RESEARCH ARTICLE

Liposome-mediated Induction of Apoptosis of Human Hepatoma Cells by C-Myc Antisense Phosphorothioate Oligodeoxynucleotide and 5-Fluorouracil

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Abstract

Background: The aim of this study was to investigate the effect of a c-myc antisense oligodeoxynucleotide and 5-fluorouracil on the expression of c-myc, invasion and proliferation of HEPG-2 liver cancer cells. Materials and Methods: HEPG-2 cells were treated with lipiosome-mediated c-myc ADSON and 5-fluorouracil. The proliferation inhibition rate and invasion were measured by MTT and invasion assay, respectively. Cell apoptosis was detected by flow cytometry and expression of c-myc by RT-PCR and immunohistochemistry. Results: The proliferation inhibition rate was significantly higher in the antisense oligodeoxynucleotide added-5-fluorouracil group than single antisense oligodeoxynucleotide or 5-fluorouracil group ($p<0.05$). G0/G1 cells in the antisense oligodeoxynucleotide group and S cells in the 5-fluorouracil groups were significantly increased than that in the control group, respectively ($P<0.01$). The amplification strips of PCR products in 5-FU, ASODN and combination groups were significantly weaker than that in the control group ($P<0.01$). The percentage of c-myc-protein-positive cells were significantly lower in antisense oligodeoxynucleotide, 5-fluorouracil and combination groups than that in the control group ($P<0.01$). Conclusions: A liposome-mediated c-myc antisense oligodeoxynucleotide and 5-fluorouracil can inhibit the proliferation and invasion of liver cancer cells by reducing the expression of c-myc. A c-myc antisense oligodeoxynucleotide can increase the sensitivity of liver cancer cells to 5-fluorouracil and decrease the dosage of the agent necessary for efficacy, providing an experimental basis for the clinical therapy of liver cancer.

Keywords: C-myc - antisense oligodeoxynucleotide - 5-fluorouracil - hepatoma carcinoma cell - apoptosis

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Introduction

Hepatoma carcinoma (HCC) is one of the most malignant carcinomas, with a very poor prognosis. Hepatoma Carcinoma is the fourth most co mmon cancer in the world and the third leading cause of cancer mortality worldwide (Parkin et al., 1997). It is estimated that there will be 28, 720 new cases diagnosed and 20, 550 deaths due to this disease in the United States in 2012 (American Cancer Society. 2012. Available online). In whom the annualized rate of HCC is 22.1 per 100,000 population among men and 8.4 per 100,000 women (Howlader et al., 2012). A major reason for this low survival rate is the insensitivity of hepatoma carcinoma to most anti-cancer therapies, including surgery liver transplantation, transcatheter arterial chemoembolization, percutaneous ethanol injection, radiofrequency ablation, microwave ablation, high intensity focused ultrasound, chemotherapy, radiotherapy, and i mmunotherapy, cryoablation therapy, etc. Most hepatoma carcinoma deaths are due to metastatic diseases, thus, the therapies that inhibit both cancer growth and metastasis are expected to be the most effectively. There is a clear need for new treatment approaches for this aggressive disease, and anti-metastatic strategies have been increasingly used in the treatment of hepatoma carcinoma patients, with the aim of decreasing the frequency of metastasis.

C-myc is a proto-oncogene, which is strongly related to cell proliferation, differentiation, apoptosis and cycle regulation, and highly expresses in most malignancies (Albihn et al., 2010, Wang et al., 2011, Barathidasan1 et al., 2013) such as liver, lung, stomach, breast, colon, and cervical cancer, etc. The relationship between the c-myc over-expression and liver cancer has been confirmed in some studies (Lin et al., 2010). Recently, gene techniques make it possible for c-myc as a target for liver cancer therapy. As an important medicinal herb, Atractylis lancea (Thunb.) has been demonstrated with anti-proliferative effects in Hep-G2 cells via down-regulation of the c-myc/hTERT/telomerase pathway (Guo et al., 2013). Some

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studies showed that antisense oligodeoxynucleotide (ASODN) aiming at c-myc could significantly decrease the expression of c-myc both at mRNA and protein levels, inhibit proliferation of cancer, induce apoptosis and increase the sensitivity of cancer cells to chemotherapy (Hayashi et al., 1994, Jin et al., 2002). ASODN in combination with chemotherapy is associated with a good efficacy (Monia et al., 1997), as compared with either of the two drugs alone. This efficacy has been proven good in melanoma (Pastorino et al., 2008). However, there are no studies on liver cancer so far.

In the present study, liver cancer cells HepG2 was treated with liposome-mediated phosphorothioate c-myc ASODN in combination with 5-fluorouracil (5-FU) to investigate effects of this combination regimen on proliferation of live cancer cells and expression of c-myc gene.

Materials and Methods

Cell Lines and Culture Conditions

Liver cancer cell lines HepG2 were provided by the Scientific Research Center, Gansu University of Traditional Chinese Medicine. They were cultured with the RPMI 1640 medium containing 10% fetal bovine serum according to the supplier's instructions. The cell lines were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

ASODN and SODN

C-myc ASODN and SODN were synthesized according to the initiation and subsequent 4 codons of the second exon (sense: 5'-ATG CCC CTC AAC GTT-3', antisense: 5'-AAC GTT GAG GGG CAT-3'). Products were treated with phosphorothioate modification to increase the stability. Products were synthesized, purified and packaged by the Sangon Biotech (Shanghai) Co., Ltd and stored at -20°C.

Lipofection reagent and liposome/c-myc ASODN compound

Cells in the logarithmic growth phase were harvested. When cells in the culture flask reached the packing density of 30% to 50%, lipofection was conducted on ASODN and SODN (1 μmol/L) using LipofectamineTM2000 (Invitrogen Co., Ltd, USA) according to the instruction.

Methods

Cells were randomized into six groups: 5-FU (10umol/L), C-myc ASODN (1 μmol/L), SODN (1 μmol/L), 5-FU (10 μmol/L)+C-myc ASODN (1 μmol/L), 5-FU (10 μmol/L)+C-myc SODN (1 μmol/L) and blank control.

Proliferation inhibition rate of HEPG-2 by MTT

Cell proliferation inhibition rate were measured by MTT. Around 1×10^4 cells were inoculated in a 96-well plate for 24 hours. Then, different drugs were added according to groups. In following 24, 48, 72 and 96 hours, the MTT solution and dissolving solution DMSO were added on an interval of 4 hours. After crystals fully dissolved, the absorption A of the product in every well was measured with the automatic enzyme-labeling instrument at the wavelength of 490 mm.

Invasion assay

The invasive capability of human liver cancer cell HEPG-2 was determined by Matrigel-coated invasion chambers (Becton Dickinson, Bedford, MA). This system is separated by a PET membrane coated with Matrigel Matrix such that only invasive cells can migrate through the membrane to the reverse side. After rehydration for 2 hours in a humidified incubator at 37°C with 5% CO_2. Cells were seeded at a density of 1×10^5 cells/well into the inner chambers of a cell culture insert and incubated at 37°C with 5% CO_2 for 24 hours with various concentration of 5-FU (10umol/L), C-myc ASODN (1 μmol/L), SODN (1 μmol/L), 5-FU (10 μmol/L)+C-myc ASODN (1 μmol/L), 5-FU (10 μmol/L)+C-myc SODN (1 μmol/L). After 24 hours incubation, no-filtering cells were removed from the upper surface of membrane by scrubbing gently with cotton-tipped applicators, the cells that invaded to the reverse side of the membrane were fixed with 70% ethanol, following, stained with Giemsa solution, and were counted in five random fields of the low filter surface under a microscope at 200× magnification.

Cell apoptosis and cycle by flow cytometry

The cell density of suspensions was estimated with the cell-counting plate. Then, the cell density was adjusted to 1×10^7/mL and treated with Coulter DNA. PrepTM Reagents Kit (Flow Cytometer Coulter Epics XL Co., Ltd, USA) according to the instruction. Cell cycle and the proportion of sub-diploids were determined by flow cytometry.

C-myc mRNA by RT-PCR

The total RNA was extracted with the Trizol RNA kit. The c-myc mRNA expression was assayed using the M-MLV Rtase cDNA synthesis Kit (code D6130). Primers of c-myc and gapdh were synthesized by the Sangon

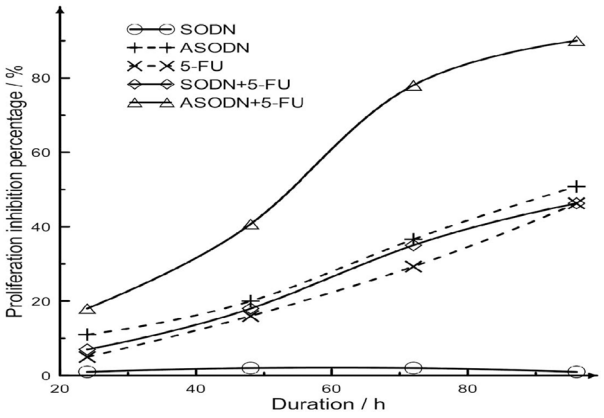

Figure 1. Effects of ASODN and 5-Fu on HEPG-2 Proliferation Inhibition. The proliferation inhibition was significantly higher in ASODN, 5-Fu and ASODN+5-FU groups than the control group (p=0.0012, 0.0041 and 0.0026, respectively) and in the ASODN+5-FU groups than the single ASODN and 5-FU groups (p=0.0072 and 0.009, respectively)

Biotech (Shanghai) Co., Ltd: for c-myc, upstream: 5'GAT TCT CTG CTC TCC TCG AC3' and downstream: 5'TCC AGA CTC TGA CCT TTT GC3' with the amplification length of 250 bp and for gapdh, upstream 5'CTG ACC TGC CGT CTA GAA A3' and downstream: 5'GTG GTG TGA CTT AGA GGG G3' with the amplification length of 380 bp. Semi-quantitative analysis was conducted with the Tanon gel analysis system. The relative level of c-myc mRNA was the ratio of the density index of electrophoretic bands of the product to internal standard gapdh in the same reaction system. Products were electrophoresed with 20 g/L agarose gel. Results were imaged.

C-myc protein by the i mmunohistochemical ABC method

Rat anti-human c-myc antibodies and the ABC i mmunohistochemical kit were purchased from the Fuzhou Maxixin Biotech Co., Ltd. Cells were colored with the DAB kit in accordance with the instruction. The proportion of c-myc-positive cells was calculated.

Statistical analysis

SPSS11.0 was used for statistical analysis. Data were expressed as the mean±standard deviation (SD). Groups were compared with the one-way analysis of variance. $p<0.05$ was considered statistically different.

Results

Effects of ASODN and 5-Fu on HEPG-2 proliferation inhibition:

To investigate the different inhibiting proliferation ability of ASODN and 5-Fu, we treated 5-FU (10umol/L), C-myc ASODN (1 μmol/L), SODN (1 μmol/L), 5-FU (10 μmol/L)+C-myc ASODN (1 μmol/L), and 5-FU (10 μmol/L)+C-myc SODN (1 μmol/L) containing the culturing media. We observed the proliferation inhibition rate was significantly higher in ASODN, 5-Fu and ASODN+5-FU groups than that in the control group (p=0.0012, 0.0041 and 0.0026, respectively) and also in the ASODN+5-FU groups than that in the single ASODN and 5-FU groups (p=0.0072 and 0.009, respectively) (Figure 1).

Involvement of ASODN and 5-Fu with invasive capabilities of liver cancer cell

After pretreated/untreated with 5-FU, C-myc ASODN, SODN, 5-FU+C-myc ASODN, and 5-FU+C-myc SODN liver cancer cells were cultured for 24 hours, the invasive capability was detected. 5-FU+C-myc ASODN, and 5-FU+C-myc SODN inhibited the invasion of liver cancer cells (p<0.01) (Figure 3).

Effects of ASODN (1 μmol/L) and 5-Fu (10 μmol/L) on HEPG-2 apoptosis

The apoptotic rate was significantly higher in ASODN, 5-Fu and ASODN+5-FU groups than that in the control group (p=0.0015, 0.0039 and 0.002, respectively) and alos in the ASODN+5-FU groups than that in the single ASODN and 5-FU groups (p=0.0061 and 0.008, respectively). Cells in the G0/G1 stage significantly increased in the ASODN group than that in the control group (p<0.01) while cells in the S stage significantly increased in the 5-FU group than that in the control group (p=0.0032) (Table 1).

Effects of ASODN (1 μmol/L) and 5-Fu (10 μmol/L) on expression of C-myc mRNA

As displayed by RT-PCR, the amplification strip of c-myc was strongly weaker in ASODN, 5-FU and ASODN+5-FU groups than that in the control group. The

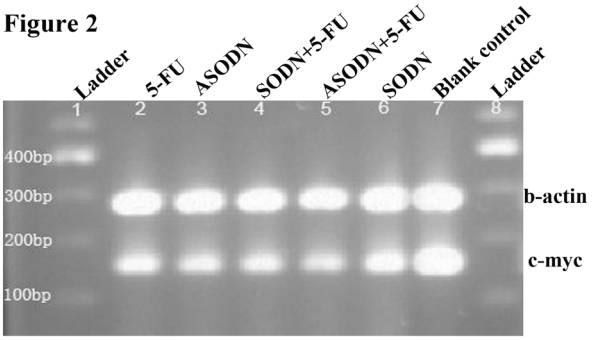

Figure 2. Effects of 5-FU and C-myc ASODN on C-myc mRNA Expression in HEPG-2. 1 and 8: DNA marker; 2: 5-FU; 3: ASODN; 4: SODN+5-FU; 5: ASODN+5-FU; 5:ASODN+5-FU; 6: SODN; 7: the blank control

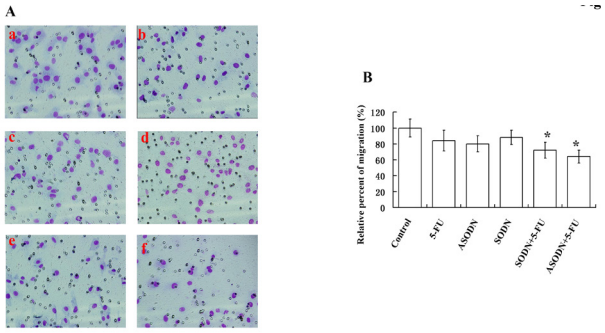

Figure 3. Effect of ASODN and 5-Fu on Invasive Capabilities Of Liver Cancer Cell. 5-FU+C-myc ASODN, and 5-FU+C-myc SODN inhibited the invasion of liver cancer cells (*p<0.01) a: Control; b 5-FU; c: ASODN; d: SODN; e:SODN+5-FU; f: ASODN+5-FU

Table 1. Cell Cycle, Apoptotic Rate and Semi-Quantitative Results of C-Myc mRNA in All Groups

Groups	n	G0/G1	S	G2/M	Apoptotic rate	Semi-quantitative RT-PCR
Blank control	5	61.8±4.19	26.1±3.78	10.1±3.91	0.8±0.27	1.026±0.14
5-FU	5	57.1±3.51	39.7±3.15[a]	5.2±4.22	12.5±1.05[a]	0.876±0.06[b]
ASODN	5	78.1±4.51[a]	21.9±3.46	6.0±4.66[b]	10.6±1.14[a]	0.874±0.04[b]
SODN	5	60.1±4.22	26.3±2.95	13.6±6.11	1.2±0.64	0.985±0.13
SODN+5-FU	5	51.6±6.71	38.6±7.90[a]	6.8±4.95	13.1±1.37[a]	0.876±0.04
ASODN+5-FU	5	79.8±3.69[a]	26.1±6.71	4.1±0.37[b]	27.4±1.48[acd]	0.776±0.05[a]

*[a]p<0.01, vs. the control group; [b]p<0.05, vs. the control group; [c]p<0.01, vs. the 5-FU group; [d]p<0.01, vs. the ASODN group

Table 2. Changes of C-Myc Proteins in HEPG-2

Groups	n	Percentage of c-myc-positive cells
Blank control	5	83.4±7.15
5-FU	5	69.1±7.44[a]
ASODN	5	62.8±5.18[a]
SODN	5	81.7±7.66
SODN+5-FU	5	69.1±4.76[a]
ASODN+5-FU	5	65.1±3.44[a]

*[a]p<0.01, vs. the blank control group

image analysis system indicated a statistical difference in the strip of the blank control group than that in ASODN+5-FU, ASODN and 5-FU groups (p=0.004, 0.02 and 0.03, respectively). The combination group showed a slightly stronger inhibition effect than the single groups but there was no statistical difference (Figure 2 and Table 2).

Effects of ASODN (1 μmol/L) and 5-Fu (10 μmol/L) on C-myc proteins

I mmunohistochemical results showed a significant decline of c-myc proteins in ASODN, 5-FU and ASODN+5-FU groups than that in the blank control group (p=0.006, 0.003 and 0.002) (Table 2).

Discussion

Recent years, more and more evidence suggested that ASODN and decoy ODNs may allow development of therapeutic and investigative tools for human malignancies. Inhibition of NF-kB by decoy ODNs has been demonstrated may offer promise as a therapeutic approach for the treatment of androgen-independent prostate cancer (Fang et al., 2011). In this study, phosphorothioate modification was adopted for ASODN to increase the stability and liposome encapsulation to increase the transfection rate and concentration in cells. After 72-hour transfection, cells well attached walls in SODN and control groups while cell colonies decreased and particles in cells increased in a concentration-dependent manner in the ASODN group. The MTT method showed that cell proliferation in the ASODN group was significantly inhibited than that in the control group. Flow cytometry indicated a significant decrease of S-stage cells and increase of G0/G1-stage cells as well as a significantly higher apoptotic rate in the ASODN group as compared with the control group. It illustrated that ASODN has an apparent inhibition effect on liver cancer cell proliferation.

The cell proliferation rate is related to the G1 phase. Inhibition of c-myc expressions can block cells from the G1 phase into the S phase, which leads to G0/G1 blockage and inhibits rapid proliferation of tumor cells thenlead to cell apoptosis (Hemann et al., 2005). Besides, inhibition of c-myc expressions may induce apoptosis via the following mechanisms: (1) it triggers the normal differentiation of cells (Jain et al., 2002). (2) C-myc blocks the potential apoptosis induced by other unknown genes (Manner et al., 2010) and after c-myc is inhibited, these genes can be activated to induce apoptosis. (3) After c-myc expressions are inhibited, cells re-recognize injured DNA, induce apoptosis of tumor cells with aberrant chromosomes (Karlsson et al., 2003, Yochum et al., 2010).

In this study, RT-PCR was used to detect changes at mRNA level of the target genes after ASODN transfection. The gray scale of the amplification strip was significantly weaker in the ASODN group than that in the SODN and blank control group. C-myc proteins were assayed with the i mmunohistochemical method and results showed a significant decrease in the ASODN than that in the control group. Results aforementioned illustrate that ASODN can inhibit c-myc expression at transcription and translation levels. Additionally, liposome is similar to the blank control in inhibiting liver cancer cell proliferation and gene expressions and MTT results also confirmed that it is no inhibition of liposome in liver cancer cells, suggesting the liposome is a safe and effective carrier of ASODN.

Existing studies demonstrated that 10 μmol/L of 5-FU could induce apoptosis of liver cancer cells. Flow cytometry showed that 5-FU could arrest liver cancer cells in the S phase. Results from assays on c-myc mRNA and proteins indicated that 5-FU also could inhibit c-myc expression, possibly contributing to inhibition of 5-FU in liver cancer cells. This illustrates that c-myc can be used as a target of multiple antitumor drugs (Wang et al., 2012, Doe at al., 2012).

In this study, MTT results displayed that the combination of 10 μmol/L of 5-FU and 1 μmol/L of c-myc ASODN could produce effects equal to 40 μmol/L of 5-FU. The proliferation inhibition rate at 48 hours after transfection was 16% in the single 5-FU group and reached 34% in the combination group. Flow cytometry also suggested a synergistic effect on apoptosis of liver cancer cells. The application of 5-FU is restrained in clinical practice for the sake of side effects (Wu et al., 2004, Yang et al., 2005). C-myc ASODN can increase the sensitivity of liver cancer cells to 5-FU, reducing the dosage of 5-FU and toxic side effects and increasing effects. Additionally, ASODN is easily synthesized with very slight side effects and can specially block the corresponding target genes (Jansen et al., 2002). Therefore, the combination of ASODN and 5-FU is of great significance in guiding the chemotherapy strategy of liver cancer and a promising alternative to current regimens.

In this study, both c-myc ASODN and 5-FU can down-regulate c-myc expressions but without a detectable synergistic effect, suggesting that other genes may be involved in inhibition of c-myc expression. For this, more studies are needed in future.

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