Antitumor effects of a polypeptide isolated from *Tegillarca granosa* linnaeus and the related molecular mechanism

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Abstract: This study is to investigate the anticancer effects and mechanisms of *Tegillarca granosa* Linnaeus-1 (TG-1) on renal carcinoma OS-RC-2 cells *in vitro*. The proliferation of OS-RC-2 cells was evaluated under various concentrations of TG-1 using MTT assay. The apoptosis of OS-RC-2 cells was analyzed using acridine orange/ethidium bromide staining. And the cell cycle distribution of OS-RC-2 cells was detected by flow cytometry. In addition, the expression level of Ki67 mRNA was examined by RT-PCR and level of casepase-3 was examined by Western blot analysis. TG-1 incubation significantly inhibited the proliferation of renal carcinoma OS-RC-2 cells and arrested cells at G_0/G_1 phase ($P < 0.05$). And TG-1 also significantly inhibited the expression of Ki67 mRNA ($P<0.05$). Additionally, TG-1 significantly promoted apoptosis and the expression of caspase-3 in cells ($P<0.05$). Moreover, the optimal effects of TG-1 was achieved at the concentration of 100 mg/L The results indicate that TG-1 has antitumor effects on renal carcinoma OS-RC-2 cells and that the underlying mechanisms may be acted through inhibiting proliferation and Ki67 mRNA expression, and promoting apoptosis and caspase-3 expression.

Keywords: The protein polypeptide; antitumor effects; renal carcinoma OS-RC-2 cells; Ki67; caspase-3.

INTRODUCTION

Renal cell carcinoma (RCC) originates in renal parenchyma layer of urinary tubule epithelial system. It accounts for 80%-90% of renal malignant tumors and is one of the common malignant tumors. (Campbell *et al.,* 2007; Jemal *et al.,* 2010) Each year about 100,000 people died of kidney cancers in the world. About one third of the kidney cancer patients have metastatic kidney cancer when they were diagnosed for the first time. (Herrmann *et al.,* 2010) The recurrence and metastasis rate of kidney cancer is still about 20%-30% even if the kidney cancer patients undergo radical surgery. (Kirchner *et al.,* 2010) Kidney cancer is not sensitive to the traditional radiotherapy and chemotherapy.

We have recently reported that chitooligosaccharides and N-acetyl-D- glucosamine induced antitumor immune responses mediated by the peripheral blood mononuclear cells. (Xu *et al.,* 2012) *Tegillarca granosa* Linnaeus-1 (TG-1), also known as *Anadara granosa or* Haishengsu, is a species of ark clam (shell fish) and a polypeptide extracted from *Tegillarca granosa* Linnaeus. It has antitumor effects and is a novel antineoplastic agent. (Liu *et al.,* 2004; Yao *et al.,* 2005) Raw material is derived from fresh *Tegillarca granosa* Linnaeus' meat and extracted at low temperature (0-10°C). Our previous studies have shown that TG-1 could inhibit the growth of human erythroleukemia K562 cell, lung adenocarcinoma epithelial A549 cells, and hepatocellular carcinoma cells. (Liu *et al.,* 2004; Yao *et al.,* 2005; Li *et al.,* 2008) TG-1 has a molecular weight of about 15 kDa.

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The aim of this study is to determine the antitumor effect and mechanism of antitumor effect of TG-1 on renal carcinoma OS-RC-2 cells *in vitro*. To some extent, tumor growth is determined by the balance between tumor cell proliferation and tumor cell apoptosis. The Ki-67 protein is a cellular marker for proliferation, the detection of which can reflect cell proliferation. Meanwhile, caspase-3 is the final executor of cell apoptosis, which can represent the extent of the cell apoptosis. Thus, to investigate the antitumor effect of TG-1, tumor cell growth was observed by MTT assay. Cell cycle distribution was detected by flow cytometry. Cell apoptosis was assessed by acridine orange/ethidium bromide (AO/EB) staining. And to further investigate the underlying mechanism, we detected the expression of cellular proliferation maker Ki67 by RT-PCR and the expression of apoptotic protein caspase-3 by Western blot analysis.

MATERIALS AND METHODS

Reagents

TG-1 was provided by the pharmaceutical department of Qingdao Haisheng Oncology Hospital (Shandong, China, batch number 990211). MTT and AO/EB kits were purchased from Sigma (St. Louis, Missouri, USA). Propidium iodide (PI) apoptosis kit was purchased from Becton Dickinson (San Jose, California, USA). RT-PCR kit was purchased from Promega (Madison, Wisconsin,

USA). Ki67 primary antibody was purchased from Wuhan Boster Experimental Equipment Co., Ltd. (Wuhan, China). Ki67 primer was synthetized by Shanghai biological engineering Co., Ltd. The primers of GADPH and β-actin and rabbit anti-caspase-3 antibody were purchased from Wuhan Boster Experimental Equipment Co., Ltd. N1-(2 tetrahydrofuryl)-5-fluorouracil (FT-207) was purchased from Qilu Pharmaceutical Co., Ltd. (Jinan, China). Other reagents were analytical pure level.

Preparation of renal carcinoma OS-RC-2 cells

The human renal carcinoma OS-RC-2 cells were purchased from the Life Science Research Institute, Chinese Academy of Sciences. The cells were cultured with DMEM medium containing 10% new bovine serum in an incubator (37°C, 5% CO_2).

MTT test

The cells (5×10^4/ml cells) were seeded into 96-well culture plates (200 μl/well). TG-1 was added at different concentrations (50 mg/L, 100 mg/L and 150 mg/L). The cells cultured with DMEM medium only were used as the negative control. The effects of various concentrations of TG-1 on the proliferation of the cells were analyzed with the MTT method on the 2nd and the 4th day of TG-1 incubation. The absorbance was measured at 570 nm on a microplate reader.

Apoptosis detection

After the cells were treated with TG-1 in 96-well plates for 3 days, 0.1 mg/ml of AO/EB dyes were added (8 μl/well). The cell apoptosis was observed under fluorescence microscopy. Living cells (VN) show green fluorescence and normal chromatin structure. The early apoptotic cells (VA) show green fluorescence and pleomorphic nuclei pyknosis. However, apoptotic cells (NVA) show orange fluorescence and pleomorphic nuclei pyknosis. The necrotic cells (NVN) show orange fluorescence and normal chromatin structure. Apoptosis rate was calculated by the formula of (NVN+NVA) /(VN+VA+NVN+NVA)×100% Flow cytometry.

The cells were digested, collected and adjusted to a density of $5x10^4$/ml. The cells were then cultured in the 25 cm^3 of cell culture flasks (3 ml of the cells per flask) for 24 h. RNase (100 μl) and PI single dye solution (25 mg/L, 200 μl) were then added to the flasks. The cells in each cell cycle phase were counted by using a flow cytometer.

RT- PCR analysis

Total RNA of the cells in each group was extracted with Trizol reagent and then 1 μg of total RNA was used for the RT - PCR analysis. Primer sequences for Ki67 (438 bp in length) were: (upstream) 5'-ACTTGCCTCCT-AATACGCC-3' and (downstream) 5'-TTACTACAT-CTGCCCATG A-3'. GAPDH (500bp in length) was used as an internal control. And primer sequences for GADPH were: (upstream) 5'-ACTGCCACCCAGAAGACT -3' and (downstream) 5'-GCTCAGTGTAGCCCAGGAT-3'. Reaction conditions of PCR were as follows: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, anneal at 53°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. After electrophoresis on agarose gel, the gray value of each mRNA band was analyzed using Quantity One software (Bio-Rad). The relative gray value of Ki67/GADPH was used as the relative expression level of Ki67 mRNA.

Western blotting

The cells were seeded into 6-well culture plates and divided into four groups (three wells per group) randomly. After the cells were treated with different concentrations of TG-1 (50 mg/L, 100 mg/L and 150 mg/L respectively) for 48 h, the proteins were extracted and quantified using BCA protein quantitative kit. The proteins were then analyzed using Western blot. Rabbit anti-caspase-3 antibody was used as the primary antibody. The blotting bands were measured with Leica Qwin image processing software. The experiment was repeated for 3 times.

RESULTS

Effect of TG-1 on the proliferation of renal carcinoma OS-RC-2 cells in vitro

To examine whether the TG-1 can affect the proliferation of renal carcinoma OS-RC-2 cells, we cultured the renal carcinoma OS-RC-2 cells with TG-1 at various concentrations. As shown in table 1, TG-1 significantly inhibited the proliferation of OS-RC-2 cells in comparison with the control (DMEM medium only) ($P< 0.05$). Moreover, the inhibition effects of TG-1 at the concentration of 100 mg/L were better than those at other concentrations ($P<0.01$).

Table 1: The effect of TG-1 on proliferation and apoptosis of kidney cancer OS-RC-2 cells *in vitro* (mean±SD, n=3).

Groups	Absorbance (A)	Apoptosis rate (%)
Control	0.4365±0.0215	4.56±1.14
TG-1 (50mg/L)	0.3927±0.024 [a]	17.43±2.31[a]
TG-1 (100mg/L)	0.2324±0.0107[b]	46.25±3.94[b]
TG-1 (150mg/L)	0.2982±0.0273[a]	28.97±4.02[a]

Note: [a], $P<0.05$, [b], $P<0.01$, vs. control.

Effect of TG-1 on apoptosis of renal carcinoma OS-RC-2 cells

To determine the effect of TG-1 on the apoptosis of renal carcinoma OS-RC-2 cells, AO/EB staining was performed

and the cells with different status were counted. As shown in table 1, the apoptotic rates of the cells treated with 50 mg/L, 100 mg/L and 150 mg/L of TG-1 were 17.43±2.31, 46.25±3.94 and 28.97±4.02, respectively. The apoptotic

Fig. 1

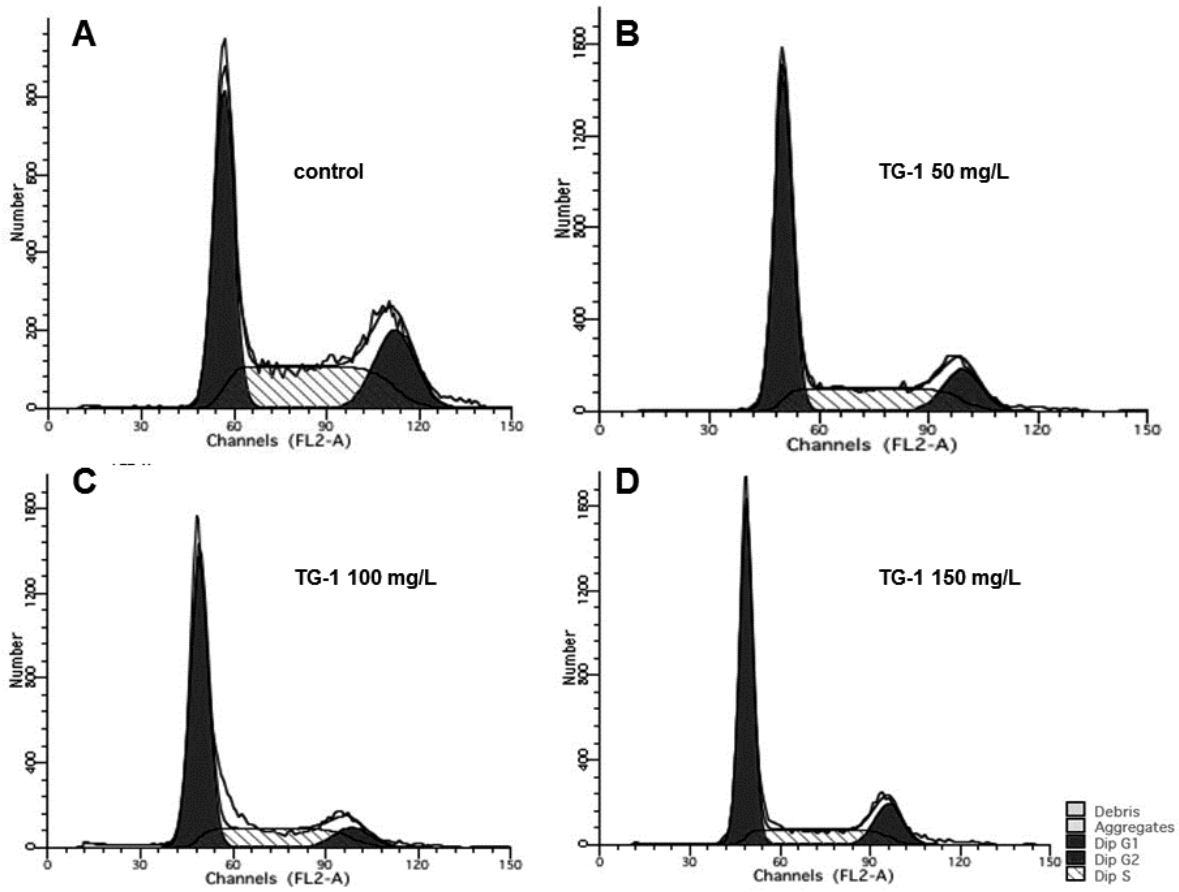

Fig. 1: The cell cycle distribution of OS-RC-2 cells detected by flow cytometry. Cell cycle distribution was analyzed using a flow cytometry. Representative flow cytometry results were shown. (A) The negative control (DMEM medium only); (B) TG-1, 50 mg/L; (C) TG-1, 100 mg/L; (D) TG-1, 150 mg/L

Fig. 2

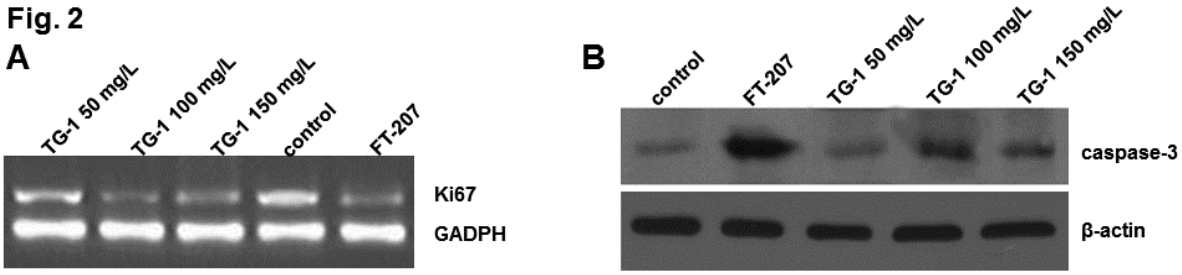

Fig. 2: The effects of TG-1 on levels of Ki67 mRNA and expression of caspase-3 in OS-RC-2 cells treated with TG-1. (A) The expression of Ki67 mRNA in OS-RC-2 cells treated with TG-1 was analyzed by RT-PCR. Amplication products were run on agarose gel and representative electrophoresis image was shown. The gray value of each mRNA band was analyzed using Quantity One software (Bio-Rad). The relative gray value of Ki67/GADPH was used as the relative expression level of Ki67 mRNA. (B) The expression of caspase-3 in OS-RC-2 cells treated with TG-1 was analyzed by Western blot. FT-207 was used as the positive control. The gray values of blotting bands were measured with Leica Qwin image processing software. The experiment was repeated for 3 times. The condition with DMEM medium only was used as the control.

rates of cells treated with TG-1 were higher than the rates in the control group.

Table 2: The influence of TG-1 on cell cycle distribution of OS-RC-2 cells as measured by flow cytometry.

Groups	G_0/G_1 phase (%)	S phase (%)	G2/M phase (%)
control	42.71	37.27	20.56
TG-1 (50 mg/L)	56.73[a]	29.24[a]	14.03
TG-1 (100mg/L)	69.04[b]	22.32[b]	8.63
TG-1 (150mg/L)	60.08[b]	26.37[b]	13.55

Note: [a], P<0.05, [b], P<0.01, vs. control.

Effect of TG-1 on cell cycle distribution of renal carcinoma OS-RC-2 cells

To determine the effects of TG-1 on cell cycle distribution of OS-RC-2 cells, flow cytometry analysis was conducted to analyze the percentage of cells in each phase. The changes of cell cycle distribution were listed in fig. 1 and table 2. Compared with the control group, the percentage of the cells increased in the G_0/G_1 phase and decreased in the S and G_2/M phase in TG-1 treated group (P<0.05) suggesting that the TG-1 inhibited the growth of OS-RC-2 cells.

Table 3: The Ki67 mRNA expression analysis in OS-RC-2 cells treated with TG-1 (mean ± SD, n=3).

Groups	Relative gray value of Ki67 to GAPDH
Control	0.7235±0.0312
FT-207 (110mg/L)	0.4217±0.0201[b]
TG-1 (50mg/L)	0.6534±0.0227[a]
TG-1 (100mg/L)	0.5439±0.0172[b]
TG-1 (150mg/L)	0.6109±0.0155[b]

Note: [a], P<0.05, [b], P<0.01, vs. control.

Effect of TG-1 on the expression of Ki67 mRNA in renal carcinoma OS-RC-2 cells

To determine if TG-1 inhibits expression of Ki67 mRNA in OS-RC-2 cells, we used RT-PCR to detect the expression levels of Ki67 mRNA in the cells treated with or without TG-1. FT-207, a clinical anti-cancer drug, was used as a positive control. As shown in fig. 2A and table 3. The expression level of Ki67 mRNA in TG-1 treated OS-RC-2 cells was significantly lower than that in the control group (P<0.05). TG-1 with the concentrations of 100 mg/L or 150 mg/L produced comparable effects on the expression levels of Ki67 mRNA as the positive control FT-207.

Effect of TG-1 on the expression of caspase-3 in renal carcinoma OS-RC-2 cells

To determine whether expression of caspase-3 in OS-RC-2 cells treated with TG-1 is changed, we detected caspase-3 levels by Western blot analysis. As shown in fig. 2B and table 4. The expression levels of caspase-3 in OS-RC-2 cells cultured with TG-1 were higher than the levels in the control group (P<0.05), but lower than the levels in the group of FT-207 (P<0.01).

Table 4: The caspase-3 protein expression in OS-RC-2 cells treated with TG-1 (mean ± SD, n=3).

Groups	Relative gray value of caspase-3 to β-actin
Control	0.1675±0.0117
FT-207 (110mg/L)	0.8814±0.0219[b]
TG-1 (50 mg/L)	0.2133±0.0324[a]
TG-1 (100 mg/L)	0.5621±0.0183[b]
TG-1 (150 mg/L)	0.2927±0.0206[a]

Note: [a], P<0.05, [b], P<0.01, vs control.

DISCUSSION

The disorder of cell growth caused by the imbalance of the positive and negative regulating signals is the main reason for tumorigenesis. The positive regulating signals can promote cell growth, proliferation and prevent terminal differentiation. The negative regulating signals can promote cell mature, differentiation and apoptosis. (Mangues *et al.,* 1999; Michor *et al.,* 2004; Shaw RJ and Cantley, 2006) Its mechanism involves proto-oncogene activation, the cancer gene inactivation, proliferation signal activation and abnormal cell cycle etc. (Hainaut P and Hollstein, 2000; Prochownik, 2008).

In this study, we investigated the antitumor effect of TG-1 in renal carcinoma OS-RC-2 cells. First we detected tumor growth by MTT assay. The results showed that growth of OS-RC-2 cells was inhibited by TG-1 and the inhibition effect at the concentration of 100mg/L was better than that at other concentrations. We also analyzed cell cycle distribution by flow cytometry. Cell cycle of OS-RC-2 cells was arrested at G_0/G_1 phase by TG-1, indicating that tumor cell growth was inhibited. Additionally, as revealed by AO-EB staining, OS-RC-2 apoptosis was increased after TG-1 treatment. Similarly, the apoptosis rate and the percentage of cells at G_0/G_1 phase were the highest when the concentration of TG-1 was 100 mg/L. Taken together, our results were consistent with previous reports and further confirmed the antitumor effect of TG-1 in OS-RC-2 cells (Liu *et al.,* 2004; Yao *et al.,* 2005; Li *et al.,* 2008).

Interestingly, in previous studies, the effect of TG-1 is dose-dependent. For example, TG-1 promoted apoptosis of drug-resistant K562/ADM tumors in mice in a dose-dependent manner. (Li *et al.*, 2013) In mice inoculated with K562/ADM cells, the inhibitory rates in the high, medium and low dose of TG-1 were 100%, 95.9%, and 44.1%, respectively. (Liu *et al.*, 2009) The cytotoxic

effects of TG-1 on two ovarian cancer cell lines SKOV-3 and OVCAR-3 were also concentration dependent (Gao *et al.*, 2009). However, in this study, the effect of TG-1 on renal carcinoma cells was not dose-dependent. A higher concentration of TG-1 (150 mg/L) had a lower effect than a lower concentration of TG-1 (100mg/L). This may be caused by the unique biological features of renal cells.

To further investigate the mechanism underlying the proliferation inhibition, we detected the expression of Ki67 mRNA in OS-RC-2 cells after TG-1 treatment. Ki67 gene is a marker of cell proliferation and is related to the proliferation of tumor cells, and its coding protein is a DNA binding protein. It expresses in G1, G2, S and M phase of the tumor cells and is an essential component. Previous studies have found that the enhanced expression of Ki67 in kidney tissue was closely related to kidney cancer staging, grading and prognosis. (Chen *et al.,* 1997; Krause *et al.,* 2000; Malmström *et al.,* 2002) In this study, we found that expression of Ki67 mRNA in OS-RC-2 cells was decreased after TG-1 treatment. This result indicates that TG-1 may inhibit proliferation of OS-RC-2 cells through suppressing Ki67 expression.

Furthermore, studies have shown that TG-1 could also exert its antitumor effect through other mechanisms such as inducing cell cycle arrest and apoptosis. (Gao *et al.*, 2009; Li *et al.,* 2008; Li *et al.,* 2010) For example, cell cycle progression was inhibited and arrested at the G0/G1 and S phases. Tumor cell apoptosis was induced with decreased expression of apoptosis suppressor gene bcl-2 and increased expression of apoptosis executor caspase-3. Caspases are a type of cysteine-aspartic acid proteases. (Martin *et al.,* 1998) It exists as an inactive precursor in normal cells. When the cells are induced to apoptosis, a variety of caspases will be activated through intracellular cascade process. (Dvorak *et al.,* 1999) They play important roles in the proteolytic process during apoptosis. In the caspase family, caspase -2, 8, 9 and 10 are upstream proteases and caspase-3, 6 and 7 are downstream proteases. Caspase-3 is the final executor of cell apoptosis, which can activate DNA enzymes, cleave DNA into pieces and finally lead to cell apoptosis. Therefore, the detection of caspases-3 activity represents the extent of the cell apoptosis. (Luo *et al.,* 1998; Ravi *et al.,* 2000; Galvan *et al.,* 2002; Brodsky *et al.,* 2007) In our study, caspase-3 expression was increased after TG-1 treatment. This data suggest that through promoting the expression of caspase-3, TG-1 induced apoptosis of OS-RC-2 cells, thus inhibiting tumor growth.

In summary, the experimental results in this study show that TG-1 inhibited cell growth of OS-RC-2 cells, arrested cells at G_0/G_1 phase and promoted apoptosis of OS-RC-2 cells. These antitumor effects may be induced through inhibiting the expression of Ki67 mRNA and inducing the expression of caspase-3 protein.

ABBREVIATIONS

RCC: renal cell carcinoma; TG-1: *Tegillarca granosa* Linnaeus-1; AO/EB: acridine orange/ethidium bromide; PI: Propidium iodide

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