

# Camptothecin inhibits the progression of NPC by regulating TGF- $\beta$ -induced activation of the PI3K/AKT signaling pathway

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**Abstract.** Nasopharyngeal carcinoma (NPC) is a type of cancer that is characterized by increased invasiveness, metastatic potential and tumor recurrence. Camptothecin has been demonstrated to exhibit anticancer activity. However, the potential underlying molecular mechanisms mediated by camptothecin in NPC cells remain elusive. In the present study, the efficacy of camptothecin for NPC was investigated *in vitro* and *in vivo*. Additionally, the potential signaling pathway mediated by camptothecin in NPC cells was also examined. The results indicated that the viability and aggressiveness of NPC cells were suppressed by camptothecin treatment in a dose-dependent manner. Camptothecin administration downregulated the expression levels of cell-cycle-associated proteins including cyclin 1, cyclin-dependent kinase (CDK)1 and CDK2 in NPC cells. Expression levels of migration-associated proteins including vimentin, fibronectin and epithelial cadherin were regulated by camptothecin treatment in NPC cells. Additionally, camptothecin inhibited the expression of transforming growth factor- $\beta$  (TGF- $\beta$ ), phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT), whereas TGF- $\beta$  overexpression abrogated camptothecin-mediated inhibition of PI3K and AKT expression and camptothecin-mediated inhibition of the viability and aggressiveness of NPC cells. Camptothecin significantly inhibited tumor growth and increased survival times in a mouse model of cancer. In conclusion, these results indicate that camptothecin treatment may inhibit the viability of NPC cells and aggressiveness by regulating the TGF- $\beta$ -induced PI3K/AKT signaling pathways, which in turn may be a potential molecular target for the treatment of NPC.

## Introduction

Nasopharyngeal carcinoma (NPC) is a type of cancer that occurs in the top and side of the nasopharyngeal cavity (1,2). Clinical observation and experimental studies have indicated that pathogenic factors of NPC are extensive, including hereditary factors, viral infections and environmental factors (3,4). A previous study has suggested that future perspectives for clinical research should include prospective and observational cohort studies, which may assess the effects of different risk factors in the development of NPC and the effectiveness of investigational treatments (5). Although previous studies have investigated numerous anticancer drugs including nimotuzumab, oxaliplatin and gemcitabine for the treatment of NPC (6-9), the therapeutic outcomes are not encouraging so far. Therefore, exploring efficient anticancer agents and the potential underlying molecular mechanisms of cellular migration and metastasis in NPC are required.

Camptothecin (C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>4</sub>) is an anticancer drug that exhibits high efficacy for the treatment of gastrointestinal, and head and neck cancer (10,11). Clinical pathologists have widely investigated the effects of camptothecin in the treatment of human cancer cells (12,13). A previous study demonstrated that camptothecin may induce human colon cancer cell death via the downregulation of mitogen-activated protein kinase phosphatase-1 and sustaining extracellular-signal-regulated kinase 1/2 activation (14). Additionally, Sun *et al* (15) have demonstrated that camptothecin may inhibit the growth and invasion of prostate cancer cells via phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT),  $\alpha\beta3/\alpha\beta5$  and matrix metalloproteinase (MMP)-2/-9 signaling pathways. Furthermore, a pharmacodynamic and pharmacogenomic study revealed that a nanoparticle conjugate of camptothecin, CRLX101, is efficient for the treatment of cancer (16). Although previous studies have indicated the efficacy of camptothecin in solid tumors (17,18), only a limited number of studies have investigated the function of camptothecin in NPC cells. To the best of our knowledge, no study has elucidated the potential molecular mechanism mediated by camptothecin in the progression of NPC.

In the present study, the inhibitory effects of camptothecin on the viability and aggressiveness of NPC cells were assessed. Potential molecular mechanisms mediated by camptothecin in NPC cells were also examined. The results of the present

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study demonstrated that camptothecin administration significantly inhibited the viability and aggressiveness of NPC cells by regulating transforming growth factor- $\beta$  (TGF- $\beta$ )-induced activation of the PI3K/AKT signaling pathway, and thus it may be a potential anticancer agent for NPC therapy.

## Materials and methods

**Ethics statement.** The present study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (19). All experimental protocols were approved by the Ethics Committee of Nanfang Hospital of Southern Medical University (Guangzhou, China).

**Cells and reagents.** NPC cell lines HK1 and C666-1 were a gift from Professor Jia-Huan Han (Department of Microbiology, Xiamen University). All tumor cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Endogenous overexpression of TGF- $\beta$ .** HK1 and C666-1 cells were cultured to 85% confluence. Cells ( $1 \times 10^7$ ) were then transfected with pcdue12.4-TGF- $\beta$  (pTGF- $\beta$ ; 100 pmol) or empty vector plasmid (control; 100 pmol) using Lipofectamine® 2000 (all Invitrogen; Thermo Fisher Scientific, Inc.). Stable TGF- $\beta$ -overexpressing HK1 and C666-1 cells were selected using a glutamine synthetase screening system (20). Cells were used for further analysis after 72 h transfection. TGF- $\beta$ -overexpressing HK1 and C666-1 were treated with 6  $\mu$ M camptothecin (pTGF- $\beta$ -CA) for 24 h at 37°C.

**MTT assays.** HK1 and C666-1 were transfected with control or pTGF- $\beta$  and cultured in a 96-well plate for 48 h at 37°C. HK1 and C666-1 cells were treated with 2, 6 or 10  $\mu$ M camptothecin and incubated at 37°C for 24, 48 and 72 h. After incubation, 20  $\mu$ l MTT (5 mg/ml) solution was added to each well and the plate was further incubated for 4 h. Medium was removed and 100  $\mu$ l dimethylsulfoxide was added to the wells to solubilize the crystals. Cell viability was determined by measuring the absorbance at 450 nm using a plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Cell invasion and migration assays.** Stable TGF- $\beta$ -overexpressing HK1 and C666-1 cells were cultured with camptothecin (2  $\mu$ M) or PBS (control) at 37°C for 48 h. Migration and invasion assays were performed in a 6-well culture plate with chamber inserts (BD Biosciences, Franklin Lakes, NJ, USA). For migration assays, HK1 and C666-1 cells ( $1 \times 10^4$  cells/well) were placed into the upper chamber (8  $\mu$ m pores) and cultured for 48 h at 37°C. For invasion assays, HK1 and C666-1 cells ( $1 \times 10^4$  cells/well) were placed into the upper chamber with the Matrigel-coated membrane for 48 h at 37°C. Migrating or invading HK1 and C666-1 cells were stained with 1% crystal violet for 30 min at 37°C and counted in at least three randomly selected fields using a light microscope (Nikon E400; Nikon Corp., Tokyo, Japan).

**Analysis of cell cycle by flow cytometry.** Flow cytometric analysis was performed to analyze the effects of camptothecin (6  $\mu$ M) on the cell cycle stages of HK1 and C666-1 cells. Exponentially grown HK1 and C666-1 cells or TGF- $\beta$ -overexpressing HK1 and C666-1 cells were treated with camptothecin (6  $\mu$ M) for 48 h. Cells were washed, trypsinized and rinsed with PBS. All cells were fixed in 75% ice-cold ethanol for 5 min and then washed with PBS three times. The fixed cells were washed with RNase A (20  $\mu$ g/ml; Fermentas; Thermo Fisher Scientific, Inc.) and stained with propidium iodide (20  $\mu$ g/ml, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 10 min at 37°C. The percentages of cells at S-phase were determined using a FACSCalibur instrument (BD Biosciences) and analyzed using BD FACSDiva™ Software (version 1.2; BD Biosciences).

**Western blot analysis.** Following treatment of HK1 and C666-1 cells with camptothecin (6  $\mu$ M) for 24 h at 37°C, cells were harvested by scraping and lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) followed by homogenization at 4°C for 10 min. Protein concentration was measured by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Proteins (10  $\mu$ g) were separated by SDS-PAGE (12% gels). The proteins were transferred onto membranes and blocked with 2% BSA (Sigma-Aldrich; Merck KGaA). Membranes were incubated with rabbit anti-human cyclin 1 (1:500; ab152116), anti-cyclin-dependent kinase 1 (CDK1; 1:500; ab18), anti-cyclin-dependent kinase 2 (CDK2; 1:500; ab32147), TGF- $\beta$  (1:500; ab92486), anti-PI3K (1:500; ab86714), anti-phosphorylated PI3K (pPI3K; 1:500; ab138364), anti-AKT (1:500; ab64148), anti-phosphorylated AKT (pAKT; 1:500; ab38449) anti-vimentin (1:500; ab92547), anti-fibronectin (1:500; ab2413), anti-E-cadherin (1:500; ab11512) or anti- $\beta$ -actin (1:500; ab32572; all Abcam, Cambridge, UK) antibody for 12 h at 4°C. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat no. 6726; Bio-Rad Laboratories, Inc.) was used at 1:5,000 dilution for 2 h at 37°C and immunoreactive protein bands were detected using a western blotting luminol reagent. The results were visualized using a chemiluminescence detection system (GE Healthcare, Chicago, IL, USA).

**Animal study.** Pathogen-free male Balb/c (8-weeks old; body weight, 25–32 g) mice were purchased from Slack Laboratory Animal Co., Ltd. (Shanghai, China). Mice were maintained on a 12 h light/12 h dark cycle with free access to diet and water. Experimental mice were implanted with HK1 cells ( $1 \times 10^8$  cells) into the groin and were divided into two groups (20 mice/group). Treatment was initiated on day 3 following tumor implantation (diameter, 5–6 mm). Tumor-bearing mice were intravenously injected with camptothecin (10 mg/kg) or PBS (control) once daily for 9 days. The tumor volumes were calculated according to a previous study (21). Mice were sacrificed when the tumor diameter reached 18 mm in each group.

**Immunohistochemical staining.** Xenograft mouse tumor tissue was fixed in 10% formaldehyde for 2 h at 37°C and embedded in paraffin. Paraffin-embedded tissue samples were cut into serial sections of 4- $\mu$ m thickness. Antigen retrieval was also performed on tumor sections. Tumor sections were

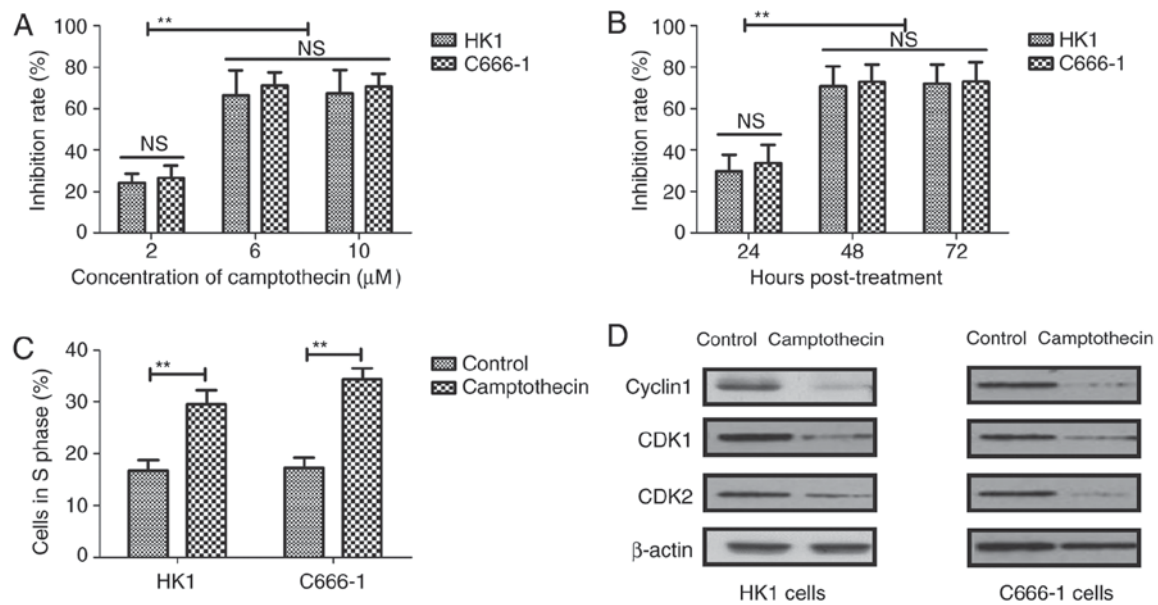


Figure 1. Camptothecin suppresses cellular viability and induces cell cycle arrest in NPC cells. (A) An MTT assay revealed that camptothecin inhibits the viability of HK1 and C666-1 cells in a dose-dependent (2, 6 and 10  $\mu$ M) manner. (B) Camptothecin inhibits the viability of HK1 and C666-1 cells in a time-dependent (24, 48 and 72 h) manner as assessed using an MTT assay. (C) Flow cytometric analysis of the cell cycle demonstrated that camptothecin (6  $\mu$ M) induces cell cycle arrest at S-phase in HK1 and C666-1 cells. (D) Western blot analysis indicated that camptothecin (6  $\mu$ M) downregulates the expression of cell-cycle-associated proteins, cyclin 1, CDK1 and CDK2 in NPC cells.  $\beta$ -actin was used as a loading control. \*\* $P < 0.01$ . NPC, nasopharyngeal carcinoma; NS, not significant; CDK, cyclin-dependent kinase.

blocked with 5% BSA for 2 h at 37°C and then incubated with rabbit anti-human TGF- $\beta$  (1:500; ab92486), anti-PI3K (1:500; ab86714) and anti-AKT (1:500; ab64148; all Abcam) antibodies for 24 h at 4°C. Tumor tissues were washed with PBS three times and incubated with goat anti-rabbit horse-radish peroxidase-conjugated anti-rabbit IgG antibodies (cat. no. 6726; Bio-Rad Laboratories, Inc.) for 24 h at 4°C. The slides were observed using a chemiluminescence detection system (Version 3.0; Sigma-Aldrich, Merck KGaA). Images were captured with an Olympus fluorescence microscope (Olympus Corporation, Tokyo, Japan).

**Statistical analysis.** Data were analyzed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 5; GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean  $\pm$  standard error of the mean. Results were analyzed by Student's t-test or one-way analysis of variance followed by Tukey's honestly significant difference test. All experiments were performed at least three times.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Camptothecin treatment suppresses viability and induces cell cycle arrest in NPC cells.** Fig. 1A indicates that camptothecin treatment (2, 6 or 10  $\mu$ M) inhibited the viability of HK1 and C666-1 cells in a dose-dependent manner after 24 h incubation. Results indicated that 6  $\mu$ M had the same inhibitory rate the viability for HK1 and C666-1 cells. Additionally, NPC cells treated with 6  $\mu$ M camptothecin displayed maximal inhibition of viability. HK1 and C666-1 cells were treated with camptothecin (6  $\mu$ M) for 24, 48

and 72 h. The results indicated that camptothecin treatment significantly inhibited the viability of HK1 and C666-1 cells in a time-dependent manner (Fig. 1B). The data identified no differences between the 48 and 72 h incubation. Next, flow cytometric analysis of the cell cycle was performed to determine whether camptothecin (6  $\mu$ M) treatment induces cell cycle arrest in HK1 and C666-1 cells after 48 h incubation. The results indicated that camptothecin treatment induced cell cycle arrest at S-phase in HK1 and C666-1 cells (Fig. 1C). Western blot analysis indicated that camptothecin downregulated the expression of cell-cycle-associated proteins, including cyclin 1, CDK1 and CDK2, in HK1 and C666-1 cells (Fig. 1D). These results indicate that camptothecin treatment may suppress cellular viability and induce cell cycle arrest in NPC cells.

**Camptothecin treatment inhibits the migration and invasion of NPC cells.** The effects of camptothecin on tumor aggressiveness were analyzed using migration and invasion assays. The results indicated that camptothecin treatment (6  $\mu$ M) significantly inhibited migration and invasion of NPC cells after 48 h of incubation ( $P < 0.01$ ; Fig. 2A and B). Additionally, western blot analysis revealed that camptothecin treatment (6  $\mu$ M) downregulated vimentin and fibronectin expression and upregulated E-cadherin expression levels in NPC cells (Fig. 2C). These results indicate that camptothecin treatment may inhibit migration and invasion by regulating the expression of migration-associated proteins, including vimentin, fibronectin and E-cadherin, in NPC cells.

**Camptothecin regulates the viability of NPC cells via the TGF- $\beta$ -induced PI3K/AKT signaling pathway.** Potential molecular mechanisms involved in camptothecin-mediated



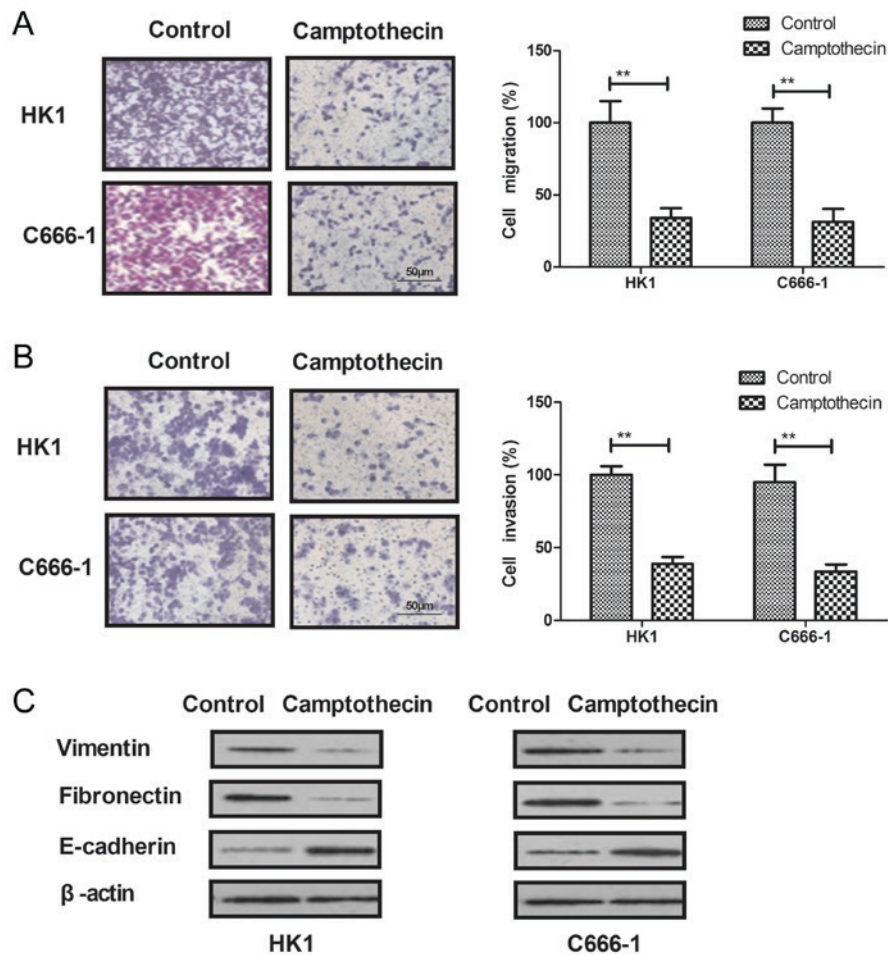


Figure 2. Camptothecin treatment inhibits migration and invasion of NPC cells. (A) At 48 h post-treatment, camptothecin (6  $\mu$ M) significantly inhibits migration (B) and invasion of NPC cells. Scale bar, 50  $\mu$ m. \*\* $P$ <0.01. (C) Western blot analysis revealed that camptothecin (6  $\mu$ M) treatment downregulates the expression of vimentin and fibronectin, and upregulates the expression of E-cadherin in NPC cells.  $\beta$ -actin was used as a loading control. NPC, nasopharyngeal carcinoma.

viability of NPC cells were investigated. Western blot analysis revealed that camptothecin treatment (6  $\mu$ M) inhibited the expression levels of TGF- $\beta$ , PI3K, AKT, pPI3K and pAKT in NPC cells (Fig. 3A). Additionally, the effects of TGF- $\beta$  overexpression in camptothecin-mediated regulation of tumor development was assessed by transfecting the cells with pTGF- $\beta$ -CA. Western blot analysis revealed that endogenous TGF- $\beta$  overexpression abrogated camptothecin-mediated inhibition of PI3K and AKT expression (Fig. 3B). The results indicated that TGF- $\beta$  overexpression abolished camptothecin-mediated inhibition of viability, migration and invasion of NPC cells (Fig. 3C-E). These results indicate that camptothecin regulates the viability of NPC cells by regulating TGF- $\beta$ -induced activation of the PI3K/AKT signaling pathway.

*Camptothecin treatment inhibits tumor growth and increases survival times in vivo.* Anticancer efficacy of camptothecin was also determined *in vivo* in tumor-bearing mice. Camptothecin treatment significantly inhibited tumor growth compared with the control group (Fig. 4A). Camptothecin significantly inhibited TGF- $\beta$ , PI3K and AKT expression in tumor sections as assessed by immunohistochemistry (Fig. 4B). Additionally, camptothecin significantly increased the survival time of

mice (120 days) (Fig. 4C). These results indicate that camptothecin treatment may inhibit tumor growth and increase the survival time of tumor-bearing mice.

## Discussion

Currently, a number of strategies that target the migration and invasion of NPC cells have been proposed for the therapy of NPC (2,8). In the present study, a camptothecin-mediated signaling pathway in NPC cells was investigated. The results indicated that 6  $\mu$ M camptothecin exhibited maximal toxicity towards NPC cells. Previous studies have revealed that NPC occurs in the epithelial lining of the nasopharynx and patients with NPC develop regional lymph node metastasis and distant metastasis (22,23). The results of the present study demonstrated that camptothecin treatment significantly inhibited the viability and aggressiveness of NPC cells and also suppressed tumor growth in tumor-bearing mice. Previous studies suggest that camptothecin is an efficient anticancer drug for the treatment of various types of cancer (24,25). Notably, the results of the present study indicated that camptothecin regulated the viability of NPC cells by regulating TGF- $\beta$ -induced activation of the PI3K/AKT signaling pathway.

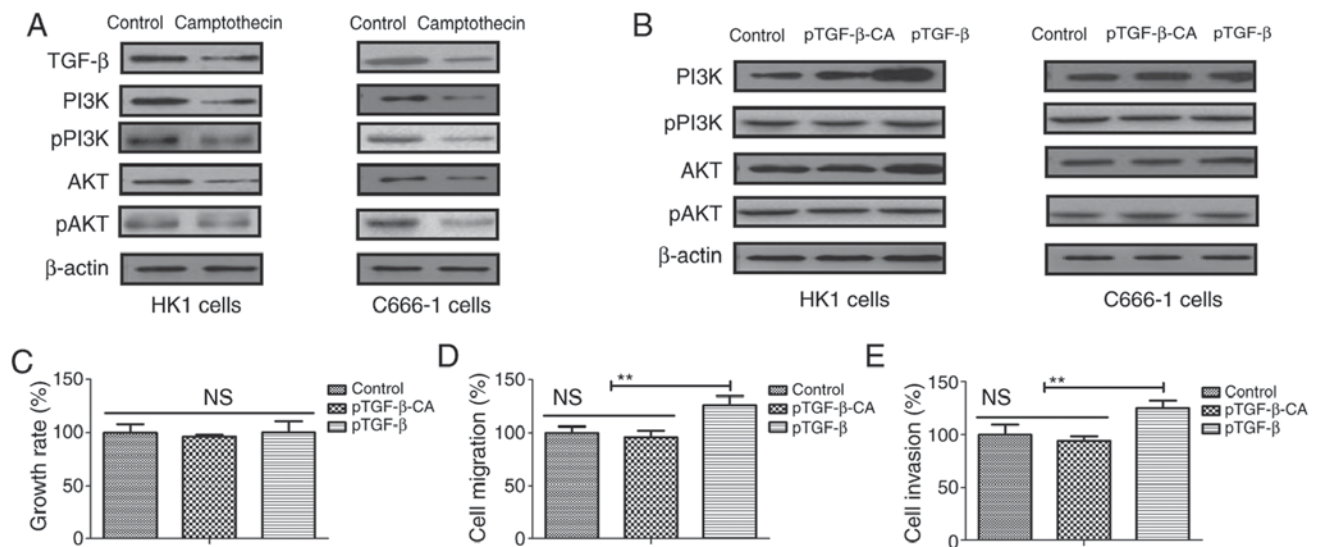


Figure 3. Camptothecin regulates the viability of NPC cells by regulating TGF-β-induced activation of the PI3K/AKT signaling pathway. (A) Western blot analysis revealed that camptothecin (6  $\mu$ M) treatment inhibits the expression of TGF-β, PI3K and AKT in NPC cells. (B) Western blot analysis demonstrated that TGF-β overexpression abrogates camptothecin-mediated inhibition of PI3K, pPI3K pAKT and AKT expression in NPC cells. (C) An MTT assay revealed that TGF-β overexpression abolishes camptothecin-mediated inhibition of viability of NPC cells. (D) TGF-β overexpression abrogates camptothecin-mediated inhibition of migration (E) and invasion of NPC cells. β-actin was used as a loading control. \*\*P<0.01. NPC, nasopharyngeal carcinoma; TGF-β, transforming growth factor-β; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; pPI3K, phosphorylated PI3K; pAKT, phosphorylated AKT; NS, not significant.

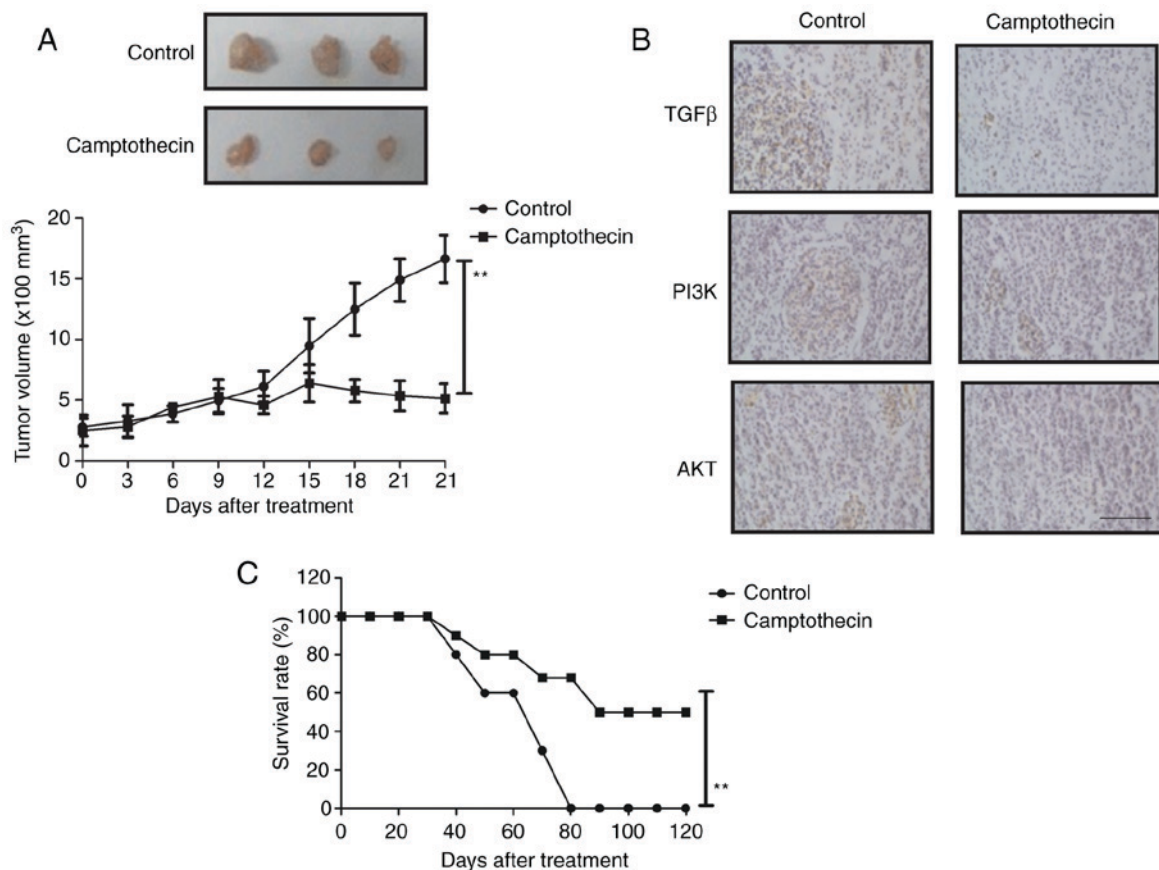


Figure 4. Camptothecin treatment inhibits tumor growth and increases survival times *in vivo*. (A) Camptothecin treatment (10 mg/kg) inhibited tumor growth in tumor-bearing mice compared with the control group. (B) Camptothecin treatment (10 mg/kg) decreased TGF-β, PI3K and AKT expression in tumor sections as assessed by immunohistochemistry. (C) Camptothecin treatment increased the survival times of mice during the 120-day treatment. Scale bars, 20  $\mu$ m. \*\*P<0.01. TGF-β, transforming growth factor-β; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B.

Increased incidence of NPC and increased mortality rates for patients with NPC have been reported in China (26-28).

The results of the present study indicated that camptothecin treatment significantly inhibited cellular migration and

invasion via regulating the expression levels of vimentin, fibronectin and E-cadherin in NPC cells. Camptothecin synergizes with cyclin-dependent kinase inhibitors, and regulates the viability and aggressiveness of small cell lung cancer cells (29). Mollica *et al* (30) suggested that camptothecin treatment led to benefits in patients with colorectal cancer. The results of the present study demonstrated that camptothecin treatment induced cell cycle arrest at S-phase via downregulating the expression levels of cyclin, CDK1 and CDK2 in NPC cells.

In the present study, the potential molecular mechanisms involved in camptothecin-mediated regulation of NPC development were investigated. A previous study reported that camptothecin and 10-hydroxycamptothecin inhibited the viability and metastasis of lung cancer cells via p38 mitogen-activated protein kinase, extracellular-signal-regulated kinase and AKT signaling pathways (31). Additionally, camptothecin suppressed platelet-derived growth factor-BB-induced proliferation of rat aortic vascular smooth muscle cells through the inhibition of the PI3K/AKT signaling pathway (32). The results of the present study indicated that camptothecin treatment inhibited TGF- $\beta$ , PI3K and AKT expression in NPC cells, whereas TGF- $\beta$  overexpression abrogated camptothecin-mediated inhibition of PI3K and AKT expression. Additionally, it has been reported that camptothecin suppressed MMP-9 and vascular endothelial growth factor expression in DU145 cells through PI3K/AKT-mediated inhibition of nuclear factor- $\kappa$ B activity and nuclear factor erythroid 2-related factor 2-dependent induction of heme oxygenase-1 expression (33). The results of the present study demonstrated that camptothecin regulated the viability of NPC cells through TGF- $\beta$ -induced activation of the PI3K/AKT signaling pathway, which led to longer survival for tumor-bearing mice.

In conclusion, a number of studies have demonstrated the anticancer efficacy of camptothecin in preclinical settings, which may contribute to the treatment of NPC. The results of the present study indicated the underlying molecular mechanism by which camptothecin may suppress the viability and aggressiveness of NPC and regulate TGF- $\beta$ -induced activation of the PI3K/AKT signaling pathway.

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#### 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

BSL and JYH performed all experiments. JG analyzed experimental data in this study. LHC designed all experiments in the present study.

#### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Nanfang Hospital of Southern Medical University (Guangzhou, China).

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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