Original article

The effect of Marine drug candidates Tagalsin on bcl–2 and caspase–3 expression in H22 tumor–bearing mice

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ABSTRACT **Objective:** To investigate the inhibitory effect of Tagalsin on H22 hepatoma cells. Materials and Methods: Animal models were established by transplanting H22 mouse hepatoma cells into mouse liver. Ten days later they were randomly divided into five groups: blank group (edible oil 0.1ml), positive group (Carmofur (HCFU)) and Tagalsin group,including low-dose, middle-dose and high-dose group. All mice were killed 24 hours after medication. Survival conditions, weight changes, tumor similar volume and spleen index of tumor-bearing mice were observed and recorded. Pathological changes of tumor were observed. The expression level of the apoptosis factors caspase-3 and bcl-2 was detected by reverse transcription polymerase chain reaction (RT-PCR). Results: Tagalsin inhibits hepatoma growth effectively without influencing spleen index,the tumor inhibitor rate of low, middle and high dose group of Tagalsin were 17.89%、63.11% and 71.78% respectively, and the tumor inhibitor rate of HCFU group was 63.12%. Apoptotic cells existed in the specimen of the positive control group and the experimental groups. The expression of caspase-3 in positive control groups and Tagalsin treatment groups was significantly enhanced, but the expression of bcl-2 was decreased compared to the control group($P< 0.05$).Conclusion: Tagalsin inhibits mouse hepatoma cells significantly; the mechanism of this anti-tumor effect might be through the up-regulation of caspase-3 expression and down-regulation of bcl-2 expression..

Key Words: Tagalsin, hepatoma, caspase-3,bcl-2, cell apoptosis

Ceriops is higher plant from the Rhizophoraceae, growing in the tropical and subtropical area. The biosynthesis pathway and enzyme reaction system of their secondary metabolite are different from others because of their special living conditions. The secondary metabolite of Ceriops tagal has novel structure and remarkable activeness. Studies have shown that the secondary metabolite has functions of stopping itchiness pain and bleeding, treating boils and malaria [1-3]. Recent studies also show that Tagalsin, the extract of Ceriops tagal, has good membrane permeability and anti-tumor activities [4], and has significant inhibition to mice subcutaneous transplantation of S180 tumor cells. However, the mechanisms therein are not quite clear yet. Our study is to investigate the anti-tumor effect of Tagalsin and its effect on caspase-3 and bcl-2 gene expression. We hope to provide some preliminary data for clinical application.

Materials and method

1.Materials

1.1Tumor cell lines: The H22 hepatoma cell lines were purchased from pharmacological research institute of Ocean University of China

1.2Animals: Sixty Kunming female mice, four weeks old and twenty nine to thirty grams, were provided by pharmacological research institute of Ocean University of China (SCXK Shandong

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1.3Drugs: Marine drug candidate Tagalsin was provided by The State Key Laboratory of Natural and Bimimetic Drugs in Peking University (GSM-01). The stock concentration of Tagalsin was 20 mg/ml. The working concentrations were0.2 mg/ ml、1.0 mg/ ml、5.0 mg/ml diluted in edible oil. Carmofur(50mg) was served as a positive control.

*1.4 Reagents:*Trizol and primers were purchased from Shanghai Clone Biology High-Tech Co. Ltd.(Shanghai, China). RT-PCR reverse transcription reagent bax were purchased from Life Technologies, Inc. (Grand Island, NY). Fetal calf serum (FCS) was purchased from Lanzhou Minhai Biologyl Engineering Co. Ltd. (Lanzhou, China), nucleic acid dye was purchased from Spain gelatin Co. Ltd. (BioKit, Spain), GoldView and markers were from Dongsheng, (Qing dao,china).
Primer sequences:
CTNNB1_forward: 5'TGCTGTCCCTGTATGCCTCT 3' Temperature 57.1 ℃
CTNNB1_reverse: 5'GATGTCACGCACGATTTCC 3' Temperature 56.5 ℃ Product length (221 base pair)
BCL2_forward: 5'GGTGGTGGAGGAACTCTTCAGGG3' Temperature 65.8 ℃
BCL2_reverse: 5'GAGACAGCCAGGAGAAATCAAACAGA 3' Tm 65.3 ℃ Product length (254 base pair)
CASP3_ forward: 5'GGAATGTCATCTCGCTCTG3' Temperature 53.0 ℃
CASP3_reverse: 5'GCAAGCCATCTCCTCATC3' Temperature 52.7 ℃ Product ength (375 base pair)

2 Methods

2.1 Model building

H22 cells (1 × 108) were injected subcutaneously into the left oxter of mice. When the subcutaneous tumor reached approximately 1 cm in length (approximately 4 weeks after injection), it was removed, minced into small pieces of equal volume (2 × 2 × 2 mm3) and to be spared. Mice were hocused through intraperitoneal injection of 10% chloral hydrate hydras, and then a 1.5 cm transverse incision was made 0.5cm below sternum inferior margin and squeezed out the liver, an about 3mm-cut was made on the left lobe of liver, the tumor mass was put in, sticking cut and then closing abdominal. Mice were dissected 10 days later.

2.2 Intervening method

Successful mouse models were randomly divided into blank group (edible oil 0.1ml), positive control group (Carmofur (HCFU)) and Tagalsin treatment groups with 12 mice in each group. The treatment groups included low-dose(0.66 mg.kg-1.d-1),middle-dose (3.30 mg.kg-1.d-1)and high-dose groups(16.50 mg.kg-1.d-1). The mice were fed with drugs by gavage for 5 days with 2 days interval

2.3 Observing indexes

2.3.1 General observing

The mice were killed 24 hours after the last medication. The length and weight of the spleen, tumor similar volume, anti-tumor rate and spleen index were recorded.

2.3.2 hematoxylin and eosin stain

Liver tissue with the size of 1 cm ×1 cm ×1 cm (including tumor tissue) was fixed by 10% methanol solution for 2 hours, and then dehydrated by general gradient alcohol, transparented by Xylene, paraffin embedding, continuous slice keep the thickness about 5um. Finally General HE dye were perfomed according to Staining Procedure :①Deparaffinize and hydrate to water ②If sections are Zenker-fixed, remove the mercuric chloride crystals with iodine and clear with sodium thiosulphate (hypo) . ③ Mayer's hematoxylin for 15 minutes ④Wash in running tap water for 20 minutes ⑤Counterstain with eosin from 15 seconds to 2 minutes depending on the age of the eosin, and the depth of the counterstain desired. For even staining results dip slides several times before allowing them to set in the eosin for the desired time ⑥Dehydrate in 95% and absolute alcohols, two changes of 2 minutes each or until excess eosin is removed. Check under microscope .⑦Clear in xylene, two changes of 2 minutes each

2.3.3 RT–PCR

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction (PCR), a laboratory technique commonly used in molecular biology to generate many copies of a DNA sequence, a process termed "amplification". In RT-PCR, however, an RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real-time PCR. In this experiment, Total RNA was extracted using Trizol reagent according to the Trizol directory. And in the second place, cDNA was synthesized when we controlling temperature at 30℃ for 10min, 42℃ for 45min,

95℃ for 5min respectively. Otherwise, β-actin was synthesized in order to served as the internal control. The procedure of the PCR is as follows: 35 circles for β-actin, and 37 circles for caspase-3 and bcl-2. The final step of PCR amplification is DNA extension from the primers. This is done with thermostable Taq DNA polymerase, usually at 72°C, the temperature at which the enzyme works optimally. PCR products were either stored at 4℃ or immediately for agarose gel electrophoresis. RT-PCR results were analyzed using imaging analysis system . The quantification was done using the Biocapt MW software.

2.3.4 Statistics

Results were expressed as mean ± SD and analyzed by statistical software SPSS15.0. Differences were considered to be statistically significant when $P < 0.05$.

Results

1 Establishment of H22 hepatoma transplantation in situ mice model

Five days after transplantation of H22 cells, we dissected the mice and saw single pale mass lesion with the size of half of a rice grain on the surface of the liver, not completely fused with the tissue around. Ten days after transplantation, pale neoplasm emerged on the surface of the mouse liver, with the size of 0.2cm and complete fusion with the tissue around. Fifteen days after transplantation, the diameter of the tumor was about 0.5 cm. At twenty-two days after transplantation, the diameter of the tumor was about 1 cm. The transplantation efficiency was 100%. Most mice had single tumor. Some mice had many sites of tumors in the livers or lungs..

2 Grow conditions of mice

During drug administration, mice of medication group had burnish hair,agile action and better diet.while the mice of blank control group was unresponsive and had sparse hair and present cachexia status.

The numbers of appearing ascites of blank, positive, low, middle and high dose groups were 6, 2, 1, 1, 1, respectively. The numbers of tumor transplantation were 4, 1, 0, 2, 1. And the numbers of deaths were 5, 1, 4, 1, 1, respectively.Comparing between before and after drug administration, weight of the mice was obviously decreased only in low and middle dose groups($P <0.01$), while the other three groups had no significant statistic difference($P>0.05$). (Table 1)

Table 1 Changes in mice weight before and after drug administration (±s)

Groups	number	Before drug (gram)	After drug(gram)	t value	P
blank control group	7	28.93±0.56	29.46±6.96	0.15	0.887
positive control group	11	28.92±0.33	29.78±0.69	2.03	0.089
low-dose group	8	28.69±0.36	24.05±1.51▲	6.96	0.002
middle-dose group	11	28.66±0.35	23.78±1.31▲	5.45	0.002
high-dose group	11	28.66±0.35	29.68±0.52	2.20	0.070

t value =5.45–6.96, P<0.01, comparison with before drug administration

3 Comparisons of Tumor weight and anti-tumor rate in each group

We found that Tagalsin, especially high doses of Tagalsin, and HCFU inhibited tumor growth significantly by calculating tumor similar volume and anti-tumor rate,. Compared to the blank control group, the tumor similar volume of middle, high dose groups and HCFU group had significant statistic difference($P <0.01$).There was no significant statistic difference between Tagalsin and HCFU as regards to the tumor similar volume($P > 0.05$). Compared among all Tagalsin groups, there was statistically significant difference between high and low dose groups($P<0.05$), while no statistic difference between high and middle groups or between low and middle groups($P > 0.05$). The anti-tumor rates of mice fed with low, middle and high dose of Tagalsin were 17.89%、63.11%、71.78% respectively, and that of HCFU-fed mice was 63.12%. (Table 2)

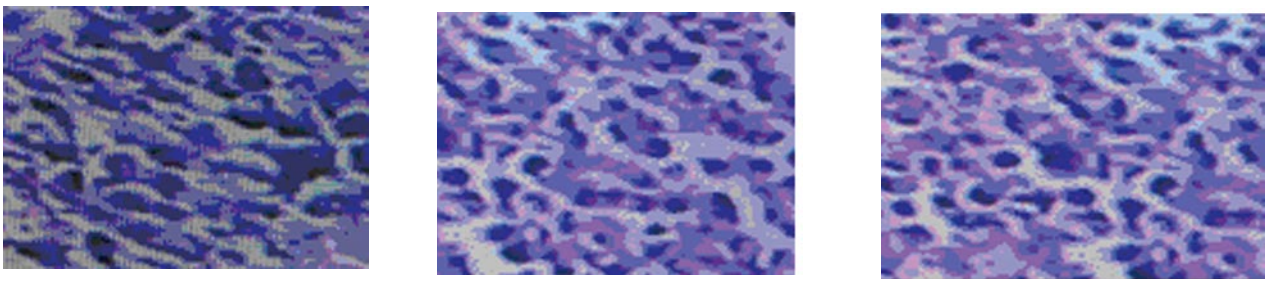
Table 2 Tagalsin' influence on tumor similar volume、anti-tumor rate and splenic index ($\bar{x}$ ±s)

Groups	number	Tumor similar Volume(mm3)	Anti-tumor rate(%)	Splenic index(mg/10g)
blank control group	7	225.80±109.73# #▲▲	—	104. 95±11. 83# #▲▲
positive control group	11	89.60±83.14**	63.12	56.23±11.42**▲▲
low-dose group	8	185.40±104.36▲	17.89	98.88±8.57# #▲▲
middle-dose group	11	89.61±71.70**	63.11	96.11±7.68# #▲▲
high-dose group	11	63.72±47.03**	71.78	75.80±9.18**# #

F =4.92、25.88,q=4.96～6.53, *P<0.01, compared to the blank control group;q=5.12~7.94, #P<0.01, compared to the positive control group; q=4.12~8.14, P< 0.05, P<0.01, compared to the high dose group

4 Histopathology Variations

While observing tumor cells of blank control group under the microscope, we saw that tumor tissue had little fibrous stroma, and tumor cells had big volume, big hyperchromatic nuclei, clearly visible nucleoli, more fission, less cytoplasm, basophilic. The characters of the tumor in the high dose Tagalsin group and positive control group are of small volume, hyperchromatic cytoplasm, karyopyknosis, unclear nucleoli, and crushed and scattered apoptosis. (Fig 1)

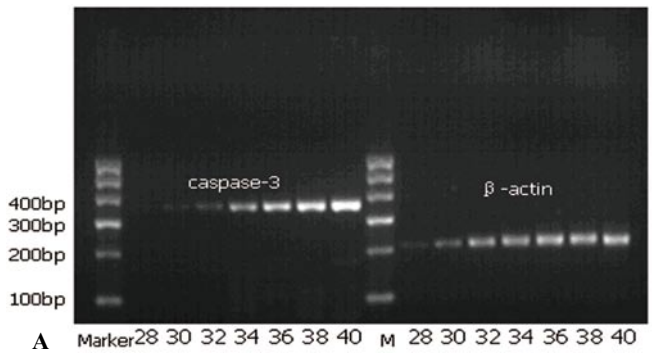


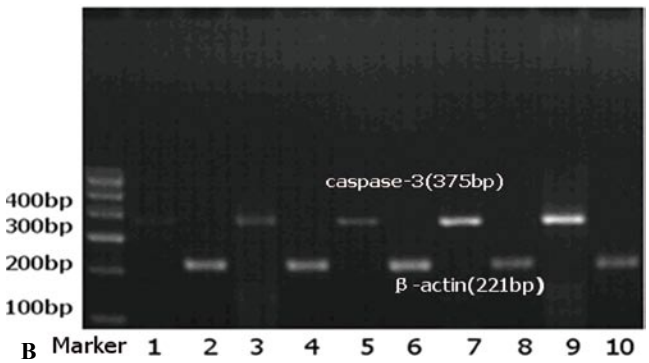
Blank control positive control high dose Tagalsin

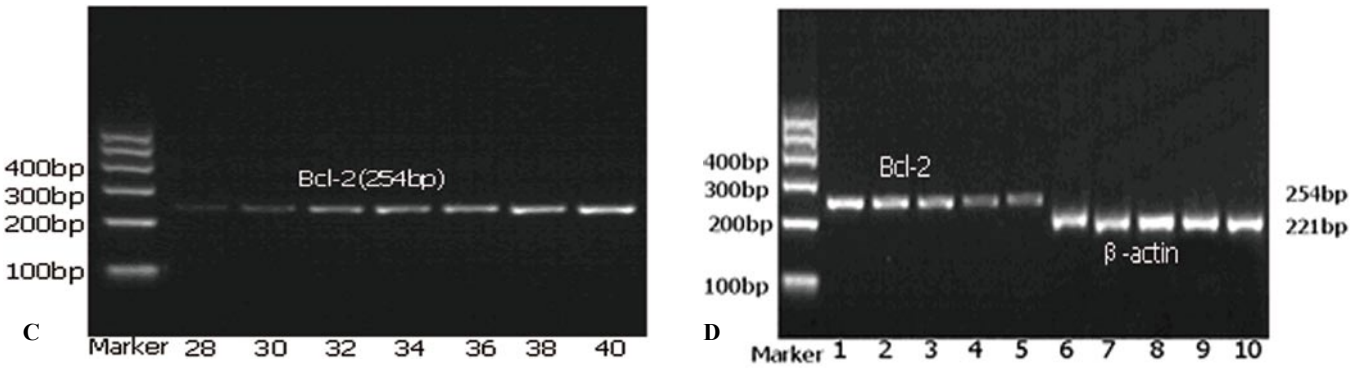
Fig 1 Histopathology morphology of blank control and high dose Tagalsin groups, HE dyeX200

5 Expression level of Caspase-3 and bcl-2 -

The mRNA level of Caspase-3 was up-regulated in all Tagalsin groups and positive control group(P<0.05), while the mRNA level of bcl-2 was down-regulated in high dose Tagalsin group and positive control group(P<0.01). (Fig 2 and table 3)







A) Expression of Caspase-3. The exponential phases of β-actin and Caspase-3 were at 36、38 cycles.

B)The electrophoretogram of Capase-3 (37 cycles). Lanes 1,3,5,7,9 are blank group, low,middle, high dose groups and positive control group respectively

C) Expression of bcl-2. The exponential phases of bcl-2 was at 38 cycles.

D) The electrophoretogram of bcl-2 (35). Lanes 1,2,3,4,5 are blank group, low, middle, high dose groups and positive control group respectively

Fig 2 The electrophoretogram of Capase-3and bcl-2

Table 3 The influence of Tagalsin and HCFU on Capase-3 and bcl-2 gene expression ($\bar{x}$ ±s)

Groups	number	Capase-3 level	bcl-2 level
blank control group	7	0.31±0.11#	1.42±0.29#
positive control group	11	0.89±0.14**	0.99±0.12**
low-dose group	8	0.56±0.11*#	0.97±0.15**
middle-dose group	11	0.65±0.14**#	0.86±0.50**
high-dose group	11	0.91±0.07**	0.62±0.16**#

F=21.58㊀12.89, q= 3.87㊀8.42 ▮ *P<0.05, **P<0.01, compared to the blank control group.q=5.22㊀7.89, #P<0.01 compared to the positive control group

Discussion

We studied the anti-tumor effect of Tagalsin in vivo by transplanting H22 cells into mice liver and fed these mice with Tagalsin at different doses.. Our data showed that Tagalsin inhibited H22 tumor effectively in a dose-dependent manner. High dose of Tagalsin-fed group and positive control group has obvious antitumor effects, their tumor inhibitor rates were 71.78%、63.12% respectively. There was no statistically significant difference between these two groups. Moreover, the ascites,tumor transfer, and death was less in high dose of Tagalsin group than in the control group. Spleen index, which is the weight of spleen per 10 grams, roughly reflects drugs' influence on immune organ [6]. This study showed that the toxicity and side-effects of each Tagalsin group were lower than those of the HCFU group. However, the spleen index of high dose group was lower than that of the low and middle dose groups (P<0.01). There was no statistically significant difference between high dose group and middle dose group or positive group as regards to the tumor similar volume, indicating that the side-effects of Tagalsin was less than those of HCFU when they has the same tumor inhibiting rate. Further study is required to determine whether high dose is the appropriate dose to treat- tumors.

Inducing cells apoptosis is the crucial pathway of anti-tumor drugs. Caspases are the nuclear executors of cell apoptosis [7]. Activation of caspase-3 is a symbol of cell apoptosis [89].

Bcl-2 is an important anti-apoptotic gene and has different subtypes. It locates in chondriosome, endoplasmic reticulum and karyolemma and inhibits apoptosis through stopping release of chondriosome cytochrome C. Besides, overexpression of Bcl-2 induces cellular glutathion (GSH), which result in nuclea redox disbalance and the decrease of caspase activity [9]. When Caspase-3 is inhibited, cell apoptosis is blocked and the dynamic balance between apoptosis and proliferation is changed, inducing further development of tumors. Our results showed that the expression of caspase-3 mRNA in high doses, middle dose and low dose Tagalsin groups was up-regulated significantly, while the mRNA level of Bcl-2 was down-regulated($P<0.05$), especially in high doses group. Our data indicate that promoting cell apoptosis might be an anti-tumor mechanism of Tagalsin.

In conclusion, Tagalsin, the extracts from the mangrove plant Ceriops tagal, inhibits H22 hepatoma cell growth in transplanting tumor in vivo, and the possible mechanism might include cellular apoptosis. The best anti-tumor drug concentration, toxic and side-effects and the detailed anti-tumor mechanism still need further investigation.

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