANOVA and a Tukey's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression levels of TOP2A in different OSCC cell lines.** TOP2A expression levels were analyzed in HOK, A-253, HSC-4, CAL-27 and SCC-4 cells via RT-qPCR and western blotting (Fig. 1). Compared with HOK cells, TOP2A expression levels were significantly upregulated in OSCC cell lines. Moreover, the expression levels of TOP2A in HSC-4 cells were significantly increased compared with the other OSCC cell lines. Therefore, HSC-4 cells were used for subsequent experiments.

**NCA increases the inhibitory effects of PYM in OSCC via TOP2A.** RT-qPCR and western blotting were performed to assess the transfection efficacy of the TOP2A overexpression plasmid (Fig. 2A and B). TOP2A expression levels were

significantly downregulated by PYM or NCA treatment compared with the control group (Fig. 2C). In addition, treatment with PYM or NCA significantly suppressed HSC-4 cell proliferation, as determined by conducting CCK-8 and colony formation assays (Fig. 3A-C). The combination of NCA and PYM treatment significantly increased the inhibitory effects induced by either treatment alone on cell proliferation. Moreover, TOP2A overexpression significantly reversed the suppressive effects of NCA and PYM on cell proliferation. Therefore, the results suggested that NCA may enhance the inhibitory effects of PYM on HSC-4 cell proliferation by regulating TOP2A.

**NCA enhances PYM-induced OSCC cell apoptosis via TOP2A.** The TUNEL staining results indicated that NCA enhanced PYM-induced OSCC cell apoptosis (Fig. 4A and B). In addition, the combination of PYM and NCA treatment significantly increased cell cycle arrest in the  $G_0/G_1$  and reduced cell levels in the S phase compared with the PYM or NCA groups (Fig. 4C and D), indicating that the combination of PYM and NCA treatment may suppress the transition from  $G_1$  to S phase. To analyze the mechanisms underlying PYM- and NCA-mediated regulation of the cell cycle, the expression levels of cell cycle-related proteins were detected via western blotting (Fig. 5A). The results indicated that the expression levels of CDK1 and cyclin B1 were significantly downregulated in the PYM and NCA groups compared with the control group. Moreover, compared with the control group, the expression levels of the anti-apoptotic protein Bcl-2 were significantly downregulated, but the expression levels of the proapoptotic proteins, Bax and caspase-3, were significantly upregulated in the PYM and NCA groups (Fig. 5A). NCA enhanced the effects of PYM treatment on cell cycle arrest and cell apoptosis, potentially by regulating CDK1/cyclin B1 expression levels and the mitochondrial-mediated apoptotic pathway in a TOP2A-dependent manner. Furthermore, when the supernatant was incubated with an anti-TOP2A antibody, CDK1 was detected in the immunoprecipitation complex, which indicated that TOP2A may interact with CDK1 (Fig. 5B).

**Effects of NCA on normal oral epithelial cells.** Subsequently, whether NCA exerted protective effects against PYM-induced oral epithelial cell injury was investigated. The CCK-8 assay and TUNEL staining results indicated that NCA markedly decreased

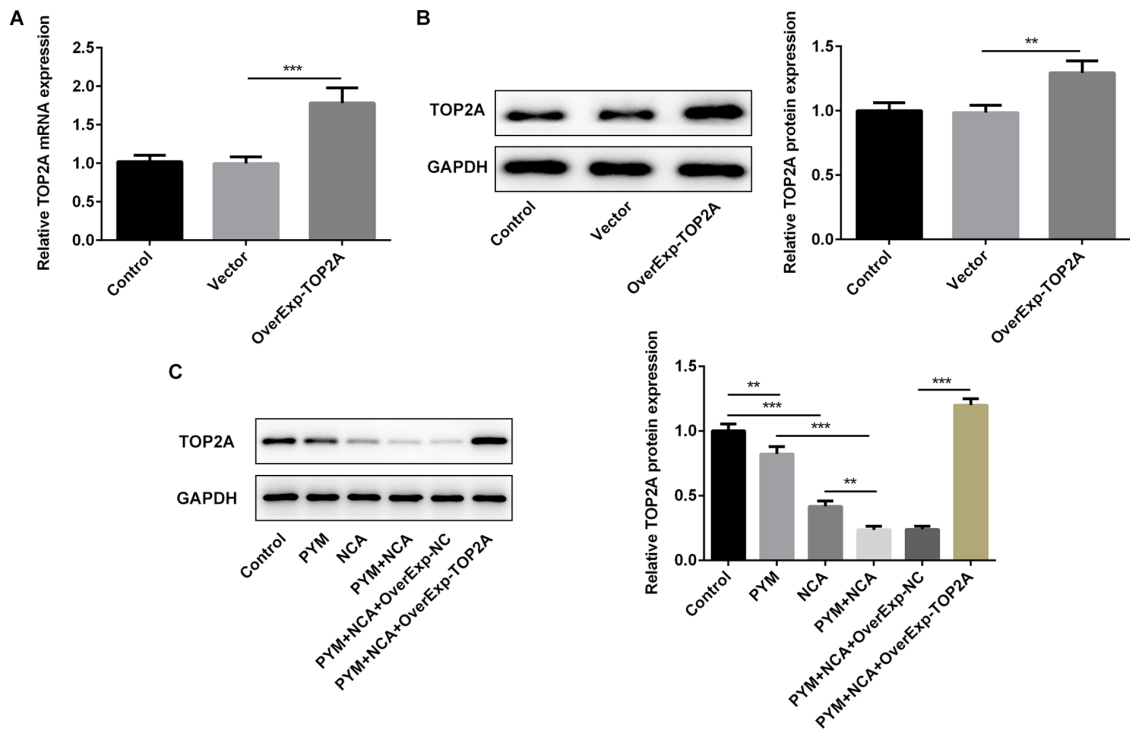


Figure 2. Transfection efficiency of TOP2A overexpression, and the effect of PYM and NCA on TOP2A expression. TOP2A (A) mRNA and (B) protein expression levels following TOP2A overexpression. (C) Effect of PYM and NCA on TOP2A expression levels. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . TOP2A, DNA topoisomerase II  $\alpha$ ; PYM, pingyangmycin; NCA, neochlorogenic acid; OverExp, overexpression; NC, negative control.

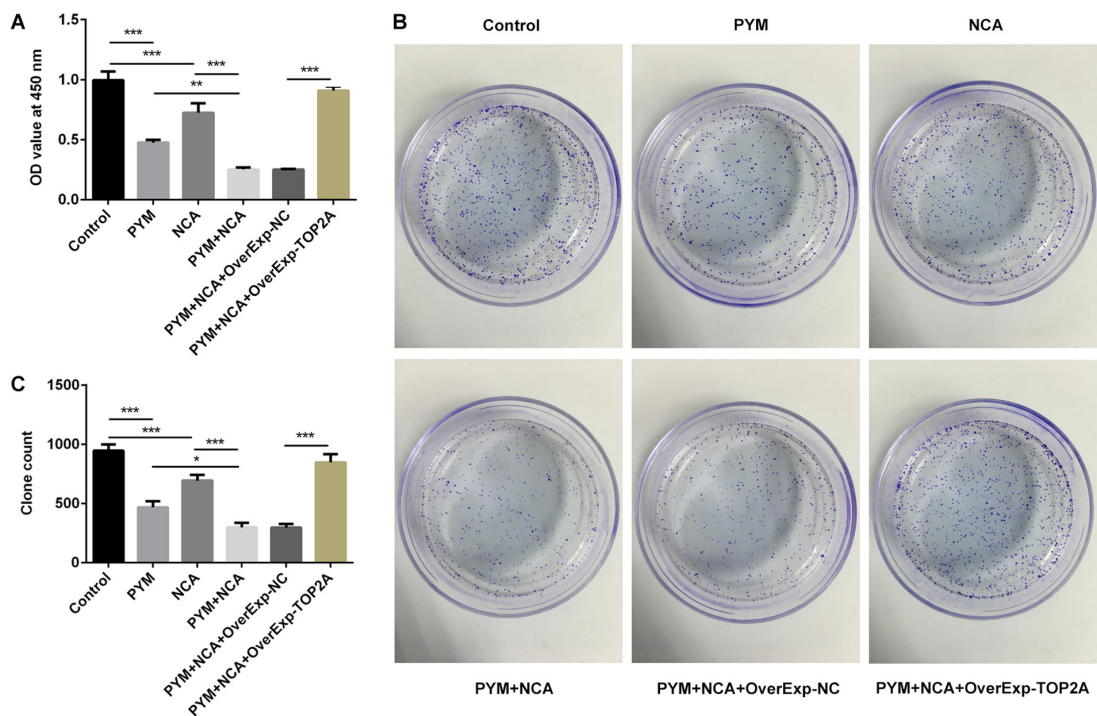


Figure 3. Effect of PYM and NCA on oral squamous cell carcinoma cell proliferation. Cell proliferation was assessed by performing (A) Cell Counting Kit-8 and (B) colony formation assays. (C) Colony count from part B. Each colony had  $>50$  cells. Magnification,  $\times 20$ . \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . PYM, pingyangmycin; NCA, neochlorogenic acid; OD, optical density; NC, negative control; OverExp, overexpression.

the toxicity of PYM in normal oral epithelial cells (Fig. 6A-C). The results also demonstrated that in normal cells, the combination of PYM and NCA significantly reduced cell apoptosis compared with PYM alone (Fig. 6B and C). Furthermore, NCA

decreased PYM-induced normal cell injury by regulating the cell cycle and apoptotic pathways (Fig. 6D and E). In HOK cells, NCA significantly upregulated TOP2A expression compared with the PYM group (Fig. 6D and E), whereas NCA displayed

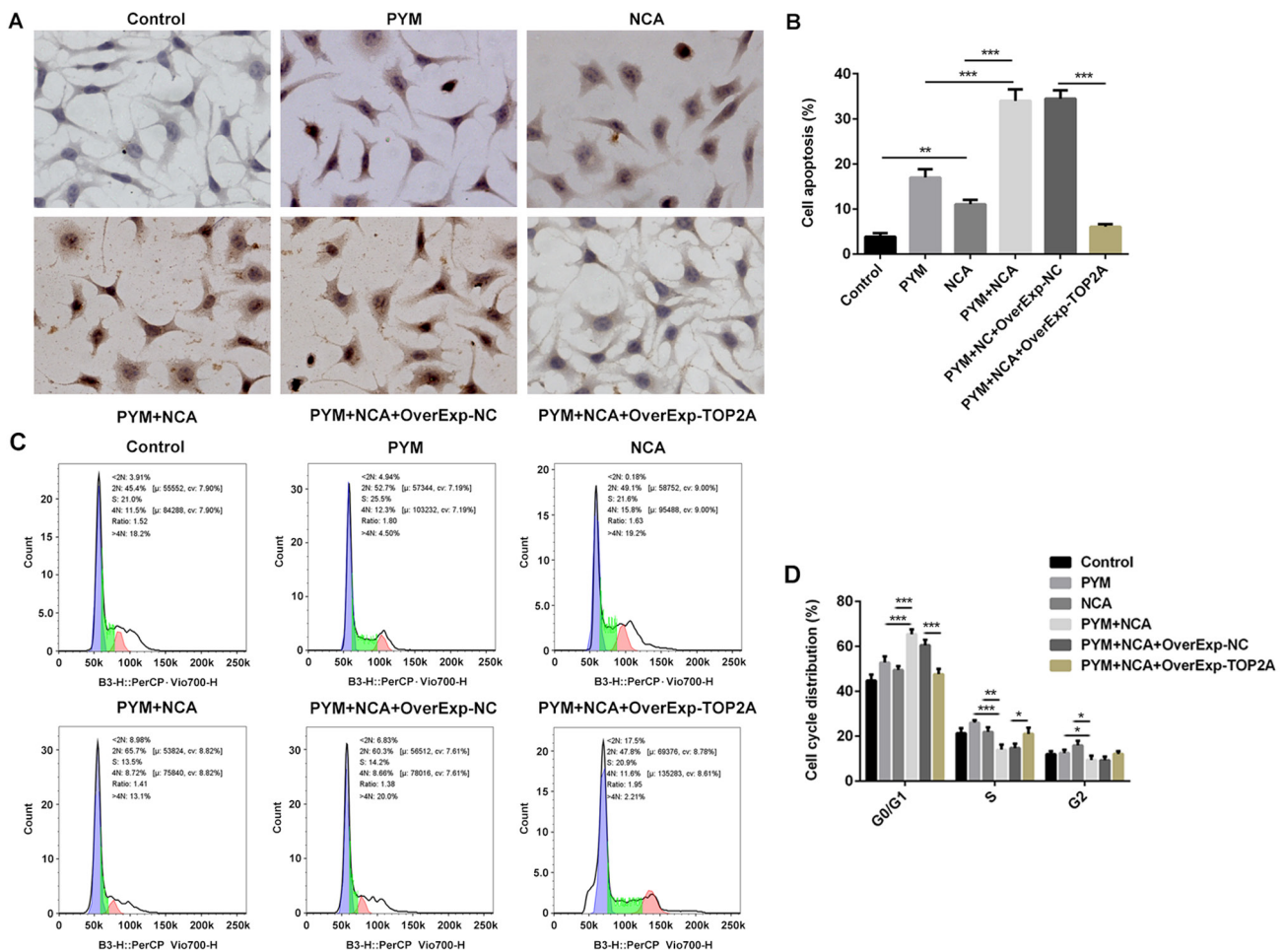


Figure 4. Effect of PYM and NCA on oral squamous cell carcinoma cell apoptosis and cell cycle distribution. (A) Cell apoptosis was assessed via TUNEL staining. Magnification, x200. (B) Semi-quantification of the results from part (A). (C) The cell cycle distribution was assessed via flow cytometry. (D) Quantification of cell cycle analysis from part (C). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. PYM, pingyangmycin; NCA, neochlorogenic acid; NC, negative control; OverExp, overexpression.

the opposite effects in HSC-4 cells (Fig. 2C). Therefore, NCA may regulate TOP2A expression in human normal oral cells and OSCC cells via different mechanisms.

**Discussion**

PYM, as one of the main chemotherapy drugs in preoperative induction chemotherapy for patients with OSCC, has achieved good efficacy; however, the drug presents with increased side effects, such as oral mucositis (19,20). The present study indicated that the combined treatment of PYM and NCA displayed improved, synergistic effects over the suppression of HSC-4 cell proliferation. OSCC is the most common malignant type of oral and maxillofacial tumor worldwide (21,22). PYM induces a common side effect of chemotherapy, oral mucositis, which can cause patients oral mucosal pain (23). Therefore, oral care has been hypothesized to serve an important role in enhancing the wound healing process during chemotherapy (24). A previous study suggested that drugs derived from natural products may provide important therapeutic effects for numerous types of human disease (25).

Honeysuckle is an effective compound that is used in a number of mouthwashes (26). Moreover, honeysuckle contains

numerous types of organic acid active ingredients, among which, chlorogenic acid has been thoroughly studied (27-29). NCA, an isomer of chlorogenic acid, displays significant anti-inflammatory, antitumor and immune-promoting effects (30-32). In the present study, the expression levels of TOP2A were significantly upregulated in OSCC cells compared with HOK cells. TOP2A and CDK1 expression levels have been reported to be upregulated in certain types of cancer, where expression is negatively associated with patient survival (33-35). In addition, TOP2A inhibited the incorrect attachment of microtubules and kinetochores, and was involved in suppressing tumor cell proliferation (16,36). Moreover, the TOP2A signaling pathway was also reported to mediate cell cycle arrest (16). The present study revealed that NCA promoted PYM-mediated cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> phase via TOP2A. The checkpoint in the G<sub>1</sub>/S phase determines whether the cell proliferates (37). Under normal conditions, G<sub>1</sub> arrest prevents damaged DNA from replication and helps to repair the damaged DNA (37). CDK1 serves an important role in determining mitotic progression (38). CDK1/cyclin complexes phosphorylate substrates that are involved in triggering centrosome separation, Golgi dynamics, nuclear envelope breakdown and chromosome condensation during the G<sub>2</sub> phase and early mitosis (37). Once

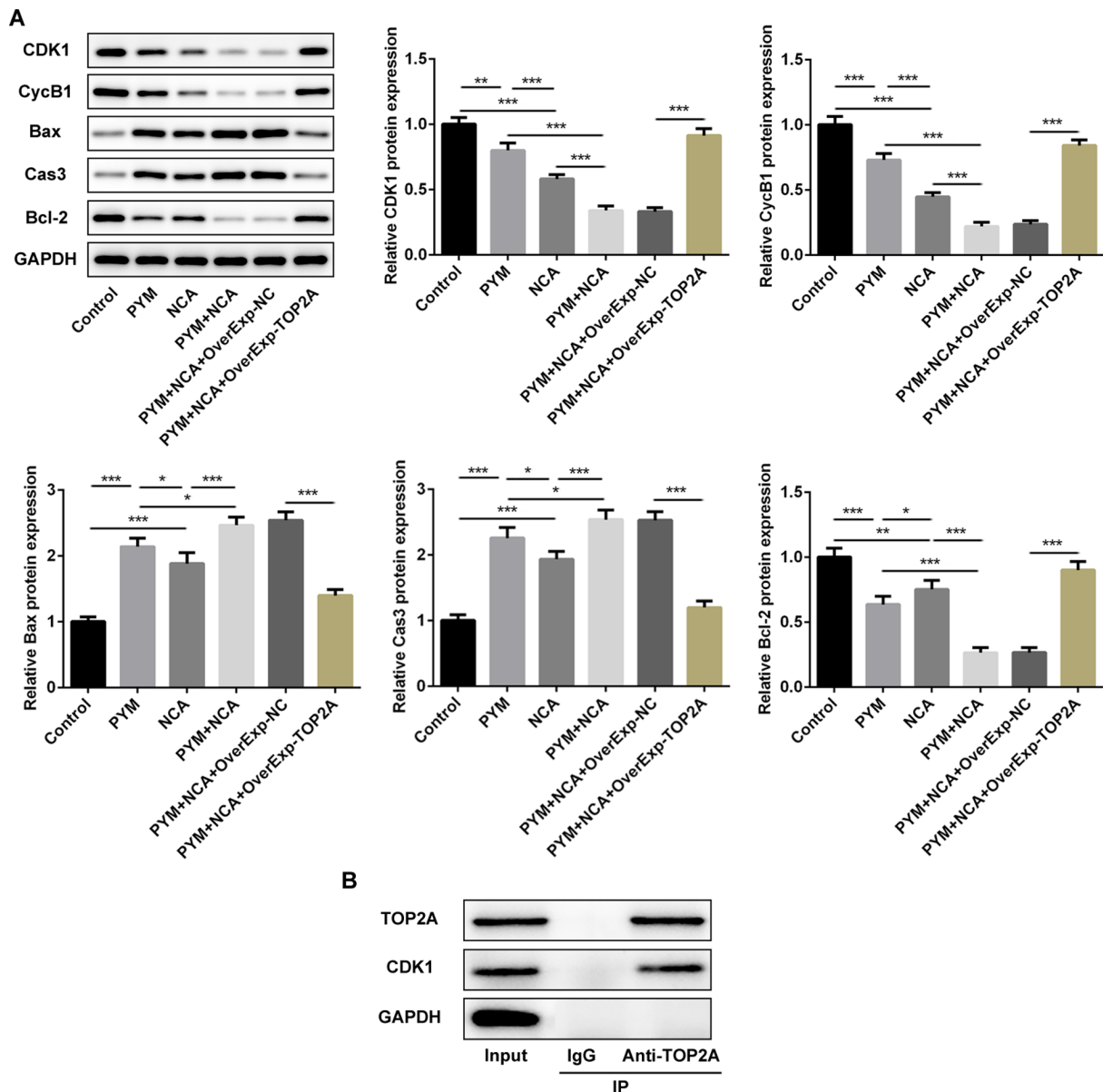


Figure 5. Effect of PYM and NCA on the expression of cell apoptosis- and cell cycle-associated proteins. (A) Protein expression levels of cell cycle- and cell apoptosis-associated proteins. (B) Co-immunoprecipitation was performed to assess the interaction between TOP2A and CDK1. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . PYM, pingyangmycin; NCA, neochlorogenic acid; TOP2A, DNA topoisomerase II  $\alpha$ ; NC, negative control; OverExp, overexpression; CDK1, cyclin dependent kinase 1; CycB1, cyclin B1; Cas3, caspase 3.

CDK1 is inactivated during cell injury, the cells are arrested at the  $G_2$  checkpoint to facilitate cell repair (37). However, a previous study has reported that the absence of CDK1 compensated the CDK2 functions by driving cells into the S phase (39). The results of the present study indicated that NCA and PYM treatment may arrest cells in the  $G_1$  phase, potentially by decreasing CDK1 and cyclin B expression levels. A previous study revealed that downregulated endogenous lysine acetyltransferase 2A expression levels promoted  $G_1/S$  transition, partially via downregulating CDK1 expression in an E2F transcription factor 1-dependent manner (40). Furthermore, CDK1 has been reported to be involved in  $G_1$  arrest (41). Previously, it was demonstrated that the promotion of  $G_1/S$  transition occurred via the interaction of ZNF1 antisense RNA 1 with the CDK1/cyclin B complex (42). The present study indicated that NCA treatment enhanced the antitumor effects of PYM by

arresting the cell cycle in the  $G_{0/1}$  phase in a TOP2A-mediated manner. In addition, both NCA and PYM treatment induced OSCC cell apoptosis, which was partially mediated via regulating the TOP2A-mediated mitochondrial-dependent apoptotic pathway. For normal cells, NCA did not inhibit proliferation, but moderately promoted it, and at the same time reduced PYM-induced apoptosis. It has been reported that NCA exerts antitumor effects in a dose-dependent manner *in vitro* and suppresses tumor growth *in vivo* in human gastric carcinoma cells (32). Moreover, NCA reduces ROS production and suppresses mTOR/PI3K/Akt signaling (32). NCA also possesses strong free radical scavenging and antioxidant activities (43). Furthermore, NCA has been reported to activate the nuclear factor erythroid 2-related factor 2 signaling pathway and induce 5'AMP-activated protein kinase phosphorylation (44). Research has demonstrated that *Apocynum venetum* tea extracts

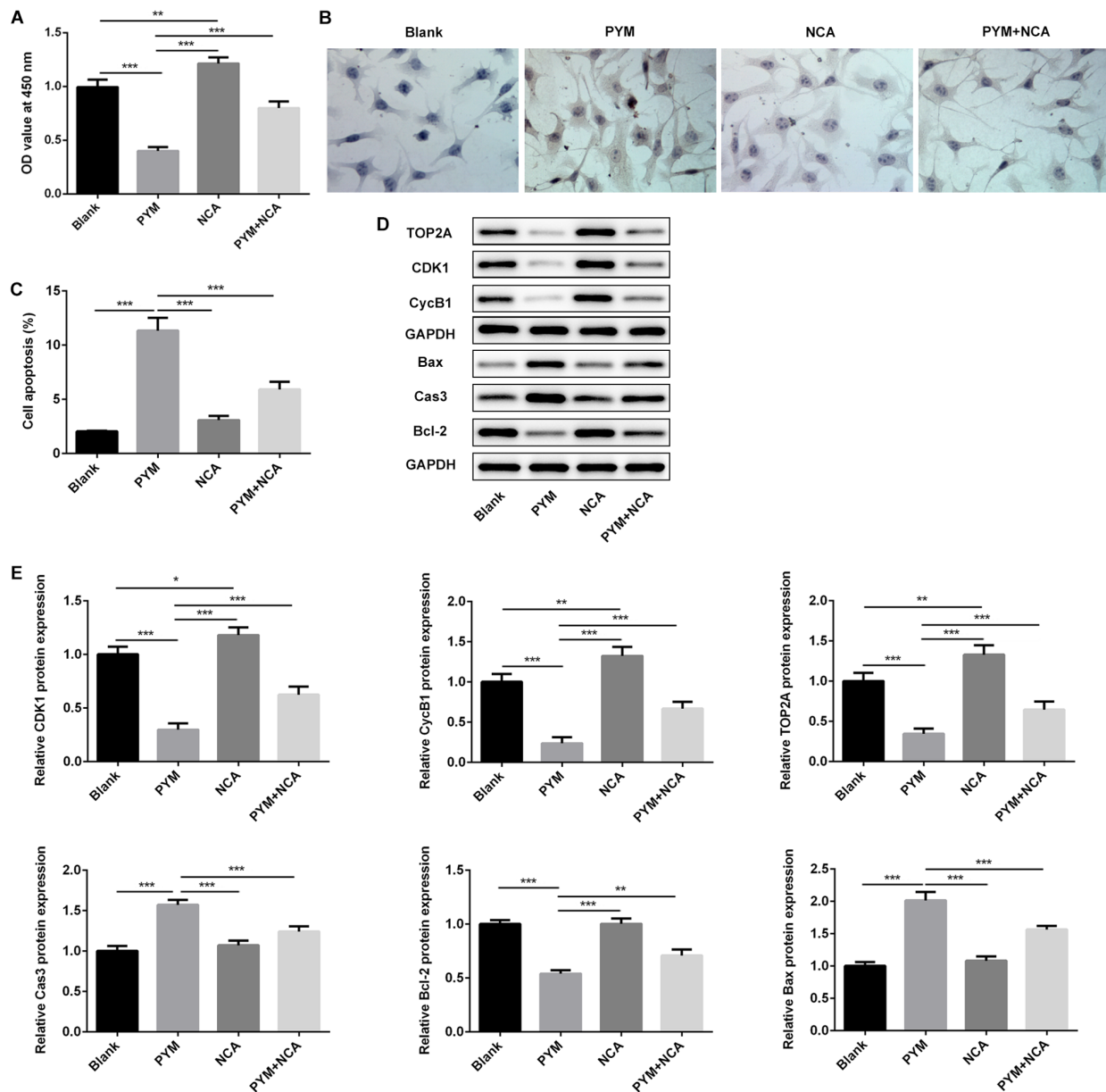


Figure 6. Effect of PYM and NCA on normal cells. (A) Cell proliferation was assessed by conducting the Cell Counting Kit-8 assay. (B) Cell apoptosis was assessed via TUNEL staining. Magnification, x200. (C) Semi-quantification of the results presented in part (B). (D) Expression levels of cell cycle- and apoptosis-associated proteins. (E) Semi-quantification of the expression levels presented in part (D). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. PYM, pingyangmycin; NCA, neochlorogenic acid; OD, optical density; TOP2A, DNA topoisomerase II  $\alpha$ ; CDK1, cyclin dependent kinase 1; CycB1, cyclin B1; Cas3, caspase 3.

(AVTEs), which contain NCA, exert antioxidation effects and promote cell survival in 293T cells, whereas in HepG2 human hepatoma cells, AVTEs display antitumor effects potentially by inducing cell apoptosis (45). Collectively, the aforementioned studies and the present study indicated that NCA could display different effects between normal cells and cancer cells via different mechanisms. The present study indicated that NCA displays antioxidant activities by promoting HOK cell survival, as indicated by the CCK-8 assay and TUNEL staining results. A previous study indicated that in etomoxir-induced oxidative stress, TOP2A expression was downregulated (46). In addition, the dose of NCA used in the present study displayed different effects in HOK and HSC-4 cells. The Co-IP results suggested that CDK1 interacted with TOP2A in HSC-4 cells, which indicated that NCA enhanced the antitumor effects of PYM partly

via regulating the interaction of CDK1 and TOP2A. TOP2A and CDK1 expression levels are upregulated and are associated with low survival in some types of cancer, such as adrenocortical (47) and hepatocellular carcinoma (48). By constructing a protein-protein interaction network, a previous study identified a potential relationship between CDK1 and TOP2A (49-51). In addition to regulating the cell cycle, CDKs possess a broad range of biological functions, including an involvement in DNA damage repair and interactions with other proteins to regulate tumor growth (52-54). In conclusion, the results of the present study suggested that NCA may enhance the antitumor effects of PYM by regulating TOP2A. Moreover, NCA may reduce the toxicity of PYM in normal oral epithelial cells. Therefore, the use of NCA may enhance the therapeutic effects of PYM chemotherapy in patients with OSCC.

## Acknowledgements

Not applicable.

## Funding

No funding was received.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

JC and DL made substantial contributions to the conception and design of the study, acquired, analyzed and interpreted the data, and drafted and revised the manuscript for important intellectual content; TZ, WL, SC, GY and XL performed the experiments and interpreted the data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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