

# Influence of norcantharidin on proliferation, proliferation-related gene proteins proliferating cell nuclear antigen and Ki-67 of human gallbladder carcinoma GBC-SD cells

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**BACKGROUND:** Gallbladder carcinoma is a highly lethal and aggressive disease with early metastasis, strong invasion and poor prognosis. Most patients with this disease are at the advanced and un-resectable stage and should be considered for palliative treatment such as chemotherapy and radiotherapy. Unfortunately, reports of chemotherapy and radiotherapy for gallbladder carcinoma are disappointing. We investigated the influence of norcantharidin (NCTD) on proliferation, proliferation-related gene proteins PCNA and Ki-67 of human gallbladder carcinoma GBC-SD cells *in vitro*.

**METHODS:** GBC-SD cell lines of human gallbladder carcinoma were cultured by the cell culture technique. The experiment was divided into NCTD group and control group. The tetrazolium-based colorimetric assay was used to evaluate cell growth. The streptavidin-biotin complex method was used to determine the expressions of proliferation-related gene proteins PCNA and Ki-67 of human gallbladder carcinoma GBC-SD cells.

**RESULTS:** NCTD inhibited the growth and proliferation of GBC-SD cells from 10 mg/L or after 6 hours in a dose- and time-dependent manner, with the  $IC_{50}$  value of 56.18  $\mu$ g/ml at 48 hours. After treatment with NCTD, the expression of PCNA ( $0.932 \pm 0.031$  vs.  $0.318 \pm 0.023$ ,  $P < 0.001$ ) and Ki-67 ( $0.964 \pm 0.092$  vs.  $0.297 \pm 0.018$ ,  $P < 0.001$ ) proteins were decreased significantly.

**CONCLUSION:** NCTD inhibits the proliferation of human gallbladder carcinoma GBC-SD cells *in vitro* and the expression of their proliferation-related gene proteins PCNA and Ki-67.

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**KEY WORDS:** gallbladder neoplasm; norcantharidin; cell culture; immunohistochemistry; cell proliferation; proliferating cell nuclear antigen, Ki-67

## Introduction

Gallbladder carcinoma is a highly lethal and aggressive disease with early metastasis, strong invasion and poor prognosis.<sup>[1-3]</sup> Most patients with the disease are at the advanced and un-resectable stage and should be considered for palliative treatment such as chemotherapy and radiotherapy.<sup>[1, 4-12]</sup> Unfortunately, reports of chemotherapy and radiotherapy on gallbladder carcinoma are disappointing, results are conflicting and most series have a small number of patients.<sup>[1, 7-12]</sup> Obviously, there is an urgent need to identify new therapeutic agents for the treatment of gallbladder carcinoma *in vivo*. We reported that norcantharidin (NCTD), a demethylated form of cantharidin, which is an active ingredient of Chinese herbal medicine *Mylabris*, was used for the treatment of human gallbladder carcinoma GBC-SD cells.<sup>[13, 14]</sup> In the present study, we investigated the influence of NCTD on proliferation, proliferation-related gene proteins PCNA and Ki-67 of human gallbladder carcinoma GBC-SD cells *in vitro* and NCTD's anticancer mechanism.

## Methods

### Cell cultures<sup>[13, 14]</sup>

GBC-SD cells of human gallbladder carcinoma (Shanghai Cell Institute Country Cell Bank, China) were cultured in RPMI-1640 medium (Gibco/BRL, MD) supplemented with 10% bovine calf serum (Hangzhou Si-jiqing Biology Co., China) in an incubator with 5% CO<sub>2</sub> at 37 °C. The medium was changed every 2 days.

When the cells became confluent, namely a 95% plating

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efficiency, they were digested with 0.25% trypsin (Gibco/BRL, MD) and observed under a inversion microscope (Chongqin Optician, China). Then the cells were returned to culture at 37 °C in 5% CO<sub>2</sub> for 24 hours, washed twice with Hanks' balanced salt solution (Gibco/BRL, MD), and they were used in the experiment.

### Inhibitory experiment of NCTD on proliferation of GBC-SD cells

The experiment was divided into NCTD group and control group. The tetrazolium-based colorimetric assay (MTT) was used to evaluate the inhibitory effect of NCTD on proliferation of GBC-SD cells *in vitro*, namely the tumor cytotoxicity test.<sup>[13, 14]</sup> After GBC-SD cells were cultured in a 96-well plate ( $3 \times 10^5$  cells  $\cdot$  100  $\mu$ l/well) in culture medium overnight, they were treated at various concentration (0, negative control; 5-100  $\mu$ g/ml, experiment groups; 6 well/per concentration) of NCTD (Beijing Fourth Pharmaceutical Works, China) in fresh culture medium at 37 °C for 48 hours. The tumor cell cytotoxicity was determined by MTT (Sigma MO., USA). The optical densities (*A* value) at 540 nm were measured using an ELISA reader (DG3032, Shanghai). The *A*<sub>540</sub> value of the experimental groups was divided by the *A*<sub>540</sub> value of untreated controls and presented as a percentage of the cells. The inhibitory percent of various concentration of NCTD on GBC-SD cells (%) =  $(1 - A_{540} \text{ value in the experimental group} / A_{540} \text{ value of control group}) \times 100\%$ . Three separate experiments were performed. The concentration of drug giving 50% growth inhibition (IC<sub>50</sub>) was obtained from the dose-response curves generated by a non-linear fitting procedure, or was calculated from the formula  $IC_{50} = \lg^{-1} [Xm - I (\sum p - 0.5)]$ .

### Immunocytochemical assay of PCNA, Ki-67

Proliferation-related gene--PCNA and Ki-67 products of GBC-SD cells were determined by the streptavidin-biotin complex method (SABC). The cells plated

on slides were treated without or with IC<sub>50</sub> dose of NCTD, and added in order with primary antibody (PCNA: mouse monoclonal antibody, 1:100, Merck Calbiochem Inc., Germany; Ki-67: mouse monoclonal antibody, 1:100, Neomarkers Co., USA), biotinylated secondary antibody (Horse serum: horse anti-mouse IgG, 1:200, Vector Inc., USA), SABC reagents (SABC kit from Boster Co., China) and 3,3-diaminobenzidine (DAB) solution (Boster Co., China), respectively. Then, they were rinsed in distilled water, dehydrated through alcohol and xylene and mounted coverslip using a permanent mount medium for analysis by microspectro-photometer (Leitz Dmrbe, Leica, Germany). For negative control, the slides were treated with PBS in place of primary antibody. Ten sample slides in each group were chosen for analysis. More than 10 visual fields were observed or more than 500 cells were counted per slide. The positive index of PCNA or Ki-67 represented expression of PCNA or Ki-67 protein. The positive index of PCNA = number of PCNA positive cells/1000 cells. The stain integral of Ki-67 protein was counted according to the positive number and the stain intensity of the cells.

### Statistical analysis

All the statistical analyses were performed using SPSS 10.0 for Windows.  $P < 0.05$  or  $F < 0.05$  was considered statistically significant.

## Results

### Inhibitory effect of NCTD on proliferation of GBC-SD cells

The effect of NCTD on cell proliferation was examined at doses between 0 and 100  $\mu$ g/ml at times between 0 and 120 hours. The inhibitory effect of NCTD on GBC-SD cells at a low concentration of 5  $\mu$ g/ml was not obvious, and when the concentration was increased, growth of GBC-SD cells was markedly inhibited by NCTD with a growth inhibition rate of 98.59% at a

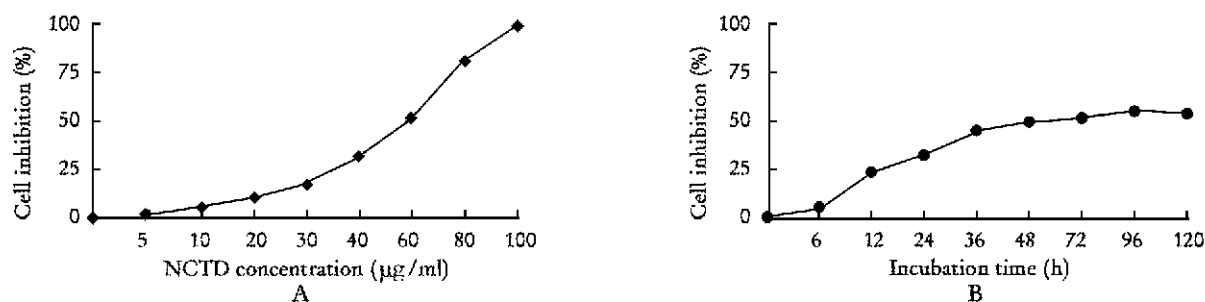


Fig. 1. A; The dose-response curves of effect of NCTD on GBC-SD cells for 72 hours. The inhibition of the growth of human gallbladder carcinoma GBC-SD cells by various concentration of NCTD. B; The time-response curves of effect of NCTD on GBC-SD cells at a concentration of IC<sub>50</sub>. Cell number was counted by the MTT method.

concentration of 100 µg/ml, in a dose-dependent manner (Fig. 1). IC<sub>50</sub> of NCTD for GBC-SD cells was 56.18 µg/ml (Fig. 2). The inhibitory effect of IC<sub>50</sub> NCTD on GBC-SD cells was shown after culture for 6 hours; moreover, the effect was markedly intensified with a growth inhibition rate of 100 µg/ml for 48 hours, in a time-dependent manner. So the effect was most obvious after 48 hours.

**Influence of NCTD on expression of PCNA and Ki-67 proteins of GBC-SD cells**

**PCNA**

The positive expression site of PCNA presented with yellow-brown reactant in cell nucleoli after immunocytochemical staining (Fig. 2). As GBC-SD cells in the control group, positive staining of PCNA was shown mostly (Fig. 2A), and the positive index of PCNA reached to 0.932±0.031. After treatment with IC<sub>50</sub> NCTD for 48 hours, the positive cells of expression of

PCNA protein was decreased significantly, the staining of cell nucleoli became light and shallow, and the positive index of PCNA was lowered to 0.318 ± 0.023 as compared to the control group (Fig. 2B; Table, P < 0.001).

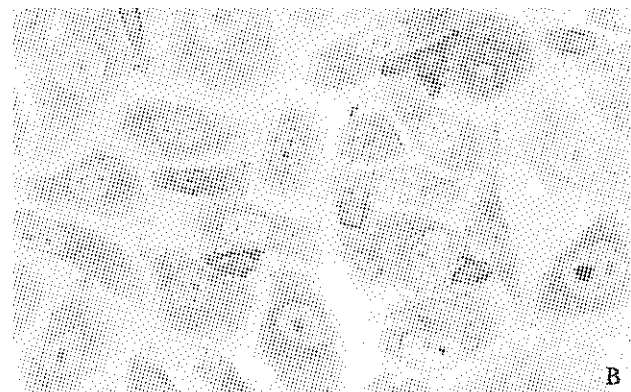
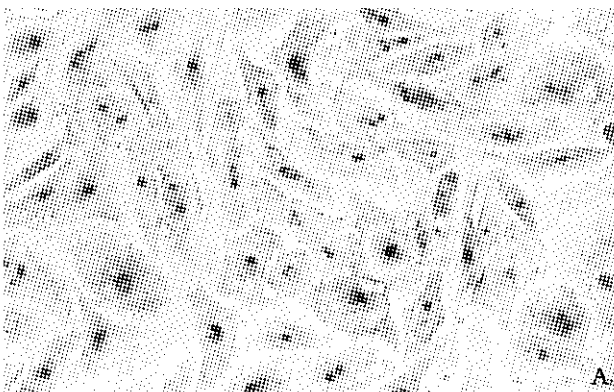
**Ki-67**

The positive expression site of Ki-67 gene protein also presented with yellow-brown reactant in cell nucleoli after immunocytochemical staining (Fig. 3). As GBC-

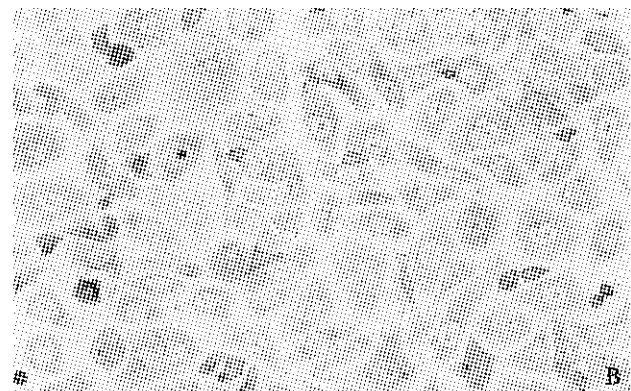
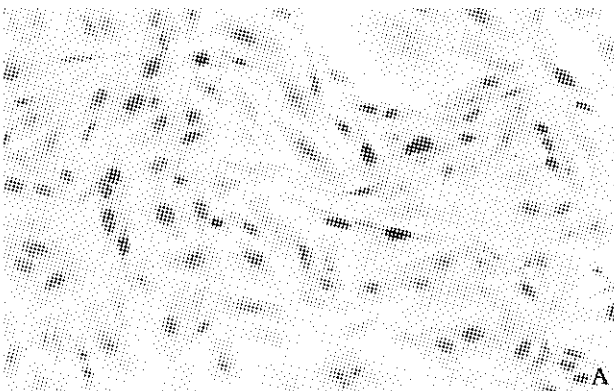
**Table.** Influence of NCTD on expression of PCNA and Ki-67 proteins of GBC-SD cells

Group	n	Positive index ( $\bar{x} \pm s$ )	
		PCNA	Ki-67
Control	10	0.932 ± 0.031	0.964 ± 0.092
IC <sub>50</sub> NCTD	10	0.318 ± 0.023 *	0.297 ± 0.018 <sup>#</sup>

\*: vs. control group, t = 46.935, P < 0.001; #: vs. control group, t = 22.008, P < 0.001.



**Fig. 2.** The positive expression occurred in cell nucleoli, with dye of brown or yellowness, of PCNA protein of GBC-SD cells (immunohistochemistry SABC method, × 200). **A**: The brown dye of PCNA was shown positively in most cells of the control group. **B**: In the experiment group with treatment of IC<sub>50</sub> NCTD for 48 hours, the positive cells of PCNA expression decreased significantly and the dye in cell nucleoli became light and shallow.



**Fig. 3.** Positive expression occurred in cell nucleoli, with dye of brown or yellowness, of Ki-67 protein of GBC-SD cells (immunohistochemistry SABC method, × 100). **A**: The brown dye of Ki-67 was shown positively in most cells of the control group. **B**: In the experiment group with treatment of IC<sub>50</sub> NCTD for 48 hours, the Ki-67 expression of positive cells decreased significantly and the dye in cell nucleoli became light and shallow.

SD cells in control group, the positive staining of Ki-67 was shown mostly (Fig. 3A), the positive index of Ki-67 reached to  $0.964 \pm 0.092$ . After treatment with IC<sub>50</sub> NCTD for 48 hours, the positive cells of expression of Ki-67 protein was decreased significantly, the staining of cell nucleoli became light and shallow, and the positive index of Ki-67 was lowered to  $0.297 \pm 0.018$  as compared to the control group (Fig. 3B; Table,  $P < 0.001$ ).

## Discussion

Gallbladder carcinoma is hitherto a highly lethal malignant neoplasm with dismal surgical result, postoperative recurrence or metastasis and poor prognosis.<sup>[1-12]</sup> Hence, the patients with gallbladder carcinoma should be considered for palliative treatment such as chemotherapy and radiotherapy. But no specific chemo-radiotherapy program for the disease has emerged as the definitive acceptable standard of care, most series have a small number of patients and there is much room for improvement.<sup>[1, 7-12]</sup> Obviously, there is an urgent need to identify new therapeutic agents for the treatment of gallbladder carcinoma.

Natural medicine, traditional Chinese medicine and their distilled analogue have been recognized increasingly. Many studies have shown that Chinese medicine contains many chemical compounds with anticancer effects. Previously we reported the effect of norcantharidin (NCTD) on human gallbladder carcinoma GBC-SD cells.<sup>[13, 14]</sup> NCTD is a demethylated and low-cytotoxic derivative of cantharidin (a 7-oxabicyclo [2.2.1] heptane-2, 3-dicarboxylic acid derivative, a natural toxin and the active ingredient extracted from Chinese medicine *Mylabris*), which is synthesized from furan and maleic anhydride via the Diels-Alder reaction.<sup>[15-18]</sup> It has been reported that NCTD inhibits the proliferation and growth of a variety of human tumor cell lines including HeLa, CHO, HL-60, K562, BEL-7402, HepG2, A2780, G401, H460 and HT29 *in vitro*, and that it is used to treat human cancers, e. g. hepatic, gastric, colorectal and ovarian carcinoma, with stimulation of bone marrow and increase of peripheral leukocyte count.<sup>[19-31]</sup> However, few reports described the effect of NCTD on human gallbladder carcinoma.

In the present study, we investigated the *in vitro* effect of NCTD on proliferation, proliferation-related gene proteins PCNA and Ki-67 of human gallbladder carcinoma GBC-SD cells. NCTD was found to be obviously effective in inhibiting the proliferation of GBC-SD cells in a dose- and time-dependent manner. The result is consistent with the inhibitory effect of NCTD on other tumor cells reported elsewhere.<sup>[19-23]</sup> It also demonstrated that NCTD as an inhibitor of protein phosphatase type 2A can inhibit the growth of human

colon cancer HT29 cells, inhibit cell growth and arrest the cell cycle at G2/M phase in K562 human myeloid leukemia cells, and inhibit DNA synthesis in HL-60 cells and induce apoptosis in human tumor cells.<sup>[22-31]</sup> Our previous studies showed that NCTD increased significantly the cells in G2/M phase and decreased significantly the cells in S phase of GBC-SD cells, with a significantly increased rate of cell apoptosis.<sup>[13, 14]</sup> The mechanisms, of which NCTD inhibits the proliferation of human gallbladder carcinoma GBC-SD cells may be correlated with inhibition of cell proliferation, arresting of cell cycle, blockage of DNA synthesis, influence of cell metabolism, and inducement of cell apoptosis.

Genesis and development of the tumor are obviously related to the proliferation and apoptosis of the tumor cells. PCNA and Ki-67, gene proteins of cell proliferation-related, are the markers for cell proliferation.<sup>[32-35]</sup> In this study, after treatment with IC<sub>50</sub> NCTD for 48 hours, the positive cells and the positive indexes of expression of PCNA and Ki-67 proteins were decreased significantly ( $P < 0.001$ ). It was indicated that NCTD could influence the expression of the proliferation-related gene proteins PCNA and Ki-67 of GBC-SD cells. This may be one of the mechanisms, by which NCTD inhibits the proliferation and growth of human gallbladder carcinoma GBC-SD cells.

## Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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