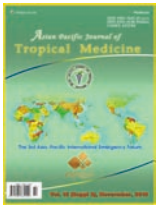

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Recombinant human calcineurin B inhibits orthotopic hepatocellular carcinoma xenograt growth in mice by promoting apoptosis

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ABSTRACT

Objective: To explore the effects of recombinant human calcineurin B (rhCNB) on hepatocellular carcinoma in mice.

Methods: An *in vivo* mouse model with hepatocellular carcinoma was established, and the mice were randomized into the rhCNB, positive control and vehicle treatments groups. Tumor growth was assessed *via* bioluminescence using a small animal imaging system. Relative tumor proliferation rate and tumor growth inhibition were calculated. The expression of p53 and caspase-9 proteins in tumors were detected by immunohistochemistry. *In vitro*, flow cytometry was used to quantify the cell-cycle stages and rate of apoptosis. Western blotting and quantitative real-time PCR assays were used to evaluate the effects of rhCNB on protein and gene expression of CDK1, cyclin B1, p53 and caspase-9.

Results: rhCNB at the higher dose significantly reduced tumor growth *in vivo* and caused tumor cell apoptosis *in vitro*. The rhCNB at the higher dose was as effective as cisplatin, and was safer.

Conclusions: rhCNB has potent pro-apoptotic effects on tumor cells *in vivo* and *in vitro* and is well tolerated *in vivo*.

1. Introduction

The World Cancer Report 2014 of World Health Organization showed that the numbers of cancer patients and cancer-related deaths are on the rise worldwide. In 2012, approximately 50% of new cancer cases occurred in Asia, mostly in China. More than 50% of the ~750 000 new cases of hepatocellular carcinoma (HCC) and ~700 000 HCC deaths in the world were found in China[1]. HCC is highly malignant and is difficult to treat[2]. The survival rate is low[3,4] resulting in a significant medical unmet need.

Calcineurin (CN) is a calcium and calmodulin-dependent serine/threonine protein phos-phatase[5], and consists of A and B subunits in a 1:1 ratio that form heterodimers. Calcineurin A (CNA) is the catalytic subunit, and calcineurin B (CNB) is the regulatory subunit

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affecting CNA activity. CN has important physiological functions in the immune system and cell apoptosis[6,7]. CNB has a low molecular weight and stable conformation, allowing for the production of recombinant human calcineurin B (rhCNB) in genetically engineered bacteria. Apart from its function in the CN holoenzyme, purified rhCNB binds to integrin αMβ2, resulting in the upregulated expression of tumor necrosis factor-related apoptosis-inducing ligand in macrophages and the induction of tumor cell apoptosis[8]. Moreover, in a yeast two-hybrid screen of a U937 cell cDNA library, CNB was shown to interact with proteasome subunit alpha type 7 and reactive oxygen species modulator 1 (ROMO1)[9]. Exogenous rhCNB increased the proteasome-associated cysteine protease activity of U937 cell lysates. In addition, it significantly inhibited the transcriptional activity of hypoxia-inducible factor 1 alpha (HIF-1) and the HIF-1α target gene, vascular endothelial growth factor in U937 cells. rhCNB interacted with ROMO1 in U937 cells and mouse leukemic monocyte macrophage RAW264.7 cells to produce reactive oxygen species. In addition, high concentrations of rhCNB increased mRNA expression of the cell-cycle-related genes *Bcl-2*, *cyclin D1*, and *p21WAF1/CIP1*. Taken together, these findings suggest that CNB is involved in the regulation of cell cycle and apoptosis. Using the MTT assay and high-content image analysis, rhCNB was shown to inhibit tumor cell proliferation and to promote tumor cell apoptosis *via* the mitochondrial pathway[10].

In this study, the effects of rhCNB on the mRNA and protein expression of cell-cycle-dependent protein kinase (CDK1), the key cell cycle regulatory protein, cyclin B1, and apop-tosis-related proteins p53 and caspase-9 were determined in human HCC (hHCC) cells.

2. Materials and methods

2.1. Experimental animals

A total of 90 male quarantine-qualified and specific pathogen-free (SPF) healthy adult Balb/c nude mice, with body weights ranging from 18 to 22 g, were provided by the Guang-dong Medical Laboratory Animal Center (Guangdong Province, China). The temperature in the animal facility was maintained at 20-26 ℃, with a relative humidity between 40% and 70%. Environmental conditions in the laboratory animal center were in line with the national standard for SPF-grade facilities. This study followed the animal protocols of the College's Animal Care and Use Committee and was ethically approved by the Hainan Medical University Committee (HYLL-2019-040) on 8 July 2019.

2.2. rhCNB, tumor cell lines, reagents, and major instruments

In this study, rhCNB was produced and supplied by Haikou Pharmaceutical Factory Co., Ltd. (Hainan Province, China). HL-7702 normal human hepatocytes and the luciferase-expressing hHCC BEL-7402 cell line were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The reagents used in this study included isoflurane (RWD Life Science, Guangdong Province, China), *D*-luciferin po-tassium salt (SynChem, Inc., Elk Grove Village, IL, USA), phosphate buffer (PBS), RPMI 1640 medium, trypsin (Gibco, Gaithersburg, MD, USA), fetal calf serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), propidium Iodide (PI; Trevigen, Gaithersburg, MD, USA), and the Annexin-V FITC Kit (Trevigen). Reagents for Western blot analysis included radio-immunoprecipitation assay buffer, bicinchoninic acid assay for protein quantification, SDS-PAGE reagent kit, rabbit anti-p53, CDK1, cyclin B1, and caspase-9 antibodies, and anti-rabbit IgG antibody were purchased from Abcam (Cambridge, UK).The catalog numbers of all antibodies were CDK1 (ab32384), P53 (ab31333), CyclinB1 (ab2949), Caspase-9 (ab32539/ab2324), β-actin (ab6721); respectively. SYBR® Premix Ex Taq™ II quantitative PCR (qPCR) reagent kit, PCR primer DNA replication, TaKaRa MiniBEST Universal RNA Extraction Kit, and the PrimeScriptTM RT reagent Kit with gDNA Eraser for reverse transcription were purchased from TakaRa (Tokyo, Japan). The PV-9000 Polymer Detection System for immunohistological staining was purchased from GBI Labs (Mukilteo, WA, USA). The instruments used included the IVIS Ki Kinetic small animal live imaging system (Caliper Life Sciences, Inc, Hopkinton, MA, USA), VIP 3000 small animal anesthesia system (Matrx, Versailles, OH, USA), Bx41 microscopy system (Olympus Co., Tokyo, Japan), YNERGY/HTX microplate reader (BioTek Instruments, Inc, Winooski, VT, USA), BD-Accuri™-C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA), DYY-6D type electrophoresis apparatus (Beijing Liuyi Biotechnology Co., Ltd., Beijing, China), Tanon-5500 chemiluminescence analyzer (Biotanon Science&Technology Co, Ltd., Shanghai, China), C-96/T/H(a) gene amplifier (Thermo Fisher Scientific, Shanghai, China), and Mx3005P Real-Time PCR system (StrataGene, Agilent, Santa Clara, CA, USA).

2.3. MTT viability assay

HL-7702 normal human hepatocytes and tumor cells were plated at 5×10^4–10×10^4 cells/mL in 96 well plates. rhCNB solution was diluted using RPMI 1640 culture medium containing 10% FBS, and RPMI 1640 culture medium containing 10% FBS was used as the vehicle control. Five wells were used per treatment condition. MTT solution was added to each well of a 96-well plate, followed by incubation for 4 h for complete crystallization. Then the supernatant was discarded, and the formazan crystals were not removed. MTT solution (150 μL) was added into each well, and then was gentle stirred on a low-speed shaker for 10 min for the complete dissolution of MTT formazan crystals. Absorbance was read at 490 nm using a microplate reader. Tumor cell inhibition rate was calculated according to the following equation: Tumor cell inhibition rate (%) $=1-(OD_{experimental}/ODc_{ontrol})\times100\%$. The IC_{50} was calculated using SPSS v22.0 (IBM Corp., Armonk, NY, USA).

2.4. Subcutaneous inoculation of tumor cells in Balb/c nude mice

The hHCC BEL-7402 cells were cultured to the logarithmic growth phase, trypsinized, and centrifuged to prepare a cell suspension at a density of 1×10^7 cells/mL. Cell suspension (0.2 mL) was injected subcutaneously in the right armpit of Balb/c nude mice. After injection, a clean and dry cotton swab was used to compress the injection site to avoid leakage of the cell suspension.

2.5. Orthotopic hHCC model in Balb/c nude mice

Subcutaneous tumor tissue was harvested from each animal and cut into 2 mm×2 mm×1 mm tissue blocks. Under anesthesia, skin and muscle layers were dissected with tissue scissors to a length of approximately 1 cm to expose the liver. A 4×10 suture needle was used to tie the tumor tissue block exactly between the right and left lobes of the liver without contact with other tissue. The abdominal cavity was sutured. All animals had wound disinfection and were carefully monitored and fed. Once tumors reached 100 to 300 mm^3 in size, mice were randomly divided into nine groups (n=10 each): rhCNB low-dose injection group (L-rhCNB, 10 mg/kg/d); rhCNB medium-dose injection group (M-rhCNB, 20 mg/kg/d); rhCNB high-dose injection group (H-rhCNB, 40 mg/kg/d); rhCNB dose escalation group (DE-rhCNB, dose increased every 3 d from the initial dose of 10 mg/kg/d to 20 mg/kg/d, 40 mg/kg/d, 80 mg/kg/d, and 160 mg/kg/d); recombinant human interleukin-2 (IL-2) injection positive control group (rhIL-2, 164 000 IU/kg/d); Low-dose cisplatin injection positive control group (L-cisplatin, 1 mg/kg/d); hydroxycamptothecin injection positive control group (HCPT, 2 mg/kg/d); and vehicle control group. rhCNB was administered daily *via* intravenous injection to the tail vein, with an administration volume of 20 mL/kg for all 3 dose groups. All groups received drug treatment daily for 15 consecutive days, followed by a recovery period of 7 d, except for the H-cisplatin group, which had a 5-day administration period. Animals were weighed before treatment initiation, twice a week during treatment, and 7 d after the last treatment administration. In addition, the tumor diameter was measured with *in vivo* imaging at the same frequency. Seven days after the last administration, animals in each group were euthanized by cervical dislocation to collect and weigh the complete solid tumor.

2.6. Detection of tumor growth in orthotopic liver transplantation in nude mice

After orthotopic transplantation of liver cancer in nude mice, inhalation depth anesthesia was performed using the Matrx 3000 VIP gas anesthesia system, with isoflurane used as the anesthetic agent. After the animals were anesthetized, the Caliper IVIS Kinetic small animal living imaging system was used to detect tumors biofluorescence in the nude mice, and the growth of tumors before and after administration was compared among the experimental groups. The first 5 min of the test was 0.1 mL/10 g body weight and 30 mg/mL of *D*-fluorescein potassium salt was injected into the abdominal cavity.

2.7. Immunohistochemistry of rhCNB-treated solid tumors

Levels of the apoptosis-related proteins, caspase-1, caspase-3, caspase-8, caspase-9, B-cell lymphoma 2 (Bcl-2), and p53 in transplanted HCC tumors were measured by immuno-histochemistry assay. After conventional paraffin embedding and tumor tissue sectioning, the tumor tissue sections were deparaffinized and hydrated. Tumor sections were incubated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After three washes in PBS for 2 min each, the tumor tissue sections were incubated with the corresponding anti-caspase-9, p53 primary antibodies at 37 ℃ for 1–2 h. After three washes in PBS for 2 min each, the Polymer Helper reagent was added to the tissue sections and incubated at 37 ℃ for 20 min. After three washes in PBS for 2 min each, polyperoxidase-anti-mouse/rabbit IgG antibody was added to the tissue sections and incubated at 37 ℃ for 20–30 min. After three washes in PBS for 2 min each, 3,3'-diaminobenzidine substrate was used for color development, followed by thorough washing in running water, counterstaining, dehydration, tissue clearing, and section mounting.

2.8. Flow cytometry

Tumor cell suspension at approximately 105 cells/mL was seeded in a new culture flask and grown for 24 h. Then the culture medium was discarded and the suspension was incubated with a new culture medium containing rhCNB (1.5 mg/mL; 3.0 mg/mL) or vehicle control for 48 h. Then cells were trypsinized and centrifuged, followed by three PBS washes. All cells were fixed in ice-cold 75% ethanol overnight at 4 ℃, followed by centrifugation and PBS washing of cell pellets three times. Cell-cycle detection solution (PI staining) was used to stain the cell suspension (at a density of 106 cells/mL) for flow cytometry. For flow cytometric quantification of the cell cycle and apoptosis, 500 μL cell suspension was stained with 5 μL phenyl isothiocyanate (PITC) dye and 5 μL PI dye in the dark for 15 min.

2.9. Western blot analyses

For Western blot analyses, tumor cells were grown to 80% confluency, then the culture medium was replaced with FBS-free culture medium to fast the cells for 24 h and synchronize their cell-cycle stage. Then the serum-free medium was replaced with fresh culture medium containing FBS and rhCNB (0 mg/mL; 2.0 mg/mL; 4.0mg/mL), and cells were incubated for 24 h followed by three washes in PBS and the addition of lysis buffer to extract total protein from the cells. Protein concentration was quantified using the bicinchoninic acid assay and microplate reader. Standard Western blotting technique was used to determine the expression levels of cell-cycle related proteins CDK1 and cyclin B1, and apoptosis-related proteins p53 and caspase-9 expression.

2.10. Quantitative RT-PCR

Bel-7402 human HCC cells were cultured to about 80% confluence and then divided into the following three groups: the blank control group (fresh FBS alone), the low-dose group (2 mg/mL rhCNB), and the high-dose group (4 mg/mL rhCNB). The next day, cells were washed once with PBS and then lysed in lysis buffer. RNA was extracted, the concentration of RNA was detected, and RNA was transcribed to cDNA. Finally, qPCR detection of target genes (*CDK1*, *cyclin B1*, *p53* and *caspase-9*) was performed. Cultured hHCC BEL-7402 cells were treated with rhCNB in fresh culture medium containing FBS for 1 d, after which cells were washed once in PBS followed by the addition of lysis buffer for RNA extraction. Quantification of total RNA and cDNA reverse transcription was performed using standard procedures. Then qPCR was used to measure *CDK1*, *cyclin B1*, *p53*, and *caspase-9* gene expression using the following conditions: 1 μL each of 10 mmol/μL forward and reverse primer, 2 μL cDNA template, 12.5 μL SYBR® Premix Ex Taq™ II, and 8.5 μL sterilized water (25 μL total reaction volume). qPCR reaction conditions were 95 ℃ denaturation for 3 min; and 45 cycles of 95 ℃ denaturation for 20 s, 60 ℃ annealing for 30 s, and 72 ℃ elongation for 30 s. Primer sequences were as follows: *p53* forward primer: 5'ACCTAGGGAAACTACTTCCTGAAA 3'; *p53* reverse primer: 5'CTGGCATTCTGGGAGCTTCA 3'; *CDK1* forward primer: 5'CTGGGGTCAGCTCGTTACTCA 3'; *CDK1* reverse primer: 5'TTTGGGAAATGTATTCTTATA 3'; *cyclin B1* forward primer: 5'GCAACCTCCAAGCCCGGACTG 3'; *cyclin B1* reverse primer: 5'AAATAGGCTCAGGCGAAAGTT 3'; *caspase-9* forward primer: 5'TGTCCTACTCTACTTTCCCAGGTTTT 3'; and *caspase-9* reverse primer: 5'GTGAGCCCACTGCTCAAAGAT 3'.

2.11. Statistical analysis

SPSS 22.0 (SPSS Inc., Chicago, IL, USA) software was used for the statistical analysis. Experimental data were analyzed by one-way analysis of variance to compare differences between drug-treatment groups and with the vehicle control group. Results are presented as the mean±standard deviation. $P<0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of tumor cell growth

Results of MTT assay showed that treatment with 3 mg/mL rhCNB resulted in >80% inhibition of hHCC BEL-7402 cell growth, with an IC_{50} of 2.33 mg/mL. At the same concentration, rhCNB injection had no inhibitory effects on HL-7702 normal human hepatocytes, suggesting that rhCNB injection has no inhibitory effects on normal human cells (Table 1).

Table 1. Tumor growth inhibition (%) of tumor cells treated with rhCNB (%, *n*=5).

rhCNB mg/mL	Tumor growth inhibition	
	hHCC BEL-7402	Human normal hepatocytes HL-7702
1 mg/mL	9.2	1.1
2 mg/mL	27.4	1.1
2.5 mg/mL	58.8	1.0
3 mg/mL	84.3	1.2
3.5 mg/mL	90.5	3.5
4 mg/mL	94.6	4.1

hHCC: human hepatocellular carcinoma; rhCNB: recombinant human calcineurin B.

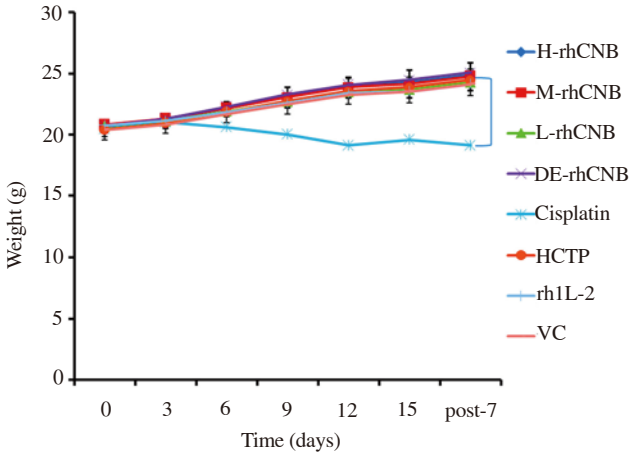

Figure 1. Animal weight gain in each group. VC: vehicle control group; DE-rhCNB, rhCNB dose-escalation group; H/M/L-rhCNBrhCNB/L-rhCNB, rhCNB high/medium/low-dose injection group; HCPT, hydroxycamptothecin injection positive control group; rhCNB: ecombinant human calcineurin B; rhIL-2, recombinant human interleukin-2.

Table 2. Effects of rhCNB on the cell cycle and the apoptosis of human hepatocellular carcinoma BEL-7402cells (x̄±SD, *n*=3).

Items	Control	rhCNB injection (1.5 mg/mL)	rhCNB injection (3.0 mg/mL)
Cell-cycle (%)			
G_0/G_1	75.50±0.83	64.51±1.11***	52.43±0.92***
S	13.85±0.24	15.17±0.82	16.51±0.70**
G_2/M	9.05±0.22	13.09±0.67***	12.32±0.85**
Apoptosis (%)			
Q1-LL	85.9±1.2	0.5±0.1	4.3±0.1
Q1-LR	84.9±0.6	0.5±0.1	6.8±0.3***
Q1-UR	62.7±1.9***	5.6±0.2***	16.9±0.5***

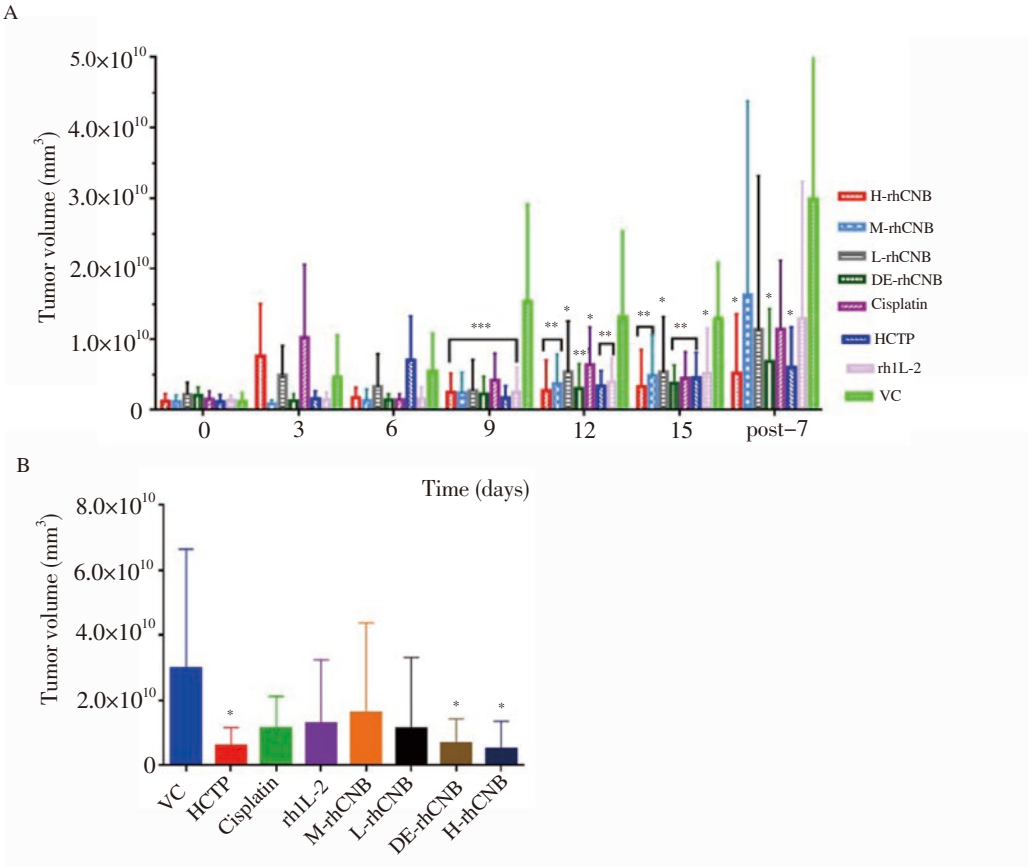

Figure 2. Tumor volume of growth over time with different treatments and quantification 7 d after treatment. A: Quantification of tumor-derived bioluminescence as an approxima-tion of *in vivo* tumor volume over time for different treatment groups. B: Quantification of tumor-derived bioluminescence as an approximation of *in vivo* tumor volume 7 d after the last treatment. VC: vehicle control group; DE-rhCNB, rhCNB dose-escalation group; H/M/L-rhCNBrhCNB/L-rhCNB, rhCNB high/medium/low-dose injection group; HCPT, hydroxycamptothecin injection positive control group; rhIL-2, recombinant human interleukin-2.

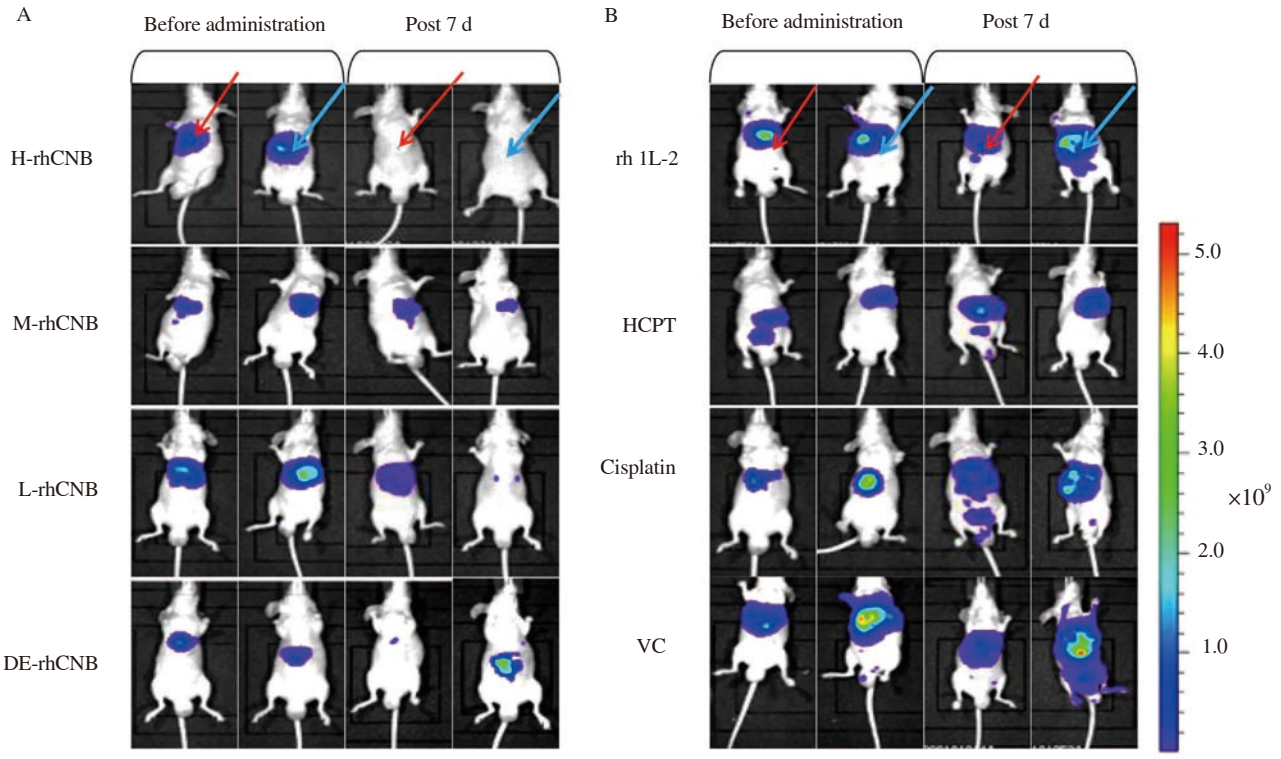

Figure 3. Bioluminescence of BEL-7402 tumor xenografts. (A-B) Color-coded bioluminescence maps of the animals in different groups before and 7 d after treatment for each animal in each treatment group. The left column in each panel shows bioluminescence before treatment, and the right column shows bioluminescence 7 d after treatment completion. A: recombinant human calcineurin B (rhCNB) high (H-rhCNB), medium (M-rhCNB), low (L-rhCNB), and dose-escalation group (DE-rhCNB), respectively; B: cisplatin positive control, hydroxycamptothecin (HCPT) positive control, recombinant human interleukin-2 (rhIL-2) positive control, and vehicle (VC) control group, respectively.

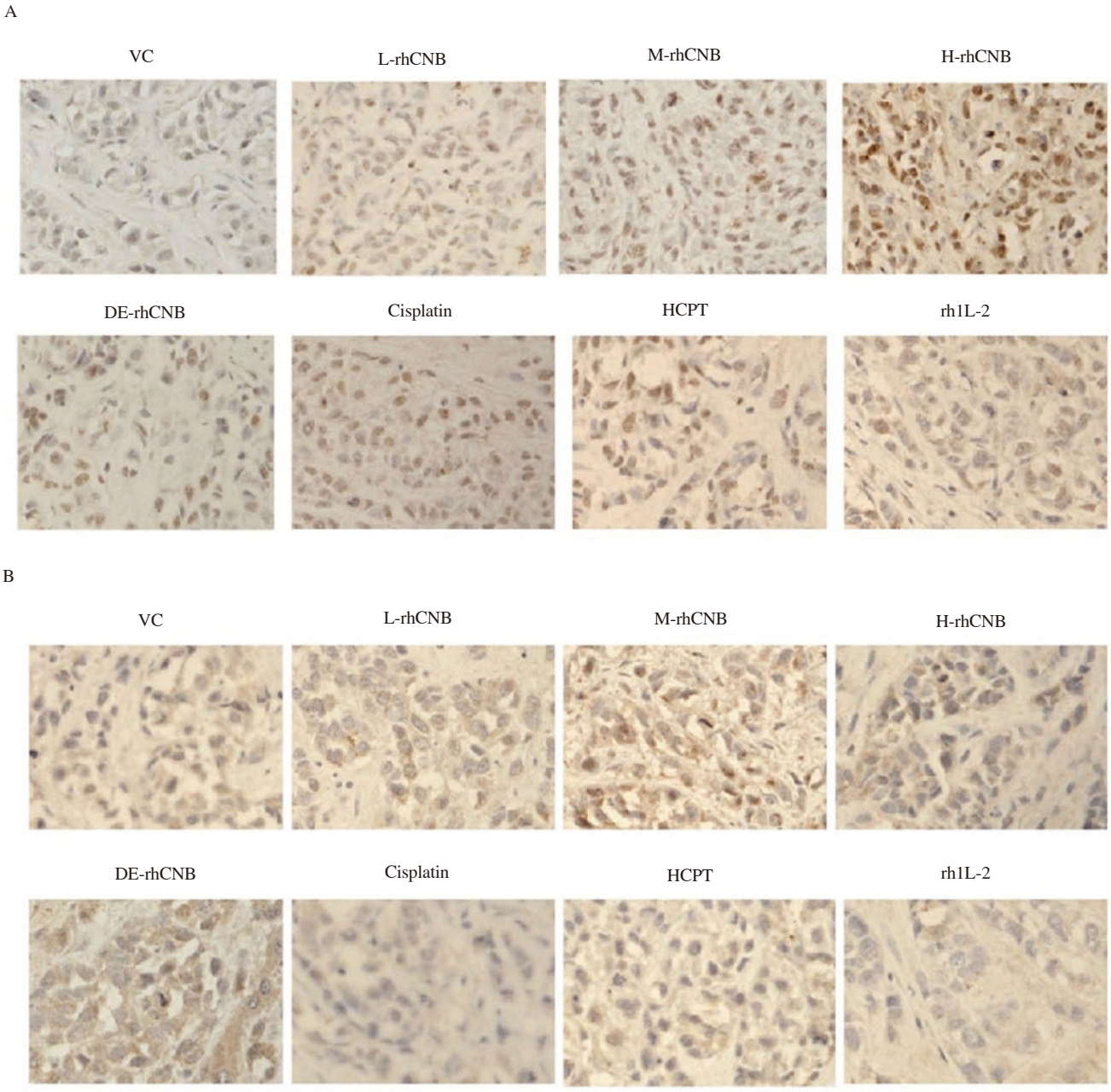

Figure 4. Immunohistochemistry result of p53 (A) and caspase-9 (B) in tumor tissue. Vehicle control (VC) group; recombinant human calcineurin B (rhCNB) low, medium, high-dose, dose-escalation groups (L/M/H/DE-rhCNB), cisplatin group; hydroxycamptothecin (HCPT) group; and recombinant human interleukin-2 (rhIL-2) group. Magnification, 400 ×.

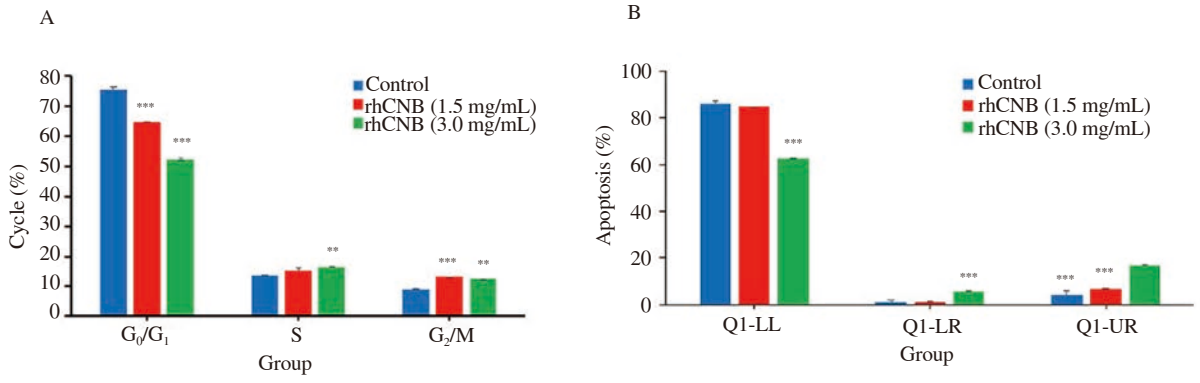

Figure 5. Flow cytometry detection of the cell cycle (A) and apoptosis (B) in hHCC BEL-7402 cells after rhCNB treatment. Cells were stained with PITC and PI 48 h after treatment. Q1-LL refers to the percentage of normal cells; "Q1-LR" refers to the percentage of early apoptotic cells, "Q1-UR" refers to the percentage of late apoptotic and dead cells. Cell cycle is "G_0/G_1", "S", "G_2/M".

3.2. Changes in body weight

Cisplatin treatment caused body weight loss (starting from day 3), compared to the vehicle control group (P<0.001). The body weight of the remaining groups did not significantly differ from the vehicle control group throughout the entire observation period (P>0.05, 7 d after the last administration), suggesting that rhCNB had no adverse effects on the weight gain of the experimental animals (Figure 1).

3.3. Tumor cell growth in the hHCC-bearing mouse model

A small animal imaging system was used to measure tumor growth *in vivo* prior to and during treatment (Figure 2). When compared with the vehicle control, significant inhibition of tumor growth was detected starting from treatment day 9 (P<0.001). After 12 and 15 consecutive days of treatment, significant inhibition of tumor growth was observed in all treatment groups. Seven days after the last treatment administration, only the high-dose rhCNB, rhCNB dose escalation, and hydroxycamptothecin (HCPT) groups significantly reduced *in vivo* tumor growth, relative to the vehicle control (P <0.05). Figure 3 demonstrate the striking reduction of tumor cell-derived bioluminescence 7 d after treatment completion, which was only seen in animals treated with high-dose rhCNB and rhCNB dose escalation. These findings were corroborated by post-mortem tumor excision. Notably, the efficacy of rhCNB after treatment completion was more sustained, when compared with the positive control groups such as those treated with cisplatin, hydroxycamptothecin, and IL-2, whereas adverse effects on body weight or obvious toxicity were not observed.

3.4. Effects of rhCNB on the expression of apoptosis-related proteins in tumors

The expression of p53 and caspase-9 was low in tumor cells of the vehicle control group and was dose-dependently increased by rhCNB treatment (Figure 4). Cisplatin also induced p53 and caspase-9 expression, while rhIL-2 did not. Hydroxycamptothecin induced p53 expression but not caspase-9 expression. No significant difference in caspase-1, caspase-3, caspase-8, and Bcl-2 expression was found between rhCNB and the ehicle control groups.

3.5. Effects of rhCNB treatment on the cell cycle and apoptosis of hHCC BEL-7402 cells

Flow cytometry analysis of DNA ploidy in hHCC BEL-7402 cells demonstrated that compared with vehicle, rhCNB dose-dependently and significantly decreased the proportion of cells in the G_0/G_1 phase, and increased the proportion of cells in the sub G_0/G_1 region (Table 2 and Figure 5A). In line with the increase of cells with sub-diploid DNA content, flow cytometric analysis confirmed that rhCNB dose-dependently and significantly increased the percentage of cells in the late stages of apoptosis (Table 2 and Figure 5B). Western blot analysis of cell cycle proteins showed rhCNB-mediated downregulation of CDK1 and cyclin B1, which are regulatory proteins of the cell cycle G_2/M phase. Cleaved caspase-9 and p53, two apoptosis-related proteins, were also downregulated by rhCNB compared with the vehicle control group. The effects of rhCNB on protein expression were also dose-dependent (Figure 6A). Expression of CDK1, cyclin B1, p53, and caspase-9 was also quantified at the mRNA level by qPCR. The results indicated that rhCNB increased the mRNA expression of *CDK1* and *p53*, and decreased the mRNA expression of *cyclin B1* and *caspase-9* (Figure 6B).

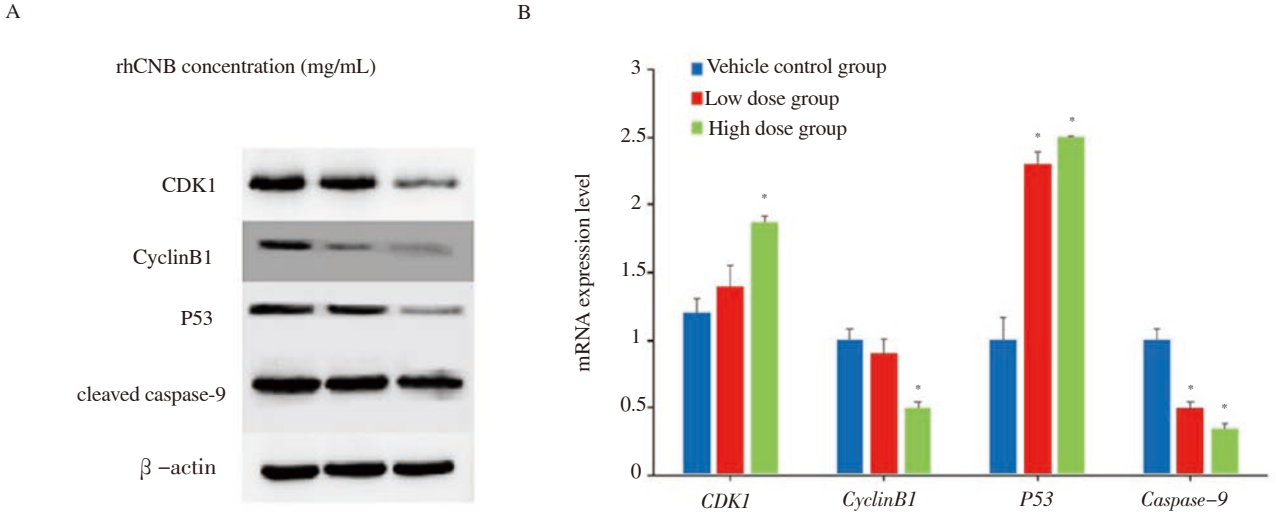

Figure 6. Western blot analysis and PCR results of rhCNB-treated hHCC BEL-7402 cells. A: Representative images of cell-cycle-dependent protein kinase (CDK1), cyclin B1, p53, cleaved caspase-9, and β-actin protein blots; B: Relative mRNA expression of cell-cycle- and apoptosis-related genes in hHCC BEL-7402 cells. mRNA expression is quantified rela-tive to vehicle control (anti-*CDK1*, *cyclin B1*, *p53*, and *caspase-9* expression, respectively).

4. Discussion

This study extends the findings of prior studies by exploring the anti-tumor activity of rhCNB in mice model of HCC with the hHCC BEL-7402 cell line. The effects of rhCNB were measured *in vivo* by quantifying bioluminescence from luciferase-expressing HCC cells in tumor transplants, as well as *ex-vivo* by weighing tumor explants. rhCNB dose-dependently reduced tumor size and growth compared to the vehicle control. It was as potent as cisplatin, and more potent than hydroxycamptothecin and rhIL-2. Importantly, unlike the other positive control treatments, the effects of rhCNB were sustained for at least 7 d after treatment was discontinued, and showed no toxicity or negative effects on body weight. In contrast, the most potent positive control group, cisplatin treatment, caused significant weight loss in treated animals. The intravenous route of administration and the recombinant formulation of rhCNB in the absence of CNA, the catalytic subunit, suggest that the anti-tumor effects of rhCNB were not mediated by the protein phosphatase activity of the CN holoenzyme. Previous studies have shown that rhCNB binds to CS11b (integrin αM) on macrophages and to toll-like receptors, and *via* these receptor pathways it can activate apoptosis and/or an immune response against tumor tissue[8,11,12]. In addition, rhCNB exerts anti-tumor effects *via* increased proteasomal degradation of HIF-1α, thereby preventing vascular endothelial growth factor expression[9]. The necrotic part considered is composed of dead cells during treatment and dead cells at the later stage of apoptosis. The induction of apoptosis is the main form of cell death. Many previous studies have proved that the induction of apoptosis is the main form of cell death. Apoptosis seems to be the main mechanism of RCNB induced cell death. In this study, rhCNB was shown to have robust pro-apoptotic effects on hHCC cells *in vitro*, as indicated by the MTT assay and flow cytometry results. These results indicate that rhCNB is capable of directly inducing apoptosis in tumor cells in the absence of immune cells. In addition, the mouse strain used in this study was immunocompromised to enable tumor transplant, and reduce the likelihood of a significant immune response contributing to the anti-tumor effects of rhCNB. Immunostaining of transplanted tumor tissue showed that rhCNB dose-dependently upregulated protein levels of the pro-apoptotic proteins p53 and caspase 9. p53 and caspase play critical roles in tumor suppression and apoptosis in multiple tumors[13-16]. While rhCNB also caused an increase in apoptosis *in vitro*, the protein levels of p53 and cleaved caspase 9 were decreased by rhCNB treatment, as assessed by Western blotting. These results suggest that at least *in vitro*, apoptosis induction may not depend on the expression of these two pro-apoptotic proteins, and instead, may be mediated by other apoptotic pathways (*e.g.*, reduced expression of Bcl-2/Bcl-x), which has been observed in HepG2 hepatoma cells upon rhCNB treatment[12]. Protein levels of the cell cycle-associated proteins CDK1 and cyclin B1 were decreased *in vitro* by rhCNB, in line with findings of reduced proliferation in the MTT assay and the increased proportion of cells with sub-diploid DNA content shown by flow cytometry. CDK1 and cyclin B1 protein levels are correlated with cell proliferation[17] and tumor cell growth[18], including in HCC[19]. At the mRNA level *in vitro*, rhCNB reduced expression of cyclin B1 and caspase 9, but increased expression of CDK1 and p53. In this study, we showed that rhCNB downregulated p53 expression in hepatoma cells, suggesting that p53 may be involved in the induction of apoptosis or cell cycle arrest in hepatoma cells. However, additional studies are needed to confirm these findings. These results indicate that for CDK1 and p53, rhCNB-mediated regulation of protein levels *in vitro* is post-transcriptional.

In summary, the results of this study provide additional evidence on the anti-tumor effects of rhCNB in an *in vivo* animal model, demonstrating its potential as an anti-cancer therapy. In addition, the study demonstrates that rhCNB has direct pro-apoptotic effects *in vitro* on cancer cells. The discrepancy between *in vivo* and *in vitro* effects of rhCNB on protein expression need to be explained in further study. Similarly, the extent to which rhCNB *in vivo* effects are direct effects on tumor cells or are mediated *via* the immune system, needs to be addressed in future experiments. Addressing these questions will help determine a rationale combination therapy that combines rhCNB with other anti-tumor agents to improve efficacy, as the combination of 5-fluorouracil with rhCNB[10].

Conflict of interest statament

The authors declare that they have no conflcit of interest.

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Authors' contribution

S.J.Z. and S.H.G. conceived and coordinated the study. X.D.Z., Z.H.H., L.P.Z., G.H.Y. and S.H.T. carried out the experiments. M.G.L. and L.Z.F. collected and analysed the data. H.H.L. and Y.D.L. contributed reagents/materials. Both S.J.Z. and S.H.G. authors contributed to the final version of the manuscript.

References

[1] Teicher BA. *Anticancer drug development guide: Preclinical screening, clinical trials, and approval* . 2nd ed. New York: Humana Press; 2004.

[2] Balogh J, Victor D 3rd, Asham EH, Burroughs SG, Boktour M, Saharia A. et al. Hepato-cellular carcinoma: a review. *J Hepatocell Carcinoma* 2016; **3**: 41-53.

[3] Soerjomataram I, Lortet-Tieulent J, Parkin DM, Mathers C, Forman D, Bray F. Global burden of cancer in 2008: a systematic analysis

of disability-adjusted life-years in 12 world regions. *Lancet* 2012; **380**(9856): 1840-1850.

[4] Altekruse SF, McGlynn KA, Reichman ME. Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. *J Clin Oncol* 2009; **27**(9): 1485-1491.

[5] Rusnak F, Mertz P. Calcineurin: form and function. *Physiol Rev* 2000; **80**(4): 1483-1521.

[6] Cheng J, Tang W, Su Z, Guo J, Tong L, Wei Q. Calcineurin subunit B promotes TNF-alpha-induced apoptosis by binding to mitochondria and causing mitochondrial Ca^{2+} overload. *Cancer Lett* 2012; **321**(2): 169-178.

[7] Musson RE, Cobbaert CM, Smit NP. Molecular diagnostics of calcineurin-related pathologies. *Clin Chem* 2012; **58**(3): 511-522.

[8] Liu L, Su Z, Xin S, Cheng J, Li J, Xu L, et al. The calcineurin B subunit (CnB) is a new ligand of integrin αM that mediates CnB-induced Apo2L/TRAIL expression in macrophages. *J Immunol* 2012; **188**(1): 238-247.

[9] Li N, Zhang Z, Zhang W, Wei Q. Calcineurin B subunit interacts with proteasome subunit alpha type 7 and represses hypoxia-inducible factor-1α activity via the proteasome pathway. *Biochem Biophys Res Commun* 2011; **405**(3): 468-472.

[10]Zhang W, Zhong Y, Cui H, Wang L, Yang R, Su Z. et al. Combination of calcineurin B subunit (CnB) and 5-fluorouracil reverses 5-fluorouracil-induced immunosuppressive effect and enhances the antitumor activity in hepatocellular carcinoma. *Oncol Lett* 2017; **14**(5): 6135-6142.

[11]Yang J, Qin N, Zhang H, Yang R, Xiang B, Wei Q. Cellular uptake of exogenous calcineurin B is dependent on TLR4/MD2/CD14 complexes, and CnB is an endogenous lig-and of TLR4. *Sci Rep* 2016; **6**: 24346.

[12]Yang Y, Yang H, Yang J, Li L, Xiang B, Wei Q. The genetically engineered drug rhCNB induces apoptosis via a mitochondrial route in tumor cells. *Oncotarget* 2017; **8**(39): 65876-65888.

[13]Wang XW, Harris CC. TP53 tumor suppressor gene: clues to molecular carcinogenesis and cancer therapy. *Cancer Surv* 1996; **28**(11): 169-196.

[14]Götz C, Montenarh M. p53: DNA damage, DNA repair, and apoptosis. *Rev Physiol Biochem Pharmacol* 1996; **127**: 65-95.

[15]Schuler M, Green DR. Mechanisms of p53-dependent apoptosis. *Biochem Soc Trans* 2001; **29**(6): 684-688.

[16]Kim B, Srivastava SK, Kim SH. Caspase-9 as a therapeutic target for treating cancer. *Expert Opin Ther Targets* 2015; **19**(1): 113-127.

[17]Porter LA, Donoghue DJ. Cyclin B1 and CDK1: nuclear localization and upstream regulators. *Prog Cell Cycle Res* 2003; **5**: 335-347.

[18]Sutherland RL, Watts CK, Musgrove EA. Cyclin gene expression and growth control in normal and neoplastic human breast epithelium. *J Steroid Biochem Mol Biol* 1993; **47**(1-6): 99-106.

[19]Park TJ, Kim JY, Oh SP, Kang SY, Kim BW, Wang HJ. et al. TIS21 negatively regulates hepatocarcinogenesis by disruption of cyclin B1-Forkhead box M1 regulation loop. *Hepatology* 2008; **47**(5): 1533-1543.