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Effects of Tyroserleutide on Gene Expression of Calmodulin and PI3K in Hepatocellular Carcinoma

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Abstract Tyroserleutide (YSL) is a tripeptide compound that has exhibited inhibitory effects on hepatocellular carcinoma in our previous research. The mechanism of this antitumor activity involves the second messenger, Ca^{2+}. Ca^{2+} influences cell function through the Ca^{2+}/calmodulin (CaM) pathway, and abnormality of the Ca^{2+}/CaM system correlates closely with the occurrence of tumors. In addition, CaM associates with phosphatidylinositol 3 kinase (PI3K), thereby enhancing the activity of PI3K, which promotes cell proliferation. In order to investigate its anti-tumor mechanism, we studied the effects of YSL on CaM protein expression and mRNA level, PI3K activity, PI3K regulatory subunit p85 protein expression and mRNA level, and the mRNA level of PI3K catalytic subunits p110α and p110γ in human hepatocellular carcinoma BEL-7402 xenograft tumors in nude mice. Our results showed that YSL decreased the mRNA level and protein expression of CaM, inhibited the activity of PI3K, and reduced the mRNA level and protein expression of the PI3K regulatory subunit p85 and mRNA level of PI3K catalytic subunits p110α and p110γ. Accordingly, it is suggestive that the anti-tumor effects of YSL may be mediated by down regulation of CaM and PI3K subunits p85 and p110, influencing the signal transduction pathway in the tumor cells and perhaps overcoming the dysfunctional PI3K activity in tumors. J. Cell. Biochem. 103: 471–478, 2008.

Key words: tyroserleutide; YSL; hepatocellular carcinoma BEL-7402; calmodulin; PI3K

Hepatocellular carcinoma is one of the most common cancers world-wide [Okuda, 2000], with an annual incidence rate of 260,000. Over 42.5% of new cases occur in China. There are about 130,000 people dying of this disease in China, accounting for over 40% of all liver cancer deaths worldwide. Alarmingly, the incidence of hepatocellular carcinoma has been on the rise every year all over the world. With the conspicuous progress in the diagnostic and therapeutic approaches for hepatocellular carcinoma in recent years, a combined therapeutic principle based on surgical intervention has been established. Nevertheless, most patients are at advanced stage when diagnosed, causing difficulty for resection and the side effects caused by chemotherapeutics must be considered as negative factors in therapeutic efficacy. Therefore, it is essential to develop new drugs with high efficacy, low toxicity, and better specificity for hepatocellular carcinoma therapy.

Our previous research indicated that YSL prolonged the survival time of BALB/c (nu/nu) mice with transplanted ascitic fluid-type liver cancer H_{22} and significantly inhibited the growth of transplanted human hepatocellular carcinoma BEL-7402 in nude mice. The mechanism

Abbreviations used: YSL, tyroserleutide; CaM, calmodulin; PI3K, phosphatidylinositol 3 kinase; SPF, specific pathogen-free; ANOVA, one-way analysis of variance; SNK, student newman-keuls; RTK, receptor tyrosine kinases; PIP3, PI(3,4,5)P3; PIP2, PI(4,5)P2.

Lan Zhao and Qian Zhao contributed equally to this paper.

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of this anti-tumor activity might be attributed to the effects of YSL on the second messenger, Ca^{2+} [Fu et al., 2005; Lu et al., 2006; Yao et al., 2006]. CaM, a ubiquitous Ca^{2+}-dependent effector protein, modulates the concentration of Ca^{2+}. An amassed body of data indicates that CaM is closely correlated with the occurrence, growth, and proliferation of tumors. Furthermore, the abnormal proliferation and cytometaplasia were shown to be caused by the change of CaM content [Hait and Lazo, 1986]. Moreover, CaM associates with SH2 domains of the 85-kDa regulatory subunit of PI3K, thereby enhancing PI3K activity; this modulates downstream signaling and induces Akt phosphorylation [Joyal et al., 1997; Nishimatsu et al., 2001; Cheng et al., 2003a], thus promoting the progression of tumors. Here we have studied the effects on CaM and PI3K of human hepatocellular carcinoma BEL-7402 tumor transplants in nude mice to investigate the anti-tumor mechanism of YSL.

MATERIALS AND METHODS

Cell Line

Human hepatocellular carcinoma BEL-7402 cells (Tumor Medicine Institute of the China Medical Academy of Science, Beijing, China) were cultured in RPMI-1640 medium (GIBCO, Invitrogen Corp., Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) in a humidified atmosphere of 5% CO_2 at 37°C.

Animals

Healthy female BALB/c (nu/nu) mice 4–5 weeks of age weighing 18–22 g (China Medical Academy of Science, Beijing, China) were used for the BEL-7402 studies. The animals were maintained under specific pathogen-free (SPF) conditions using a laminar air-flow rack. The light/dark cycle, temperature, and humidity were controlled, according to the operating instructions of the laboratory animal department of Tianjin Medical University. The animals had continuous access to sterilized food and autoclaved water. Experiments were started after 1 week of acclimatization.

Human hepatocellular carcinoma BEL-7402 cells growing in log phase were suspended in RPMI-1640 medium with 10% FBS and the cell concentration was adjusted to 1×10^8/ml. BEL-7402 cells (0.1 ml) were subcutaneously inoculated into the right flank of each nude mouse. When the tumors became visible, nude mice bearing BEL-7402 carcinoma were randomized into one of three groups: YSL (Shenzhen Kangzhe Pharmaceutical Co. Ltd, China) 320 μg/kg/day dose group, YSL 160 μg/kg/day dose group, or a saline (Otsuka Pharmaceutical Co. Ltd, China) group. Drug was administered i.p. once daily in 0.2 ml saline for 50 days.

Real-Time Quantitative PCR

Tumors without necrosis were detached from nude mice, prepared as described above, and cut into about 100 mg pieces. The pieces were then flash-frozen in liquid nitrogen and stored until extraction of RNA or protein. Total RNA was extracted with Trizol (Invitrogen, Inc.) as described by the manufacturer. The concentration and purity of the RNA was quantified using a Smart Spec^{TM} spectrophotometer (Bio-Rad, Hercules). 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) primers and an M-MLV reverse transcription kit (Promega Co., Madison). Real time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City) and the 7500 System Software (Applied Biosystems). The reaction system conformed to the Real Time PCR Master Mix (Toyobo, Osaka, Japan) procedure provided by the manufacturer. 10 μl cDNA (diluted 20 times) was used for the real-time PCR according to the absolute quantitation 2 (-delta delta C(T)) method [Schostak et al., 2006]. This method allows simultaneous target gene and standard amplification in independent reactons and for the measurement of CaM, PI3K p85α, p110α, and p110γ message levels. The RT-PCR primers for CaM, PI3K p85α, p110α, and p110γ amplification were selected as shown in Table I, with β-actin as an internal reference. The amplified target gene cDNA was normalized against amplified β-actin to compensate for any changes due 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: 30 s at 95°C, 30 s at 57°C, and 45 s at 72°C. Fluorescence signal was collected at every extension stage (72°C). The C_T value represents the number of cycles required for the fluorescence signal reach a threshold for each reacton ($\Delta C_T = C_T$ (target gene) $- C_T$ (β-actin)). The relative expression

TABLE I. Real-Time Quantitative PCR Primer Sequences

Prime	Upstream	Downstream
CaM	5′-AGCAGCAGCAGCAGCAGCAT-3′	5′-TTCAGCAATCTGTTCTTCGGTCAG-3′
PI3K p85α	5′-GCCTCCTAAACCACCAAAACC-3′	5′-TCCCGTCTGCTGTATCTCGAA-3′
PI3K p110α,	5′-CCCAGGTGGAATGAATGGCT-3′	5′-AGCACCCTTTCGGCCTTTAAC-3′
PI3K p110γ	5′-TCTGATGGATATTCCCGAAAGCC-3′	5′-CTCACCCACTGGAAGTTTT TGAT-3′
β-actin	5′-TTGCCGACAGGATGCAGAAGGA-3′	5′-AGGTGGACAGCGAGGCCAGGAT-3′

level of CaM, PI3K p85α, p110α, and p110γ were calculated as $2^{-\Delta C_T}$ [Livak and Schmittgen, 2001].

Protein Extraction

Tumor tissues were homogenized on ice in RIPA protein lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) supplemented with PMSF (Sigma). 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), and diluted to 5 μg/μl for immunoprecipitation and Western blotting.

Immunoprecipitation and ELISA

Samples (cell lysates) were incubated overnight with anti-PI3K p85 (Table II) at 4°C. After adding Protein A agrose (Upstate cell signaling solutions) and shaking for 1 h at 4°C, the samples were centrifuged for 5 s at 14,000*g*. Immunoprecipitates were washed with cleaning solutions. After centrifugation for 30 s at 14,000*g*, antigen-antibody complexes were used for the detection of PI3K activity by PI3-Kinase ELISA kit (Echelon Bioscience incorporated).

Western Blot

Samples were separated by SDS–PAGE (PowerPac Basic, Bio-Rad) and transferred to PVDF membranes (Immobilon- P^{SQ}, Millipore Corporation) using protein transfer equipment (MiniProtean, Bio-Rad, Inc.). The membranes were blocked in 5% fat-free milk, which was prepared in a TBST buffer (50 mM tris, pH 8.0, and 150 mM NaCl, containing 0.05% Tween-20) and incubated overnight at 4°C. Sections were subsequently incubated with antibodies for 3 h at room temperature. The antibodies for CaM and PI3K p85 were used to probe the western blot and β-actin served as an internal reference (Table II). After washing, the membranes were incubated with HRP-conjugated antibody (Table II) for 1 h. The reactions were detected with a chemiluminescent substrate and were captured on Kodak XAR film. The products are reported as the target gene/β-actin densitometric ratio calculated by the TotalLab software to compute the relative expression of proteins.

Statistical Analysis of Data

Experimental data were analyzed using one-way analysis of variance (ANOVA). The differences between groups were assessed by the student newman-keuls (SNK) test or independent-samples *t* test. Data was considered statistically significant if *P* values were <0.05.

RESULTS

Inhibitory Effects of YSL on the Protein Expression and mRNA Level of CaM

CaM regulates multiple processes in cells, and plays important roles in cell proliferation and tumor progression. In this, CaM level has a positive correlation with disordered cell proliferation, especially with malignant tumor cell proliferation. After protein extraction from human hepatocellular carcinoma BEL-7402 tumors transplanted into nude mice, CaM protein levels were probed by Western blot, revealed that the YSL (320 μg/kg/day) and YSL (160 μg/kg/day) groups were significantly lower

TABLE II. Antibodies and Dilutions for Western Blot

Antibody	Producer	Dilution
Mouse anti-Calmodulin	Zymed	1:1,000
Rabbit anti-PI3 Kinase p85	Upstate cell signaling solutions	1:1,000
Mouse anti-β-actin (AC-15)	Sigma	1:4,000
Goat anti-mouse IgG HRP conjugated	Upstate cell signaling solution	1:6,000
Goat anti-rabbit IgG HRP conjugated	Upstate cell signaling solution	1:6,000

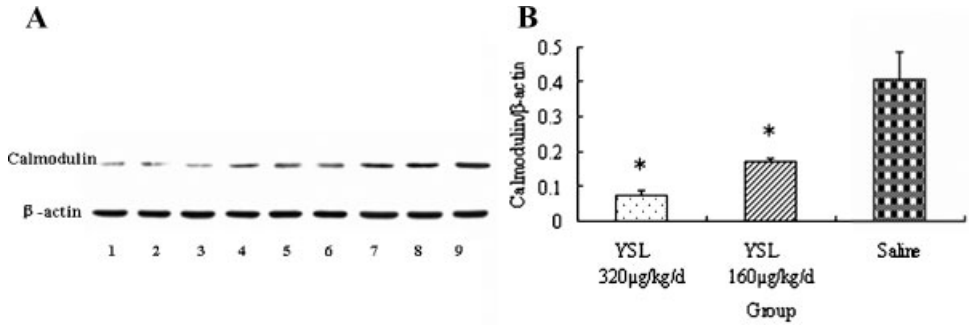

Fig. 1. Western blot for CaM in extracts from human hepatocellular carcinoma BEL-7402 in nude mice. Expression of CaM protein was assessed by Western blot in extracts from human hepatocellular carcinoma BEL-7402 transplanted into nude mice that were treated with YSL or saline control. Blots were reprobed for expression of β-actin to control for loading and transfer. **A**: CaM expression: YSL (320 μg/kg/day) group (lanes 1–3), YSL (160 μg/kg/day) group (lanes 4–6), saline group (lanes 7–9). **B**: Representative data showing analysis of CaM expression in BEL-7402 tumors from nude mice (N = 3, mean number of ratio ± SD; *, compared to saline group, $P < 0.05$. Bars indicate SD).

than saline group (Fig. 1). Similarly, mRNA levels of CaM, determined by RT-PCR, indicated that the YSL (320 μg/kg/day) and YSL (160 μg/kg/day) groups were significantly lower compared to the saline group (Fig. 2).

Effects of YSL on PI3K Activity

CaM associates with the p85 regulatory subunits of PI3K, thereby enhancing PI3K activity. PI3K influences cell growth and tumor progression when activated by receptor tyrosine kinases (RTK) and G protein coupled receptors. Abnormal increases of PI3K activity have been found in multiple human malignant tumors. After the protein extraction from the transplanted tumors, PI3K activity was determined by ELISA. As PI(3,4,5)P3 (PIP3) is the product of PI(4,5)P2 (PIP2) phosphorylation by PI3K, PIP3 content reflects the PI3K activity after enzymatic reaction. The result suggested that the PIP3 content of the YSL (320 μg/kg/day) group was significantly lower than the saline group, indicating that YSL significantly inhibited the activity of PI3K in tumor cells (Fig. 3).

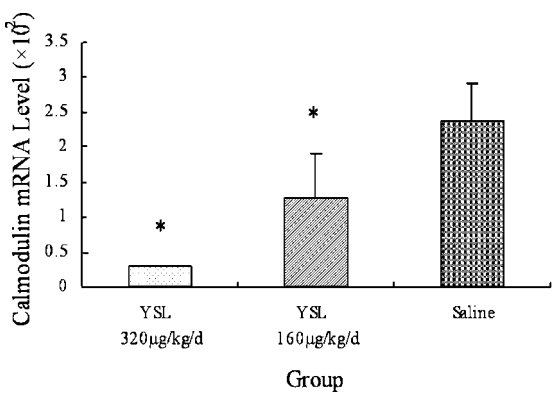

Fig. 2. RT-PCR analysis of CaM mRNA level in extracts from BEL-7402 xenografts. RT-PCR analysis for CaM in extracts from BEL-7402 transplanted into nude mice treated with YSL or saline control. RT-PCR was performed on total RNA for detection of CaM mRNA level. The relative expression level of CaM was calculated as $2^{-\Delta C_T}$. The products were quantified using β-actin as an internal reference (N = 5, mean number of ratio ± SD; *, compared to saline group, $P < 0.05$. Bars indicate SD).

Inhibitory Effects of YSL on Protein and mRNA Expression of the PI3K Regulatory Subunit, p85

PI3K is composed of regulatory a subunit (p85) and catalytic subunit (p110). The p85

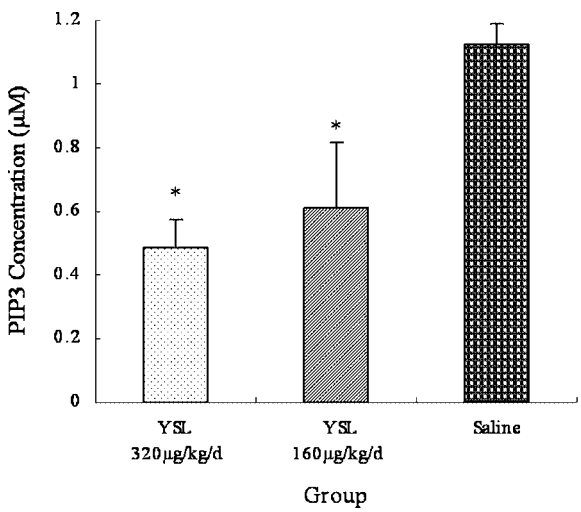

Fig. 3. PI3K activity in BEL-7402 xenograft extracts from nude mice. PI3K activity was assessed by immunoprecipitation and ELISA in extracts from the implanted tumors that were treated with YSL or saline. PI3K activity was calculated by the concentration of PIP3, a product of PI3K enzymatic activity (N = 3, mean number of ratio ± SD; *, compared to the saline group, $P < 0.05$. Bars indicate SD).

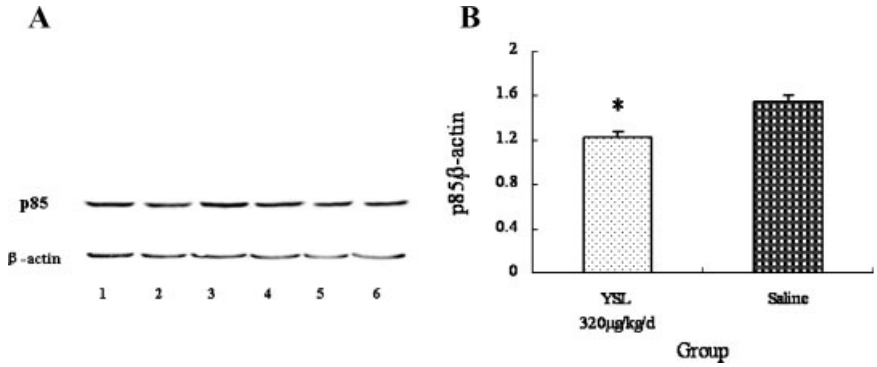

Fig. 4. Western blot of PI3K p85 regulatory subunit in tumor xenograft extracts. Expression of the p85 regulatory subunit protein was assessed by Western blot in extracts from human hepatocellular carcinoma BEL-7402 transplanted into nude mice that were treated by YSL or saline. Blots were re-probed for expression of β-actin to control for loading and transfer. **A**: PI3K regulatory subunit p85 expression: YSL (320 μg/kg/day) group (lanes 1–3), saline group (lanes 4–6). **B**: Representative analysis of p85 expression in BEL-7402 tumors (N = 3, mean number of ratio ± SD; *, compared to saline group, $P < 0.05$. Bars indicate SD).

regulatory subunit of PI3K was recently found to be directly involved in tumor occurrence. Here, after extracting the protein from BEL-7402 transplants, the p85 protein level was studied by Western blot. We found that the YSL (320 μg/kg/day) group was significantly lower than the saline group (Fig. 4). The PI3K p85 family consists of homological subunits p85α, p85β, and p85γ. Among them, p85α, a known oncogene, can be found aberrantly expressed in many malignant tumors. Analysis of p85α by RT-PCR indicated that the YSL (320 μg/kg/day) group significantly inhibited mRNA levels compared to the saline group (Fig. 5).

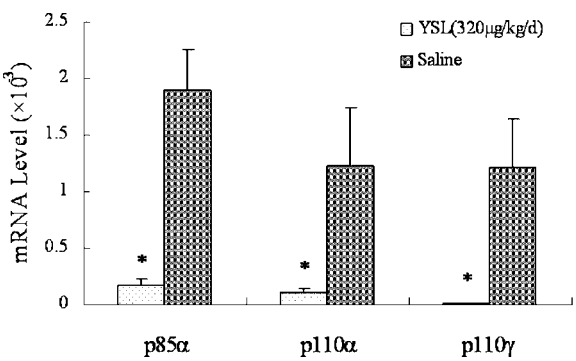

Fig. 5. RT-PCR analysis of PI3K p85α, p110α, and p110γ subunit mRNA expression in BEL-7402 tumor extracts. RT-PCR analysis for PI3K p85α, p110α, and p110γ subunits in extracts from YSL or control treated human hepatocellular carcinoma BEL-7402 tumors. The relative expression level of p85α, p110α, and p110γ was calculated as $2^{-\Delta C_T}$. The products were quantified using β-actin as an internal reference (N = 5, mean number of ratio ± SD; *, compared to saline group, $P < 0.05$. Bars indicate SD).

Inhibitory Effects of YSL on Protein Expression and mRNA Level of the PI3K Catalytic Subunits p110α and p110γ

The PI3K regulatory subunit p85 regulates activity of the p110 catalytic subunit when combined with phosphorylation of receptor tyrosines, thereby p110 regulates cell growth and metabolism by substrate phosphorylation. The PI3K catalytic subunit p110 consists of the homologous subunits, p110α, p110β, p110γ, and p110δ. Recently, both the expression level and structural mutation of p110α and p110γ were found to participate in tumor development. Analysis of p110α and p110γ by RT-PCR indicated that the high-dose YSL (320 μg/kg/day) group significantly inhibited mRNA level compared to the saline control (Fig. 5).

DISCUSSION

As demonstrated in our previous work, YSL inhibited human hepatocellular carcinoma through up regulation of Ca^{2+} content, destruction of calcium homeostasis in BEL-7402 cells. CaM, a second messenger important for receptor signaling, regulates the physiological function of target proteins to influence the cellular activity [Zhao et al., 2007]. CaM undergoes a conformational change when bound to Ca^{2+} that allows it to bind to target effector proteins and stimulate their activities, which participate in Ca^{2+} regulation and cell proliferation in physiological conditions. The CaM level is known to correlate positively with disordered

cell proliferation, particularly with malignant tumor cell proliferation [Rasmusen and Means, 1990]. Consistent with this notion, CaM levels were elevated in Morris hepatoma carcinoma cells compared to normal or fetal liver cells. This had a positive relation with hepatoma carcinoma growth speed. Similarly, in pulmonary carcinoma cells, CaM was higher than in innocuous pulmonary diseases or normal pulmonary cells, and exhibited a positive correlation with histopathology grading and classification of tumor node metastases in pulmonary tissues [Liu et al., 1996]. In this study, YSL decreased CaM protein expression by down regulation of CaM mRNA level in human hepatocellular carcinoma. Many other studies have found that the activity of several drugs is related to inhibitory effects on cell proliferation though CaM inhibition. For example, W7 inhibited the growth of B16 melanoma [Mac Neil et al., 1984]; berbamine inhibited human leukemia K562 cells in vitro [Sun et al., 2006]; and trifluoperazin inhibited granulocytic monocytic leukemia (WEHI3) and human pulmonary PLA801 cell proliferation. Thus we presume that YSL may inhibit malignant tumor cell proliferation through mechanisms involving both calcium overcapacity caused by Ca^{2+} increasing and inhibition effects on CaM.

CaM associates with SH2 domains on the 85-kDa regulatory subunits of PI3K, thereby enhancing PI3K activity, which, in turn, induces Akt phosphorylation. In this manner, PI3K can be activated through the CaM pathway in hepatocytes [Benzeroual et al., 2000]. PI3K/Akt plays an important role in multiple events, including regulation of cell proliferation, resistance to apoptosis, and angiogenesis [Yao and Cooper, 1995; Kureishi et al., 2000]. Abnormally increased of PI3K activity was detected in multiple human malignant tumors, such as colon carcinoma, bladder carcinoma, and ovarian carcinoma [Jimenez et al., 1998; Phillips et al., 1998; Benistant et al., 2000]. The activity of PI3K in human hepatocellular carcinoma BEL-7402 nude mouse transplants can be reduced significantly by YSL treatment. PI3K is composed of a p85 regulatory subunit and p110 catalytic subunits. Interestingly, p85 was found to relate directly with tumor progression. As Bavelloni suggested, down-regulation of p85 expression or specific PI3K inhibitors might lead to accumulation of cells in G_1 phase and prevent the differentiation in erythroleukaemia cells [Bavelloni et al., 2000]. The PI3K regulatory subunit p85 is composed of homologous subunits p85α, p85β, and p85γ. Among these, p85α is a potential oncogene in many malignant tumors, and was found to be involved in the migration and metastasis of mammary adenocarcinoma [Sliva et al., 2002]. Importantly, in this study, YSL decreased the mRNA and protein expression level of p85. Therefore, we suggest that YSL inhibits the progression of human hepatocellular carcinoma by down regulation of p85α mRNA level and p85 protein expression, thereby reducing PI3K activity.

The PI3K catalytic subunit p110 is comprised of homological subunits p110α, p110β, p110γ, and p110δ. Researches have suggested that specific inhibitors of PI3K result in induction of apoptosis in hepatoma, mammary adenocarcinoma, colon carcinoma, pancreatic carcinoma, and gastric carcinoma cells cultured in vitro [Izuishi et al., 2000; Sliva et al., 2002; Cheng et al., 2003b]. It has also been implicated in the up regulation of p110α (PI3KCA) that can promote angiogenesis in ovarian carcinoma [Zhang et al., 2003]. In addition, p110γ was found to contribute to the growth, progression, metastasis, and poor differentiation of colorectal cancers; while PI3K signal transduction pathways were blocked by p110γ inhibition during tumorigenesis and tumor progression [Semba et al., 2002]. YSL decreased the mRNA level of p110α and p110γ in human hepatocellular carcinoma BEL-7402 nude mouse transplants. The PI3K catalytic subunit p110α is regulated by receptor tyrosine phosphorylation and its catalytic activity depends upon coexpression of p85. When cells are stimulated through a tyrosine kinase, high affinity is generated between the SH2 domains of the 85-kDa regulatory subunits of PI3K and specific tyrosine phosphorylation sequences. Then p110 is recruited to cell membrane, where PIP3 is produced from PIP2 by kinase activity. YSL reduced both p85 and p110α content, thus inhibiting PI3K kinase activity, decreased the production of PIP3, and ultimately blocked PI3K signal transduction pathways. It also stimulated apoptosis of human hepatocellular carcinoma BEL-7402 cells. YSL can inhibit the mRNA level of, not only p110α, but also another PI3K catalytic subunit, p110γ. This subunit (p110γ) exercises catalytic activity without the p85 regulatory subunit. In the absence of help from Ras, GTP, and other cell membrane lipids,

PI3K promotes the interaction between p110γ and regulatory submit, induces p110γ to enter the nucleus directly, and activates signal transduction pathways downstream of PI3K. An unidentified serum factor is responsible for inducing the movement of p110γ through the nuclear membrane, activation of PI3K, and promotion of the growth of human hepatocellular carcinoma HepG2 tumor transplants. The inhibitory effects of YSL on p110γ reduced the abnormal activity of PI3K in these tumors, inhibiting the proliferations of BEL-7402 cells.

In conclusion, the results are suggestive that the inhibitory effects of YSL on human hepatocellular carcinoma (BEL-7402) tumors in nude mice occurs by down regulation of the Ca^{2+} receptor, CaM, disruption of PI3K signaling though down regulation of the principal regulatory (p85α) and catalytic subunits (p110α and p110γ), thus reducing the protein expression of p85 and p110 and inhibiting the abnormal activity in tumor cells. Furthermore, the alterations to PI3K activity caused by YSL treatment shifts the expression ratio and activity of correlated oncogenes, as well as downstream anti-oncogenes of the PI3K signal transduction pathway, and therefore exhibits anti-tumor effects on human hepatocellular carcinoma.

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