# Effects of a novel tripeptide, tyroserleutide (YSL), on cell cycle progression of human hepatocellular carcinoma

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**The objective of this study was to develop a new small molecular peptide, tyrosyl-seryl-leucine (tyroserleutide, YSL), as an anticancer drug. Our study investigated the effects of YSL on human hepatocellular carcinoma and cyclin, and explored its antitumor mechanism *in vitro*. In-vitro effects of YSL on human hepatocarcinoma cell BEL-7402 were assayed by the MTS (dimethylthiazol-carboxymethoxyphenyl-sulfophenyl – tetrazolium inner salt) method. The ultrastructure of tumor cells was observed by electron microscopy. DNA ladder was used to investigate apoptosis of BEL-7402 cells. The effects of YSL on the cell cycle of BEL-7402 cells were determined by flow cytometry. Expression of PCNA, P21, and P27 were investigated by real-time PCR and western blot in BEL-7402 cells. YSL inhibited the proliferation of BEL-7402 cells *in vitro*, induced DNA fragmentation, and changed their ultrastructure evidently, resulting in the necrosis and apoptosis of tumor cells. YSL interrupted cell cycle of tumor cells at $G_0/G_1$ and postponed their proceedings. YSL markedly enhanced the mRNA and protein expression of P21 and P27, and decreased the expression of PCNA of tumor cells. In conclusion, YSL significantly inhibited the growth of human hepatocellular carcinoma BEL-7402 cells and its anti-tumor effects may result from the upregulation of cyclin P21 and P27, and downregulation of cyclin PCNA. *Anti-Cancer Drugs* 20:534–542 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Liver cancer is the sixth most common cancer worldwide and the third most common cause of death from cancer [1]. Over 80% of new cases occur in developing countries. In China alone, liver cancer accounts for over 53% of all liver cancer cases and deaths worldwide. Incidence of liver cancer rises every year in countries all over the world. Surgery, radiation, and chemotherapy are options for treatment, with the difficult early discovery and low rate of surgery exairesis, and prognosis for patients with liver cancer is very poor [2]. Therefore, it is essential to find new drugs for liver cancer therapy.

The spleen contains many immune cells such as macrophages, reticuloendothelial cells, lymphocytes, and dendritic cells. These cells and the molecules they produce may modify signal transduction of tumor cells and tumor cell killing. Many different peptides with anticancer activity or activating immune system have been identified from splenic tissues [3–5]. This provides a rationale to explore the spleen for peptides that target cancer. Accordingly, we explored the biological character of 132 small molecule peptides, which were extracted from the spleen of pigs. One peptide, tyrosyl-seryl-leucine (tyroserleutide, YSL) is a tripeptide consisting of three natural amino acids, L-tyrosine, L-serine, and L-leucine, with the molecular structure $C_{18}H_{27}N_3O_6$, and molecular weight of 381.42. Earlier, YSL has been shown to have antitumor effects, with the inhibition ratio of human hepatocellular carcinoma BEL-7402 tumor in nude mice greater than 40%, though unknown mechanism [6–8]. Research has shown that loss of control of cell growth and differentiation is the essential characteristic of tumor cells compared to normal cells. Disorders of the cell cycle may be the main mechanism of excessive proliferation and insufficient apoptosis [9]. Cyclin P21, P27, and PCNA (proliferating cell nuclear antigen) have an influence on the growth and proliferation of tumor cells by regulating the cell cycles. In this study, we investigated the effects of YSL on cell growth, cell cycle, apoptosis, and the expression of cyclin P21, P27 and PCNA.

## Materials and methods

### Cell line

The human hepatocellular carcinoma BEL-7402 cell line [10] was provided by the Tumor Medicine Institute of the China Medical Academy of Science (Beijing, China).

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The human hepatocellular carcinoma BEL-7402 cell line was cultured in RPMI-1640 medium (GIBCO, Invitrogen Corp., Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah, USA) in a humidified atmosphere of 5% $CO_2$ at 37°C.

### MTS proliferation analysis

The concentration of BEL-7402 cells growing in log phase was adjusted to $1 \times 10^5$/ml in RPMI-1640 medium with 10% FBS. BEL-7402 cells were placed in a 96-well cell-culture plate at 100 µl/well. The cells were incubated for 24 h at 37°C under 5% $CO_2$. The experiment included four groups treated with different doses of YSL (Shenzhen Kangzhe Pharmaceutical Co. Ltd., Shenzhen, China), and a negative control group. Each group consisted of eight parallel wells. YSL (100 µl/well) was added to the wells of the treatment group, and plain RPMI-1640 (100 µl/well) to those of the negative control, respectively. The final concentrations of YSL were 0.2, 0.4, 0.8, and 1.6 mg/ml. The cells were incubated for 48 h at 37°C, 5% $CO_2$, and then pelleted by centrifugation at 150*g* for 10 min. The supernatants were discarded, 20 µl/well MTS (dimethylthiazol-carboxymethoxyphenyl-sulfophenyl – tetrazolium inner salt) (Promega Co., Madison, USA) and 100 µl RPMI-1640 was added to the cell pellets. Incubation was continued for 2 h. An ELISA (enzyme-linked immunosorbent assay) reader (Model 550; Bio-Rad Inc., Hercules, USA) was used to measure the optical density (OD value at 490 nm) of each well. The following formula was used to calculate the inhibition of cell growth: growth inhibition (%) = [(mean OD value of control group – mean of OD value of treatment group)/mean OD value of control group] × 100%.

### Electron microscopy

The concentration of BEL-7402 cells growing in log phase was adjusted to $5 \times 10^4$/ml in RPMI-1640 medium with 10% FBS. The cells were placed in 60 mm cell-culture dishes at 4 ml/dish. The cells were incubated for 24 h at 37°C under 5% $CO_2$. The experiment included three groups treated with different doses of YSL and a negative control group. Each group contained three parallel dishes treated similarly. YSL (4 ml/dish) was added to the treatment group dishes, and plain RPMI-1640 (4 ml/dish) was added to the negative control, respectively. The final concentrations of YSL were 0.4, 0.8, and 1.6 mg/ml. The cells were incubated for 48 h at 37°C under 5% $CO_2$. The cells were immediately fixed in 2.5% glutaraldehyde solution and were glued to blank epon/araldite blocks with fast cyanoacrylate adhesive. The thin sections were taken with a diamond knife and were picked up on formvar-coated slot grids and post-stained with uranyl acetate and lead citrate. The ultrastructures of the cells were observed with a JEOL-100CX-type transmission electron microscope (JEOL Ltd., Tokyo, Japan).

### DNA ladder

The concentration of BEL-7402 cells growing in log phase was adjusted to $2 \times 10^5$/ml in RPMI-1640 medium with 10% FBS. The cells were placed into 100 mm cell-culture dishes at 10 ml/dish. The cells were incubated for 24 h at 37°C under 5% $CO_2$. The experiment included four groups treated with different doses of YSL and a negative control. Each group contained three parallel dishes treated similarly. YSL (10 ml/dish) was added to the treatment group dishes, and plain RPMI-1640 (10 ml/dish) was added to the negative control, respectively. The final concentrations of YSL were 0.2, 0.4, 0.8, and 1.6 mg/ml. The cells were incubated for 48 h at 37°C under 5% $CO_2$. By following the instruction the manufacturer's protocol of ApopLadder Ex kit (Takara Bio Inc., Shiga, Japan), extracted DNA were electrophoresed on a 1.5% agarose gel containing ethidium bromide 2 mg/ml. DNA fragments were visualized by an ultraviolet transillumination gel imaging system (Gel Doc EQ, Bio-Rad Inc.).

### Flow cytometry

The concentration of BEL-7402 cells growing in log phase was adjusted to $1 \times 10^5$/ml in RPMI-1640 medium with 10% FBS. The cells were placed in 25 $cm^2$ Cell Culture Flasks (Corning Inc., New York, USA) at 10 ml/bottle. The cells were incubated for 24 h at 37°C under 5% $CO_2$. The experiment included three groups treated with YSL and a negative control group. Each group contained three parallel bottles treated similarly. YSL (10 ml/bottle) was added to the treatment group bottles, and plain RPMI-1640 (10 ml/bottle) was added to the negative control, respectively. The final concentrations of YSL were 0.4, 0.8, and 1.6 mg/ml. After incubated for 48 h at 37°C under 5% $CO_2$, the cells were fixed in cold 70% ethanol, and stained with PI solution (70 µmol/l propidium iodide, 38 mmol/l sodium citrate, 20 µg/ml RNase A). Then the cell cycle was detected and analyzed by flow cytometry (FASCalibur, BD, San Jose, California, USA).

### Quantitative real-time PCR

The concentration of BEL-7402 cells growing in log phase was adjusted to $2 \times 10^5$/ml in RPMI-1640 medium with 10% FBS. The cells were placed into 100 mm cell-culture dishes at 10 ml/dish. The cells were incubated for 24 h at 37°C under 5% $CO_2$. The experiment included two groups treated with different doses of YSL and a negative control group. Each group contained three parallel dishes treated similarly. YSL (10 ml/dish) was added to the treatment group dishes, and plain RPMI-1640 (10 ml/dish) was added to the negative control, respectively. The final concentrations of YSL were 0.4 and 0.8 mg/ml. The cells were incubated for 48 h at 37°C under 5% $CO_2$. Total RNA of human hepatocellular carcinoma BEL-7402 cells was extracted with Trizol (Invitrogen Corp., Carlsbad, USA) as described by the manufacturer. The concentration and purity of the RNA

**Table 1 Quantitative real-time PCR primer sequence**

| Prime | Upstream | Downstream |
|---|---|---|
| P21 | 5′-AGCAGAGGAAGA CCATGTGGAC-3′ | 5′-GGCGTTTGGAGTGGT AGAAATC-3′ |
| P27 | 5′-TAATTGGGGCTCCGG CTAACT-3′ | 5′-TTGCAGGTCGCTTCC TTATTC-3′ |
| PCNA | 5′-TCCCACGTCTCTTTGGTG C-3′ | 5′-TCTTCGGCCCTTAGTGTAA TGAT-3′ |
| β-actin | 5′-TTGCCGACAGGATGC AGAAGGA-3′ | 5′-AGGTGGACAGCGAGG CCAGGAT-3′ |

were quantified using Smart Spec and spectrophotometer (Bio-Rad Inc.). The RNA was diluted to 0.2 μg/μl and 2 μg of total RNA was then used for reverse transcription using oligo dT (12–18) as primer and an M-MLV reverse transcription kit (Promega). Real-time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA) and a 7500 System Software (Applied Biosystems). The reaction system conformed to the Real-Time PCR Master Mix (Toyobo, Osaka, Japan) procedure provided by the manufacturer. cDNA of 10 μl (diluted 20 times) was used for real-time PCR (RT-PCR) by absolute quantitation $2^{-\Delta\Delta C_T}$ method [11]. This method for accomplishing measurement of mRNA levels of P21 and P27 allows simultaneous amplification of target gene and standard in independent reactions. The primers for RT-PCR P21, P27 and PCNA amplification were selected as follows, and β-actin was used as an internal reference (Table 1). The amplified target gene cDNA was normalized against amplified β-actin to compensate for any changes owing to RNA degradation or amplification efficiency. The reaction mixture was incubated at 95°C for 1 min, followed by 40 amplification cycles at the following conditions: 15 s at 95°C, 15 s at 57°C, and 45 s at 72°C. The fluorescence signal was collected at every extension stage (72°C). The $C_T$ value represents the number of cycles during the progress of fluorescence signal reach a limitation in each reaction. $\Delta C_T = C_T$ (target gene) – $C_T$ (β-actin). The relative expression level of P21, P27, and PCNA were calculated as $2^{-\Delta C_T}$ [12].

### Western blot

The concentration of BEL-7402 cells growing in log phase was adjusted to $5 \times 10^5$/ml in RPMI-1640 medium with 10% FBS. The cells were placed into 145 mm cell-culture dishes at 20 ml/dish. The cells were incubated for 24 h at 37°C under 5% $CO_2$. The experiment included two groups treated with different doses of YSL and a negative control group. Each group contained three parallel dishes treated similarly. YSL (20 ml/dish) was added to the treatment group dishes, and plain RPMI-1640 (20 ml/dish) was added to the negative control, respectively. The final concentrations of YSL were 0.4 and 0.8 mg/ml. The cells were incubated for 48 h at 37°C under 5% $CO_2$. Total protein of human hepatocellular carcinoma BEL-7402 cells was extracted with RIPA protein lysis buffer (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) supplemented with PMSF (Sigma, Saint Louis, Missouri, USA). After centrifugation for 15 min at 12 000*g* at 4°C, the cleared supernatant was quantified by BCA protein assay kit (Pierce Inc., Rockford, USA), and was diluted into 5 μg/μl for western blot. Samples were separated by SDS–polyacrylamide gel electrophoresis (PowerPac Basic; Bio-Rad Inc.) and transferred onto PVDF membranes (Immobilon-P$^{SQ}$, Millipore Corporation, Bedford, Massachusetts, USA) by protein transfer equipment (MiniProtean; Bio-Rad Inc.). The membranes were blocked in 5% fat-free milk, which was prepared in a TBST buffer (50 mmol/l tris, pH 8.0, and 150 mmol/l NaCl, containing 0.05% Tween 20) and were left overnight at 4°C. Sections were subsequently incubated with antibodies for 2 h at room temperature. The primary antibodies include Mouse anti-P21 (CP74) (Sigma), Rabbit anti-P27 (C-19) (Santa Cruz Biotech Inc.), and Mouse anti-PCNA (CP74) (NeoMarkers, Fremont, California, USA). Mouse anti-β-actin AC-15 (Sigma) was used as an internal reference. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated antibody [goat anti-mouse IgG HRP-conjugated (KPL Ltd., Gaithersburg, Maryland, USA) or goat anti-rabbit IgG HRP-conjugated (Upstate Ltd., Dundee, UK)] for 1 h and the reactions were detected in the blots by a chemiluminescent substrate and were captured on Kodak XAR film (Kodak, Rochester, New York, USA). The products are reported as the target gene/β-actin densitometric ratio conducted by TotalLab software (Nonlinear Dynamics Ltd., Newcastle, UK) to compute the relative expression of protein.

### Statistical analysis of data

Experimental data were analyzed using one-way analysis of variance and the differences between groups were assessed by the Student–Newman–Keuls test. Data above were considered statistically significant if $P$ values were less than 0.05.

## Results

### Inhibitory effects of YSL on human hepatocellular carcinoma BEL-7402 cells *in vitro*

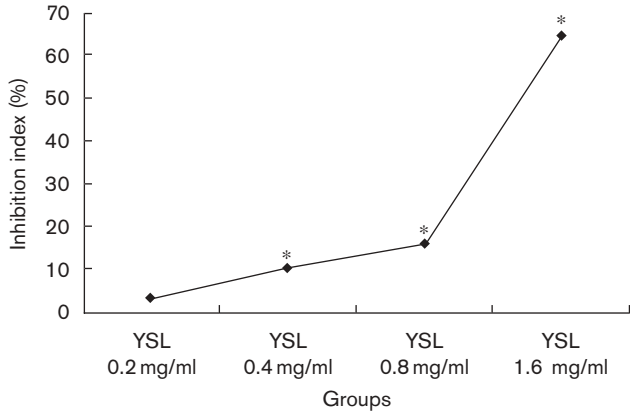
Cell viability can be detected by MTS, and MTS detection reflects the proliferation of cells indirectly. After incubated for 24 h and then together with YSL for 48 h, the growths of human hepatocellular carcinoma BEL-7402 cells were inhibited by YSL *in vitro*, with the dose-dependent relationship presentation. The inhibition index of YSL (0.4, 0.8, and 1.6 mg/ml) was significantly different from that of the control group ($P < 0.05$) (Fig. 1).

### Electron microscopic analysis of the effects of YSL on the ultrastructure of human hepatocellular carcinoma BEL-7402 cells *in vitro*

The typical characteristics of apoptosis, including cytoplasmic pyknosis, concentrated and half-moon chromatin

adjacent to the nucleolemma, nuclear fragmentation, and apoptotic body, could be revealed under electron microscopy, which provided reliable evidence for apoptosis.

**Fig. 1**



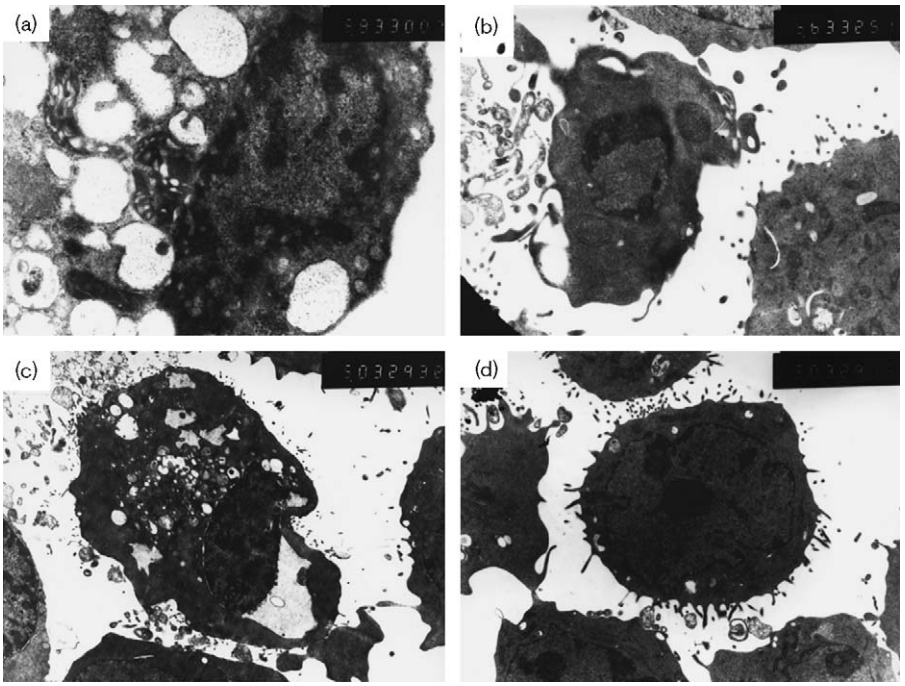

Inhibition effects of YSL (tyroserleutide) against human hepatocarcinoma cell line BEL-7402 *in vitro*. After incubated together with YSL for 48 h, the growth of human hepatocellular carcinoma BEL-7402 cells ($1 \times 10^5$/ml) was detected by dimethylthiazol-carboxymethoxyphenyl-sulfophenyl (MTS). The rates of inhibition were 64.47, 16.13, 10.25, and 3.34% for YSL 1.6, 0.8, 0.4, and 0.2 mg/ml, respectively ($N=8$, mean number of ratio ± SD; $^*P<0.05$, compared with control group.

Some injuries were observed in chondriosomes and rough endoplasmic reticulum in the cells, including disintegrated totally, dissolved, denaturalized, and vacuolization. Some euchromosomes in the centers of the nuclei were extensively dissolved, some had disappeared, and some formed a loose net. Lowered electron density was apparent. The endomembranes adjacent to nuclei, and the nucleoli and chromatin at the centers of nuclei were concentrated and contracted, and typical apoptosis appearance could be viewed. The shrinkage and darkening of the whole hepatocellular carcinoma cells were seen (Fig. 2).

### Apoptosis induction effects of YSL on human hepatocellular carcinoma BEL-7402 cells *in vitro*

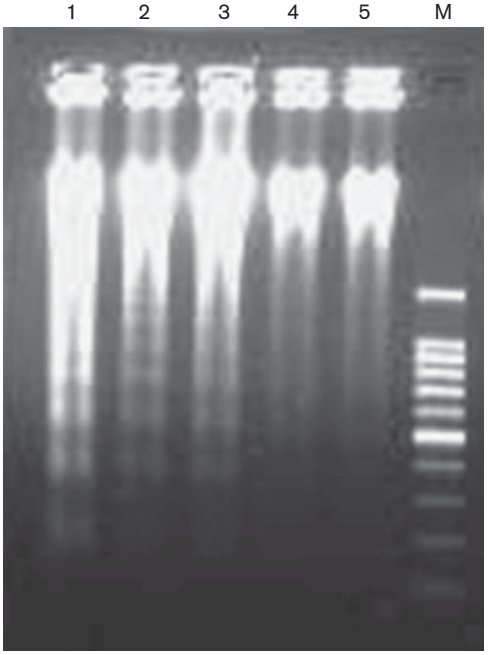
In apoptosis cells, DNA can be dissected into mononucleotide or oligonucleotide fragments of integer times of 180–200 bp among nucleosomes with the effects of endogenous endonuclease. These fragments manifest the distinctive 'ladders' by agarose gel electrophoresis, which can be an important biochemical indicator of apoptosis. After being incubated with YSL for 48 h, DNA was extracted from human hepatocellular carcinoma BEL-7402 cells. YSL (0.4, 0.8, and 1.6 mg/ml) could increase

**Fig. 2**




Effects of YSL (tyroserleutide) on the ultrastructure of human hepatocellular carcinoma BEL-7402 cells *in vitro*. After cells had been incubated with YSL for 48 h and fixed in 2.5% glutaraldehyde solution, the ultrastructure of human hepatocellular carcinoma BEL-7402 cells ($5 \times 10^4$/ml) were observed under electron microscopy. (a) YSL (0.4 mg/ml). Expanding vacuolization of rough endoplasmic reticulum, darkened chondriosome, oncoides of intercristal space, and articulo mortis ($\times$ 12 450). (b) YSL (0.8 mg/ml). Apoptosis of tumor cells. Cytoplasmic pyknosis, concentrated and half-moon chromatin adjacent to the nucleolemma and nuclear fragmentation ($\times$ 9900). (c) YSL (1.6 mg/ml). Apoptotic cancer cell ($\times$ 7500). (d) Negative control group. Hepatocellular carcinoma cells have the characteristics of tumor cells, two large nucleoluses, nuclear chromatin is widely distributed, polyribosome, and microvilli on the cell surface are not evenly distributed ($\times$ 7500).

**Fig. 3**




DNA ladder pattern of human hepatocellular carcinoma BEL-7402 cells as shown by agarose 1.5% electrophoresis. After cells had been incubated with YSL (tyroserleutide) for 48 h, DNA extracted from human hepatocellular carcinoma BEL-7402 cells ($2 \times 10^5$/ml) were electrophoresed on a 1.5% agarose gel containing ethidium bromide 2 mg/ml. Lanes 1–5: YSL (1.6 mg/ml), YSL (0.8 mg/ml), YSL (0.4 mg/ml), YSL (0.2 mg/ml), and negative control. M, DNA markers. Compared with the negative control, DNA ladder appeared more pronounced for the treatment with YSL (1.6, 0.8, and 0.4 mg/ml; lanes 1–3). From top to bottom, the patterns represent DNA fragments of 1000, 900, 800, 700, and 600–100 bp, respectively (lane M). The experiment was repeated three times under identical conditions.

the DNA ladders, which presented the typical pattern of apoptosis (Fig. 3).

### Repression effects of YSL on the cell cycle of human hepatocellular carcinoma BEL-7402 cells *in vitro*

During every stage of the cell cycle ($G_0$, $G_1$, S, $G_2$, M), the content of DNA changes periodically. Marked with a nucleic acid stain, the distribution of each stage can be obtained by flow cytometry. The distribution of cell cycle, proliferation activity of cells, and precise staging can be made by calculation of $G_0/G_1$, S, and $G_2$/M. After the cells had been incubated with YSL (0.4, 0.8, and 1.6 mg/ml) for 48 h, the proportion of S-phase (proliferative phase) human hepatocellular carcinoma BEL-7402 cells was decreased, and the proportion of $G_0/G_1$ (DNA-synthesis resting phase) cells was increased (Fig. 4).

### The effects of YSL on protein synthesis and mRNA transcription of PCNA in human hepatocellular carcinoma BEL-7402 cells *in vitro*

Expression of PCNA is elevated in the nucleus during late $G_1$ phase immediately before the onset of DNA synthesis, becoming maximal during S-phase and declining during $G_2$ and M phases. Its level correlates directly with rates of cellular proliferation [13,14]. After the cells had been incubated with YSL for 48 h, the PCNA protein level of human hepatocellular carcinoma BEL-7402 cells was studied by western blot. We found that YSL (0.4 and 0.8 mg/ml) decreased the expression of PCNA compared with that in the control group (Fig. 5). Total RNA of human hepatocellular carcinoma BEL-7402 cells was extracted with Trizol, and gene expression was accomplished by RT-PCR. YSL (0.4 and 0.8 mg/ml) significantly decreased the mRNA level of PCNA in human hepatocellular carcinoma BEL-7402 cells compared with the control group (Fig. 6).

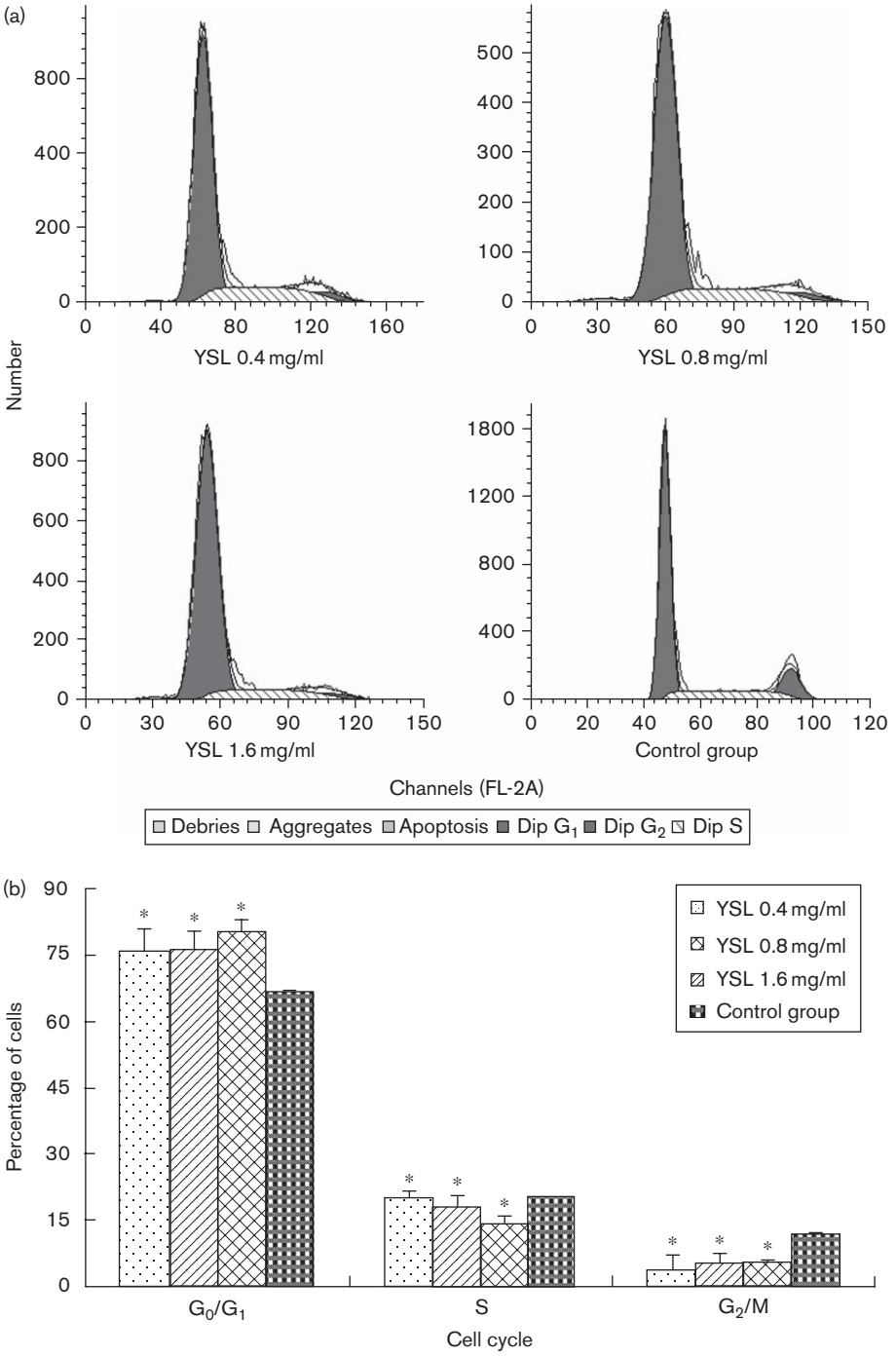
### The effects of YSL on protein synthesis and mRNA transcription of P21 in human hepatocellular carcinoma BEL-7402 cells *in vitro*

Several studies described the cyclin-dependent kinase inhibitor, P21, as a potent inhibitor of cell proliferation in various cell culture models, which is positively correlated with a delay in $G_1$ progression [15]; and the functional incapacitation of P21, either caused by gene mutation or other factors, results in abnormal cell proliferation and deprivation of normal cell cycle control. After cells had been incubated with YSL for 48 h, RIPA protein lysis buffer was used to separate tissue protein, and protein expression was accomplished by western blot. We found that YSL (0.4 and 0.8 mg/ml) significantly increased the protein expression of P21 in human hepatocellular carcinoma BEL-7402 cells compared with the control group (Fig. 5). Total RNA of human hepatocellular carcinoma BEL-7402 cells was extracted with Trizol, and the analysis of P21 by RT-PCR indicated that the YSL significantly increased mRNA levels compared with the control group (Fig. 6).

### The effects of YSL on protein synthesis and mRNA transcription of P27 in human hepatocellular carcinoma BEL-7402 cells *in vitro*

P27 is a cell cycle inhibitor that prevents the commutation of $G_1$/S cell cycle. Importantly, overexpression of P27 induces $G_1$ arrest, and P27 degradation is enhanced in many aggressive human tumors. Observations indicate that loss of P27 may confer a growth advantage to cancers [16]. After incubated together with YSL for 48 h, RIPA protein lysis buffer was used to separate tissue protein, and protein expression was accomplished by western blot. We found that YSL (0.4 and 0.8 mg/ml) significantly increased the protein expression of P27 in human hepatocellular carcinoma BEL-7402 cells compared with the control group (Fig. 5). Total RNA of human hepatocellular carcinoma BEL-7402 cells was extracted with Trizol, and the analysis of P27 by RT-PCR showed that the YSL significantly increased mRNA levels compared with the control group (Fig. 6).

**Fig. 4**

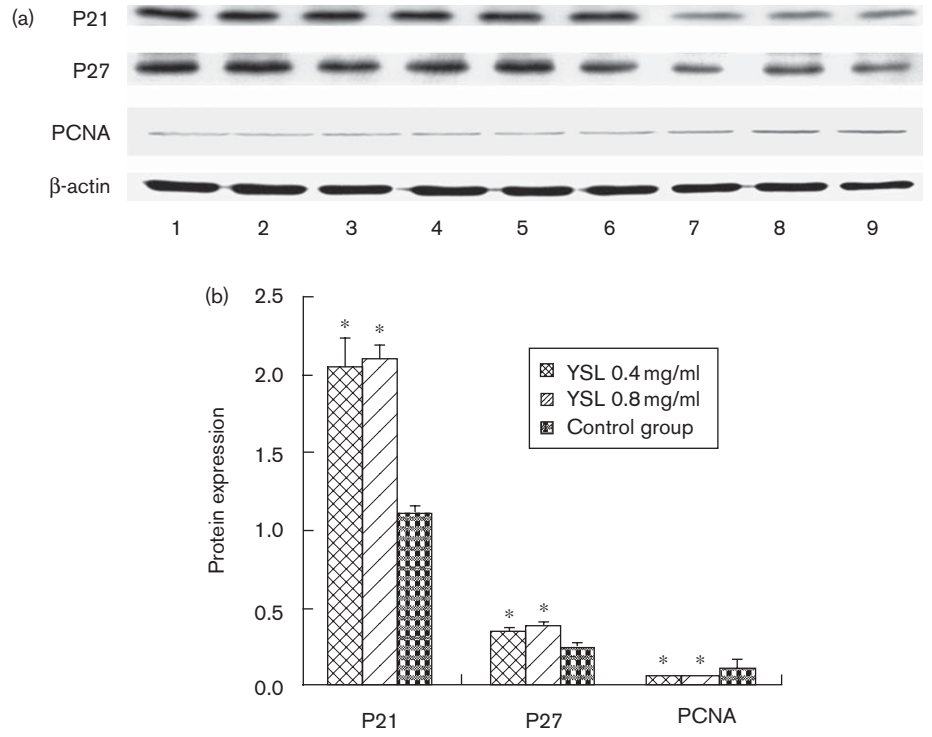



Repression of cell cycle effects of YSL (tyroserleutide) on human hepatocellular carcinoma BEL-7402 cells. Inhibitory effects of YSL on human hepatocellular carcinoma BEL-7402 cells by flow cytometry *in vitro*. After cells had been incubated with YSL for 48 h, fluorescence cell sorting and cell cycle analysis were used to generate cell cycle profiles for BEL-7402 cells ($1 \times 10^5$/ml). The cells were stained with propidium iodide. After sorting, fluorescence data were plotted as DNA content versus cell number, and cell cycle modeling was performed and the percentage of cells in $G_0/G_1$, S and $G_2/M$ phase was calculated by flow cytometry. (a) The histograms of cell cycle analysis by flow cytometry. (b) Representative data showing the analysis of cell cycle effects of YSL on human hepatocellular carcinoma BEL-7402 cells ($N=3$, mean number of ratio ± SD; $^*P<0.05$, compared with control group. Bars indicate SD).

## Discussion

YSL (0.2–1.6 mg/ml) is tested to have an inhibition effect on the growth of human hepatocellular carcinoma BEL-7402 cells *in vitro* by MTS, and the inhibition rate is enhanced when increasing the dose of YSL. MTS reflects the quantity of live cells and the proliferation of cells

**Fig. 5**

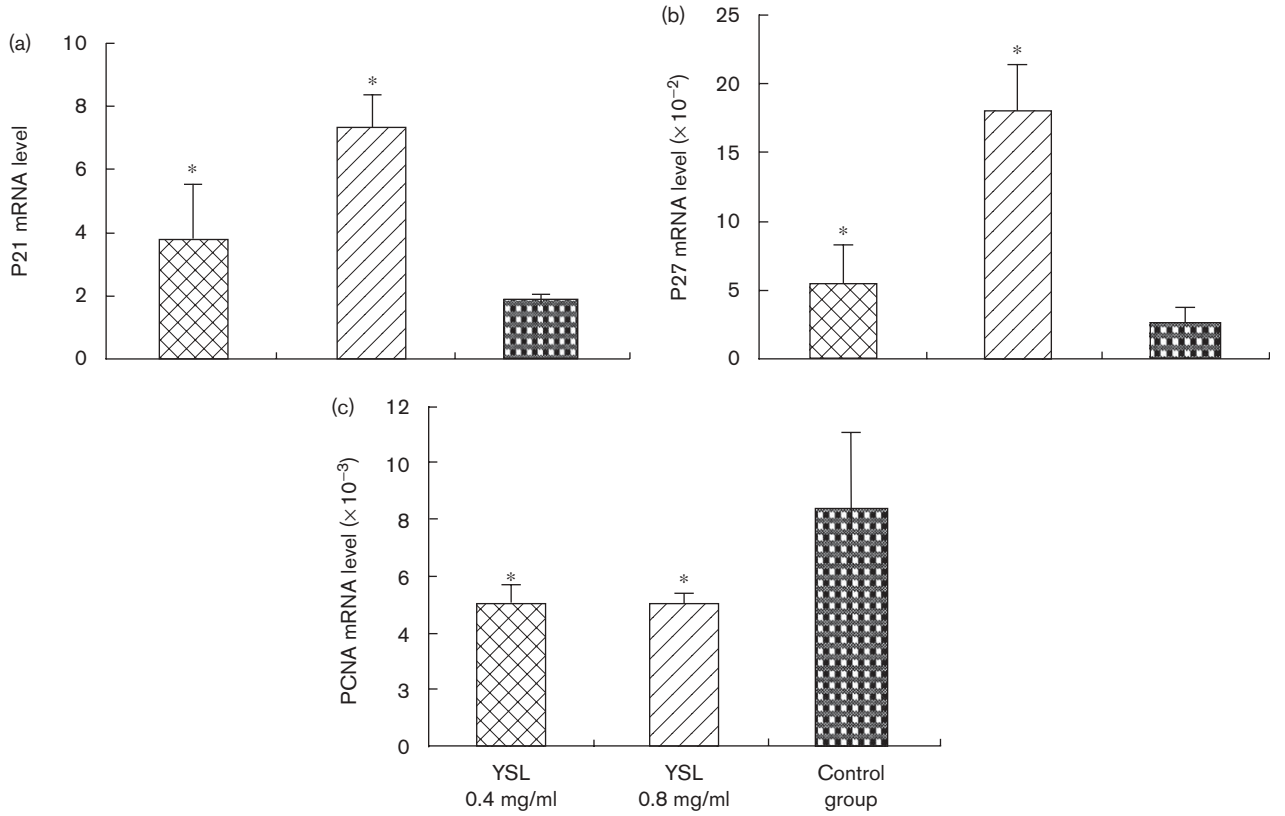



Western blot of PCNA, P21, and P27 in human hepatocellular carcinoma BEL-7402 cells. Expression of PCNA, P21, and P27 protein was assessed by western blot in human hepatocellular carcinoma BEL-7402 cells that were treated with YSL (tyroserleutide) or negative control. Blots were reprobed for expression of β-actin to control for loading and transfer. Western blot analysis was performed with antibody against target protein. Chemiluminescence was used for the protein expression. (a) PCNA, P21, and P27 expression: YSL group (0.8 mg/ml; lanes 1–3), YSL group (0.4 mg/ml; lanes 4–6), and control group (lanes 7–9). (b) Representative data showing analysis of PCNA, P21, and P27 expression in BEL-7402 cells ($N=3$, mean number of ratio ± SD; $^*P<0.05$, compared with control group. Bars indicate SD).

indirectly. Electron microscopic assay shows that chromatinolysis, swelling, and degeneration of chondriosome and endoplasmic reticulum are promoted by YSL *in vitro*. The apoptosis and necrosis induced by YSL were seen in most tumor cells. Simultaneously, YSL (0.4–1.6 mg/ml) evidently induces typical DNA ladders of human hepatocellular carcinoma BEL-7402 cells *in vitro* by DNA ladder detection. The proportion of S-phase human hepatocellular carcinoma BEL-7402 cells decreased, and the proportion of $G_0/G_1$ cells increased with the treatment of YSL by flow cytometry. At present, research suggests that there is an exceptional ability of proliferation and differentiation in tumor cells, and these tumor cells are deprived of the ability of cell cycle control. Some check points in the cycle modulate the progress of cell cycle. Among those check points, $G_1/S$ and $G_2/M$ are the most important. Crossing these checkpoints successfully is vital in the commutation among different phases of cell cycle [17–19]. YSL can reduce the transition of cell from $G_0/G_1$ stage into S stage by interrupting the cell cycle at $G_0/G_1$, and thus inhibit the growth of tumor cells and induce apoptosis.

YSL decreases the mRNA level and protein expression of cyclin PCNA, and increases the mRNA level and protein expression of cyclin P21 and P27 in human hepatocellular carcinoma BEL-7402 cells. PCNA is an auxiliary subunit of DNA polymerase-δ, which helps the synthesis of leading chain and lagging chain of DNA during the period of cell division. The expression of PCNA is at S stage, and there is no expression of PCNA at $G_0$ and $G_1$ stage. When the activity of PCNA is inhibited, normal disintegration and proliferation of cells stops [20,21]. YSL evidently decreases the expression of PCNA in the control group, and thus inhibits the growth of human hepatocellular carcinoma BEL-7402 cells. P21 and P27, negative cell cycle modulins, belong to the Cip/Kip (P21, P27, P57) protein family that inhibit Cyclins-CDK (Cyclins-cyclin-dependent kinase) by competing with cyclins or binding with Cyclins-CDK directly, and prevent the commutation of $G_1/S$ cell cycle [22–25]. Infinite cells proliferation and tumor will appear when there is an abnormal expression in P21 and P27. YSL is able to increase the expression of P21 and P27. P21 can bind with every kinds of Cyclins-CDK, and dephosphorylate both Rb and P53 and ultimately inhibit the entry of the cell into S phase of the cell cycle. P21 can also inhibit the combination of PCNA and DNA polymerase, thus inhibiting the extension of DNA [26,27], which inhibits the

**Fig. 6**




Real time (RT)-PCR analyses for mRNA expression of PCNA, P21, and P27 in human hepatocellular carcinoma BEL-7402 cells. RT-PCR analysis for PCNA, P21, and P27 in human hepatocellular carcinoma BEL-7402 cells transcripts using the primer pairs described in the Materials and methods treated by YSL (tyroserleutide) or negative control. Trizol was used for total RNA extracting, and RT-PCR was used for the detection of PCNA, P21, and P27 mRNA level. The relative expression level of PCNA, P21, and P27 was calculated as $2^{-\Delta C_T}$. The number of cycles during the progress of fluorescence signal reach a limitation in each reaction was represented as $C_T$ value. $\Delta C_T = C_T$ (target gene) $- C_T$ (β-actin). The products were quantified using β-actin as an internal reference ($N=3$, mean number of ratio ± SD; *$P<0.05$, compared with control group. Bars indicate SD). (a) P21 mRNA level; (b) P27 mRNA level; and (c) PCNA mRNA level.

cell cycle and cause apoptosis of tumor cells. P27 can inhibit proliferation of cells through modulating the proceeding of cell cycle restrictedly. P27 inhibits the activity of CDKs in response to a variety of growth-modulating signals [28], through which cell cycle is interrupted, and thus cell proliferation is inhibited. Some data suggest that the content of P27 changes according to the alteration of cell cycle progression. P27 is higher at $G_0$ stage, lower at $G_1$ stage, and almost could not be found at S stage [29]. Results from the current study show that YSL enhances the expression of both P21 and P27, and inhibits the expression of PCNA. The effects on the expression of these proteins may result in an interruption of the cell cycle at $G_0/G_1$ and the induction of necrosis and apoptosis of tumor cell proliferation, and ultimately inhibit the growth of human hepatocellular carcinoma BEL-7402.

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