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Full Length Research Paper

Effects of RNAi-mediated cathepsin L gene silencing on bionomics of hepatoma carcinoma cells

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This study aimed to explore the effects of RNAi-mediated cathepsin L gene silencing on bionomics of hepatoma carcinoma cells. In this study, cathepsin L siRNA silencing group (silencing group), blank hepatoma carcinoma cells group (blank group) and the siRNA fluorescin group (fluorescence control group) were set. The observing time include 1, 3 and 6 days after RNA interference for cathepsin L. The transfection efficiency of each group was observed. The expression of cathepsin L in hepatoma carcinoma cells was detected by immunofluorescence, reverse transcription polymerase chain reaction (RT-PCR) and western blot. Cell viability was detected by methyl thiazolyl tetrazolium (MTT) assay. The changes of cell cycle and apoptosis were observed by flow cytometry. The changes of invasiveness of hepatoma carcinoma cells were detected by Boyden chamber. Compared with the blank group and fluorescence control group, mRNA and protein level of cathepsin L decreased significantly, and cell growth was inhibited. Meanwhile, the proliferation index decreased significantly, while the apoptosis index increased significantly in the experimental group. The invasiveness of hepatoma carcinoma cells was also found to decrease. The study indicated that RNA interference could inhibit cathepsin L expression, and decrease cell proliferation and cell invasiveness of hepatoma carcinoma cells efficiently.

Key words: RNA interference, liver cancer; Cathepsin L.

INTRODUCTION

Cathepsin L is one of the lysosomal cysteine protease family, and an eosinophilic lysosomal protease. Cathepsin L is widely distributed in normal cells and cancer cells. Recently, numerous researchers investigate the relationship between cathepsin L and cancer. Results showed that cathepsin L is highly expressed in prostate cancer, melanoma, gastric cancer, and pancreatic cancer. The main function of cathepsin L is to break apart other proteins, cleave precursor proteins (proenzyme and prohormone) resulting in suppression of protein activetion, while activating other proteinases involve in a variety of physiological processes such as antigen processing, activation of hormone precursors, oogenesis and spermatogenesis, degradation of bone matrix, keratinocyte differentiation, growth cycle of the hair follicle and maturation and development of the central nervous system (Colella et al., 2004). In addition, over-expression of cathepsin L has been found to be related to some pathological processes including glomerulonephritis, osteoporosis, rheumatoid arthritis and invasion and metastasis of cancers (Stabuc et al., 2006; Caserman et al., 2006).

Nowedays, surgical resection is still the best treatment for primary hepatic carcinoma. However, the 5-year recurrence rate is still high after radical resection of liver cancers, so it is very necessary to understand the molecular mechanism of the invasion and metastasis of liver

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Abbreviations: RT-PCR, Reverse transcription polymerase chain reaction; **MTT,** methyl thiazolyl tetrazolium; **RNAi,** RNA interference; **DMEM,** Dulbecco's modified Eagle's medium; **GFP,** green fluorescent protein; **DMSO,** dimethyl sulfoxide; **PBS,** phosphate buffer; **PI,** propidium Iodide; **FCS,** fecal calf serum; **PCI,** protein C inhibitor; **FCM,** flow cytometric.

Table 1. PCR primer sequences.

Amplified genes	Primer Sequences	Length	Tm
Cathepsin L	Sense : 5'- CCG TGA GCC TCT GTT TCT-3′ Antisense: 5'- TTT GAC GAG CCA ATA CTT-3'	583bp	52°C
GAPDH	Sense: 5'-AAT CCC ATC ACC ATC TTC C-3' Antisense: 5'- CAT CAC GCC ACA GTT TCC-3'	382bp	54.9°C

cancer (Lu et al., 2004). It has been confirmed that cathepsin L gene expressed highly in tumor cells, but further questions need to be answered for example what role does cathepsin L gene play in hepatoma carcinoma cells and which effects for the proliferation and growth of hepatoma carcinoma cells may be produced if the expression of cathepsin L gene is blocked (Hashimoto et al., 2006). RNA interference (RNAi) technology has been successfully applied in various areas of biological research, and its main principle is that through the siRNA-mediated recognition of homologous target mRNA molecule, the corresponding target gene mRNA is cut under RNA-induced silencing complex (RISC) to play the gene silencing effects (Krueger et al., 2007). Through inhibiting the gene expression of cathepsin L in cultured hepatoma carcinoma cells using RNAi technology, the effects of Cathepsin L for hepatoma carcinoma cells *in vitro* were observed in our study.

MATERIALS AND METHODS

Main reagents and instruments

Main reagents and instruments used in our study included: Cathepsin L siRNA, cathepsin L rabbit anti-human antibody, TRITC labeled goat anti-rabbit immunoglobulin (IgG) (Santa Cruz, USA), Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA), methyl thiazolyl tetrazolium (MTT, Amresco Company), CO_2 incubator (Heraeus, Germany) and flow cytometer (BD Company, USA). Primers for Cathepsin L were synthesized by TaKaRa Company, and they are shown in Table 1

Experimental grouping and gene silencing

Experimental object

Human hepatoma carcinoma cells (HepG2 cell line was stored in our laboratory).

Grouping

The silencing cathepsin L siRNA transfection group (silencing group) was the experiment group. The blank hepatoma carcinoma cells group (blank group) and the siRNA transfection fluorescin group (fluorescence control group) were control groups.

Methods of Cathepsin L gene silencing by siRNA

The cell suspension was adjusted to a density of 2×10^5 cells/mm^3, of which 2 ml for each well was added into a 6 - well culture plate, and then it was placed and cultured in CO_2 incubator at 37°C. The cell growth was observed and then siRNA transfection was carried out when the cell confluence reached 60-80%.

Preparing reagent A

In each transfection, 2 - 8 µl of double-strands siRNA (about 0.25 - 1 µg or 20 - 80 pmols siRNA) was mixed with 100 µl of siRNA transfection dilution sc-36868. In each transfection, 2 - 8 µl siRNA transfection reagent sc-29528 was mixed with 100 µl siRNA transfection dilution sc-36868. Reagent A was added into reagent B by a suctionpipe. It was shaked up to misce bene and then reacted for 15 - 45 min at room temperature. It was washed by 2 ml of siRNA transfection dilution sc-36868, and then the exsuction liquid was used directly in the next step. 0.8 ml siRNA transfection solution was added into the mixed transfection solution in each transfection. After mixing lightly, the liquor was added onto the cell surface. It was incubated in CO_2 incubator at 37°C for 5 - 7 h. 1 ml of common culture fluid was added into the transfection solution. The observation of significant cytotoxicity led to the removal of the transfection solution and the addition of common medium, con-tinuing cell culture for 18-24 h. Then the common culture fluid was renoveatured. Corresponding cell detection was carried out after 48 h of cell culture. In the fluorescence control group, green fluore-scent protein (GFP) siRNA transfection was performed. Green fluorescently-labeled siRNA gene silencing was observed under a fluorescence microscope (Green fluorescence was positive signal). Five fields (× 200) were selected randomly, and the efficiency of gene silencing was calculated.

mRNA level of cathepsin L gene

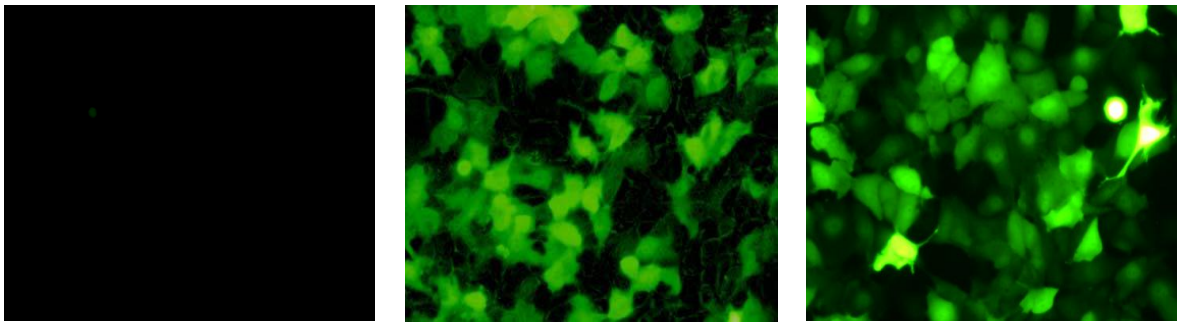
Total RNA was extracted by TRIzol. OD260 and OD280 were measured, and the purity of RNA was assessed ($OD_{260/280}$ ratio was 1.6 - 2.0), and the concentration of total RNA was calculated with an ultraviolet spectrophotometer. RT-PCR was carried out by rou-tine method. The products were separated by 2% agar gel electrophoresis and then analyzed by gel imaging system. The results are represented by OD ratio (target fragment /GAPDH).

Cathepsin L expression detected by immunofluorescence

Cathepsin L in hepatoma carcinoma cells was detected by routine immunofluorescence method. It was incubated by cathepsin L antibody (1:200) at 4°C and stayed overnight. After incubating with the second antibody working fluid (1:200 TRITC labeled goat anti-rabbit IgG working fluid) for 30 min, the results were analyzed.

Cathepsin L expression detected by western blot

Cells of each phase were obtained form each group. Total proteins were extracted and then the protein level was detected. Routine



Blank group, 3d. Fluorescence control group, 3d. Silencing group, 3d.

Figure 1. Results of green fluorescent labelled protein after cell transfection × 200.

western blot was used for detection. The primary antibody was diluted by 1:1000 and the second antibody was by 1:2000 in normal saline. Chemoluminescence was used for colouration and then cathepsin L/GAPDH ratio was calculated.

Cell viability detected by MTT method

Hepatoma carcinoma cells in logarithmic growth phase were obtained and the cell density was adjusted at 1×10^5/ml. It was inoculated in a 96-well culture plate, with 100 µl in each well. Transfection was performed after cell adhesion. After refreshing the medium, the cells continued to culture for 1, 3 and 6 d. Then, the supernatant fluid was removed, and 20 µl of MTT (10 mg/ml) added into each well. The medium was removed 4 h later, and 150 µl of dimethyl sulfoxide (DMSO) was added into each well and swung for 10 min. The absorbance value was measured at 490 nm by enzyme mark instrument. The actual absorbance value was obtained by the absorption value of each well after subtracting blank absorbance value.

Cell cycle and apoptosis

Hepatoma carcinoma cells were obtained by digestion, and then centrifuged for 5 min in a 10 ml centrifuge tube (4°C, 1000 rpm). These were washed by 0.1 mol/L phosphate buffer (PBS), and then as added into 70% alcohol for fixation. Staining was done in the dark at 4°C using propidium Iodide (PI) for 30 min. The cell proliferations and apoptosis index were tested by flow cytometer. Average value was calculated after repeated test for 5 times. The formulas are as follow:

Proliferation index (%) = (S + G2/M) / (G0/G1 + S + G2/M) × 100%

Apoptotic index (%) = subdiploid / (subdiploid+diploid+multiploid) × 100%

Cell invasiveness

Cells in logarithmic growth phase were obtained and adjusted as 2×10^5 /ml single-cell suspension, which was added into the upper invasion chamber, and DMEM and 20% fecal calf serum (FCS) were added into the underlayer. Corresponding gene silencing experiments were carried out respectively. The invasion chamber was taken at observation time, and then scoured the artificial matrigel in upper surface by cotton swab.

It was washed by PBS for 5 min × 2 times, then formalin fixed and hematoxylin stained mildly. It was soaked and washed by tap water for 15 min, and then anhydrous alcohol dehydrated. The invasion chamber membrane was obtained by girdling, and then immersed faceup in dimethyl benzene for 2 min. Some neutral resin was dropped on the slide, the invasion chamber membrane placed faceup in the resin, and then, some neutral resin was dropped again and mounted with a cover slip. Five fields including upper, lower, left, right, center were observed under light microscope (× 200). The cell number at the lower surface of the filter membrane was recorded, and then the average calculated, which represented the cell invasiveness.

Statistical analysis

Date was represented as means ± standard deviation ($\bar{x} \pm s$). Statistical package for the social sciences (SPSS) 12.0 software was used for student's t test, with $P < 0.05$ as statistically significant.

RESULTS

Gene silencing efficiency of cathepsin L siRNA

In the silencing group as well as the fluorescence control group, through detecting green fluorescent labelled protein, we found that gene transfection rate of hepatoma carcinoma cells increased gradually, which was more than 30% the first day, and more than 90% 3 days later (Figure 1).

Effects on cathepsin L gene expression in hepatoma carcinoma cells

The cathepsin L mRNA expression was reduced significantly, which indicated that cathepsin L siRNA gene silencing might inhibit cathepsin L mRNA expression

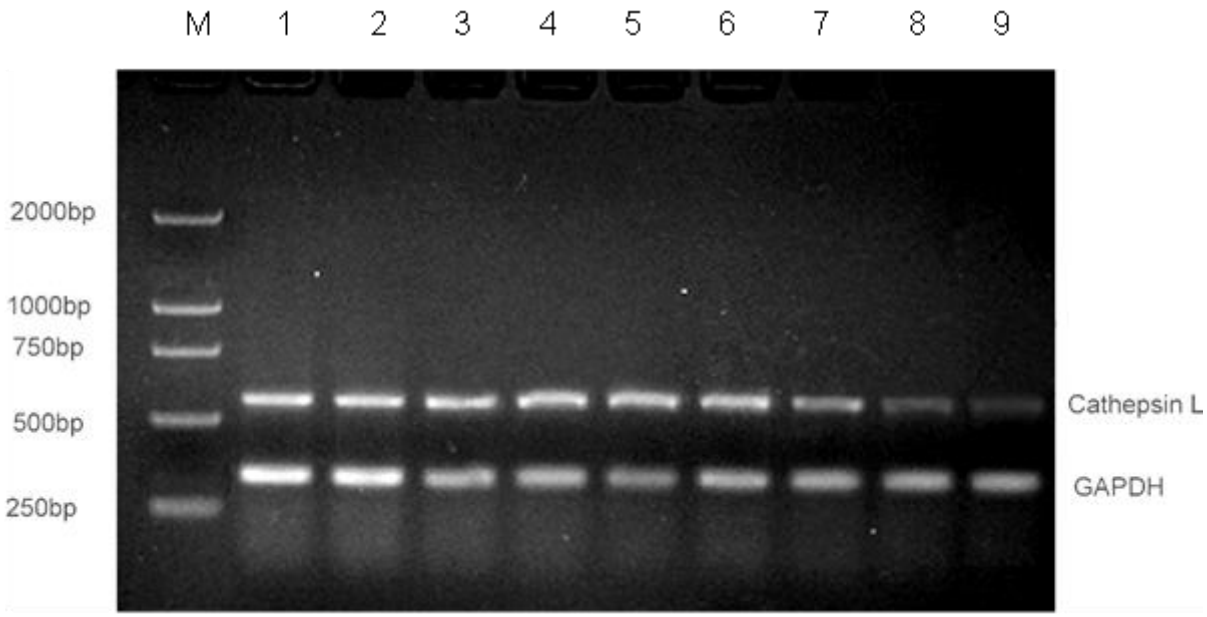


Figure 2. Results of Cathepsin L RT-PCR in hepatoma carcinoma cells. M: Marker 1 + 2 + 3, blank group 1, 3, 6 days, 4 + 5 + 6; fluorescence control group 1, 3, 6 days, 7 + 8 + 9; silencing group, 1, 3, 6 days.

Table 2. Results of RT-PCR in cathepsin L siRNA transfected hepatoma carcinoma cells (OD ratio, Cathepsin L/GAPDH, n=5).

Group	1d	3d	6d
Blank	0.515±0.045	0.519±0.042	0.529±0.031
Fluorescence	0.527±0.028	0.500±0.035	0.511±0.050
Silencing	0.259±0.037▲▲**	0.141±0.013▲▲**	0.090±0.009▲▲**

Compared with the blank group, *P < 0.05, ** P<0.01; compared with fluorescence control, ▲P < 0.05; ▲▲P < 0.01.

markedly (Figure 2 and Table 2).

Immunofluorescent result of cathepsin L in siRNA tranfected hepatoma carcinoma cells

Cathepsin L expressed in cytoplasm of hepatoma carcinoma cells showed no signficant difference at each time point in the blank group. However, its expression was reduced with time in the silencing group, and there was no significant change in the fluorescence control group (Figure 3 and Table 3).

Western blot result of cathepsin L in siRNA transfected hepatoma carcinoma cells

Cathepsin L expression was decreased with time in the silencing group, and there was no significant change in the fluorescence control group. Through statistical analysis, we found that there were significant differences among groups (Figure 4 and Table 4).

Cell viability

There was no significant change based on the absorbance value of cathepsin L siRNA detected by MTT with time in the silencing group. However, it was increased in the blank group and fluorescence control group. Through statistical analysis, we found that there was significant difference between groups (Table 5).

Result of cell cycle and proliferation index

In the silencing group, the proportion of G_1 phase cells increased, however, the proportion of S phase cells reduced significantly, indicating the cell proliferation index reduced significantly. There was no significant change in the fluorescence control group and the blank group (Figure 5 and Table 6 and 7).

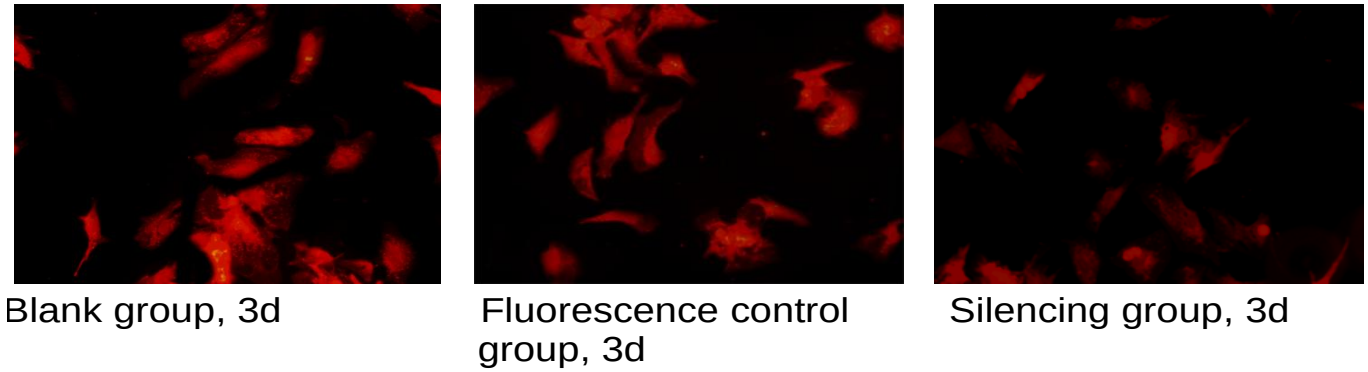


Figure 3. Immunofluorescent result of cathepsin L expression × 200.

Table 3. IOD value of cathepsin L expression by immunofluorescent staining in hepatoma carcinoma cells ($\times 10^3$, n=5).

Group	1d	3d	6d
Blank	22.25±1.82	22.77±2.01	22.22±2.92
Fluorescence	20.88±2.24	22.12±2.64	21.84±1.78
Silencing	10.95±1.03**▲▲	7.54±0.56**▲▲	4.06±0.54**▲▲

Compared with blank group,*P < 0.05, ** P < 0.01; compared with fluorescence control group,▲,P < 0.05; ▲▲,P < 0.01.

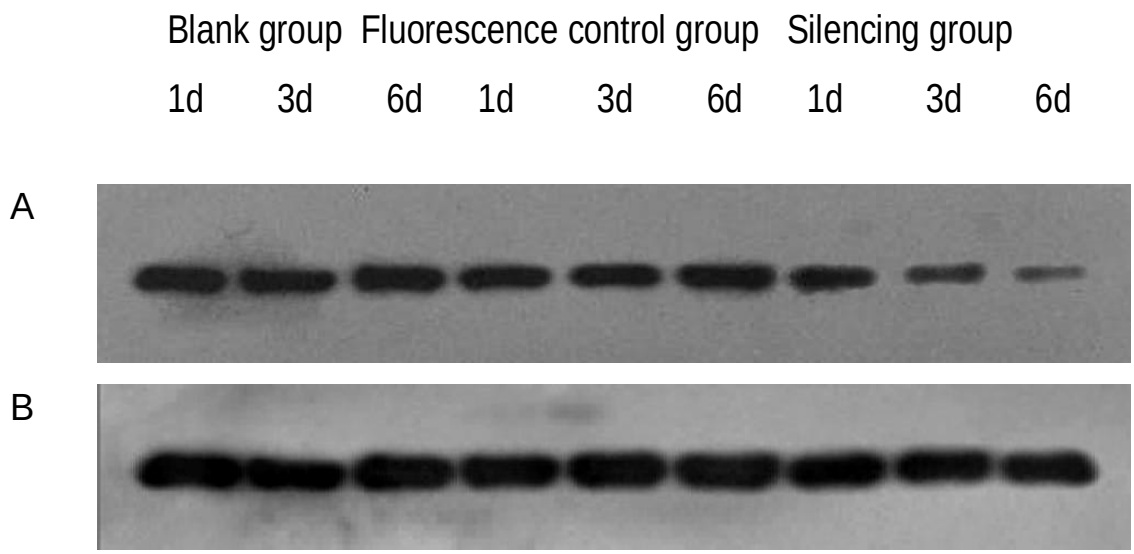


Figure 4. Western blot result of cathepsin L expression. A: Cathepsin L; B: GAPDH.

Table 4. Western blot result of cathepsin L in hepatoma carcinoma cells (Cathepsin L /GAPDH, n=5).

Group	1d	3d	6d
Blank	0.758±0.066	0.767±0.072	0.770±0.061
Fluorescence	0.762±0.090	0.778±0.060	0.778±0.084
Silencing	0.428±0.048**▲▲	0.225±0.032**▲▲	0.110±0.013**▲▲

Compared with blank group,*P < 0.05, ** P < 0.01; compared with fluorescence control group, ▲P < 0.05, ▲▲P < 0.01.

Table 5. Cell viability detection by MTT method in hepatoma carcinoma cells (absorbance,n=5).

Group	1d	3d	6d
Blank	0.440±0.033	0.542±0.050	0.787±0.071
Fluorescence	0.434±0.042	0.564±0.040	0.792±0.071
Silencing	0.298±0.021**▲▲	0.335±0.031**▲▲	0.399±0.030**▲▲

Compared with blank group,*P < 0.05, ** P < 0.01; compared with fluorescence control group, ▲P < 0.05, ▲▲P < 0.01.

Result of apoptotic index detected by flow cytometer

The apoptotic index of hepatoma carcinoma cells was increased significantly in the silencing group while there was no significant change in the fluorescence control and blank group (Table 8).

Detection result of cell invasiveness

There was no significant change in the fluorescence control and blank group. However, cell invasiveness in cathepsin L siRNA transfected hepatoma carcinoma cells was significantly lower than that in the fluorescence control and blank group (Figure 6 and Table 9).

DISCUSSION

When double-stranded RNA which was homologous with the endogenous mRNA coding region was imported into cells, sequence-specific RNA degradation process could be activated to result in gene silencing (Holmes and Cohen, 2007; Matsui et al., 2007). The mechanism of RNA interference included initial step and effecting step. The initiate step was that, by a specific RNase Dcer, double-stranded RNA molecules were combined to the nucleinase complex to form RNA-induced silencing complex (RISC). RISC was activated by uncoiled small interfering RNAs double-stranded molecules, and mRNA was dissected from the termination of siRNA through base complementary and binding target sequence (Colmenares et al., 2007). Small interfering RNAs (siRNAs) are becoming an important research tool in oriented areas of gene silencing (Akhtar and Benter, 2007).

A small library of 36 functionalized benzophenone thiosemicarbazone analogs has been prepared by chemical synthesis and evaluated for their ability to inhibit the cysteine proteases cathepsin L and cathepsin B. Inhibitors of cathepsins L and B have the potential to limit or arrest cancer metastasis (Kishore Kumar et al., 2010). Consequently, inhibiting cathepsin L by serpins like protein C inhibitor (PCI) may be a new pathway of regulating hemostasis, cardiovascular and metastatic diseases (Fortenberry et al., 2010). In our study, hepatoma carcinoma cells were transfected by cathepsin L siRNA successfully, and high efficient gene silencing was obtained. Compared to the green fluorescent protein and blank carrier, we found that the siRNA gene silencing rate in hepatoma carcinoma cells at the third day could be higher than 90%. We detected the cell proliferation status by MTT and flow cytometric (FCM), and the results showed that the cell viability of siRNA transfected heaptoma carcinoma cells reduced significantly. There was no significant change on the absorbance value of cathepsin L siRNA detected by MTT with time in the silencing group, however, it was increased in the Blank group and fluorescence control group, which indicated that the hepatoma carcinoma cells were not affected and cell proliferation was normal in the blank and Fluorescence control group. In the silencing group, the cell proliferation was inhibited, however, the cell viability did not change significantly. The FCM results shows that, the cell proliferation index in cathepsin L siRNA transfected hepatoma carcinoma cells was decreased significantly. However, there was no significant change in the fluorescence control and blank group, which indicated that the cell cycle of hepatoma carcinoma cells was inhibited while cells in quiescent stage, increased. That is, the proportion of G_1 phase cells increased, however, the proportion of S phase cells reduced significantly in the silencing group. The apoptosis test results described the effect of cathepsin L siRNA transfection in hepatoma carcinoma cells from another aspect. The apoptotic index of hepatoma carcinoma cells was increased significantly in the silencing group, while there was no significant change in the fluorescence control and blank group. It was obseverd that there was significant difference between groups, which indicated that the increase of apoptosis was one of the ways of death for inhibited hepatoma carcinoma cells.

Cathepsin L which is an eosinophilic lysosomal proteinase is a member of lysosomal cysteine protease family. Cathepsin L is extensively distributed in virous kinds of human tissue and tumor cells, and it is highly expressed in many malignant tumors such as prostate cancer, melanoma, stomach cancer, pancreatic cancer, etc. Stabuc et al. (2006) investigated the differences in cathepsin L concentrations in primary cutaneous malignant melanoma stage I and normal skin and correlated these values with well-established malignant melanoma prognostic factors, and they found that higher cathepsin L concentrations in early primary malignant melanomas

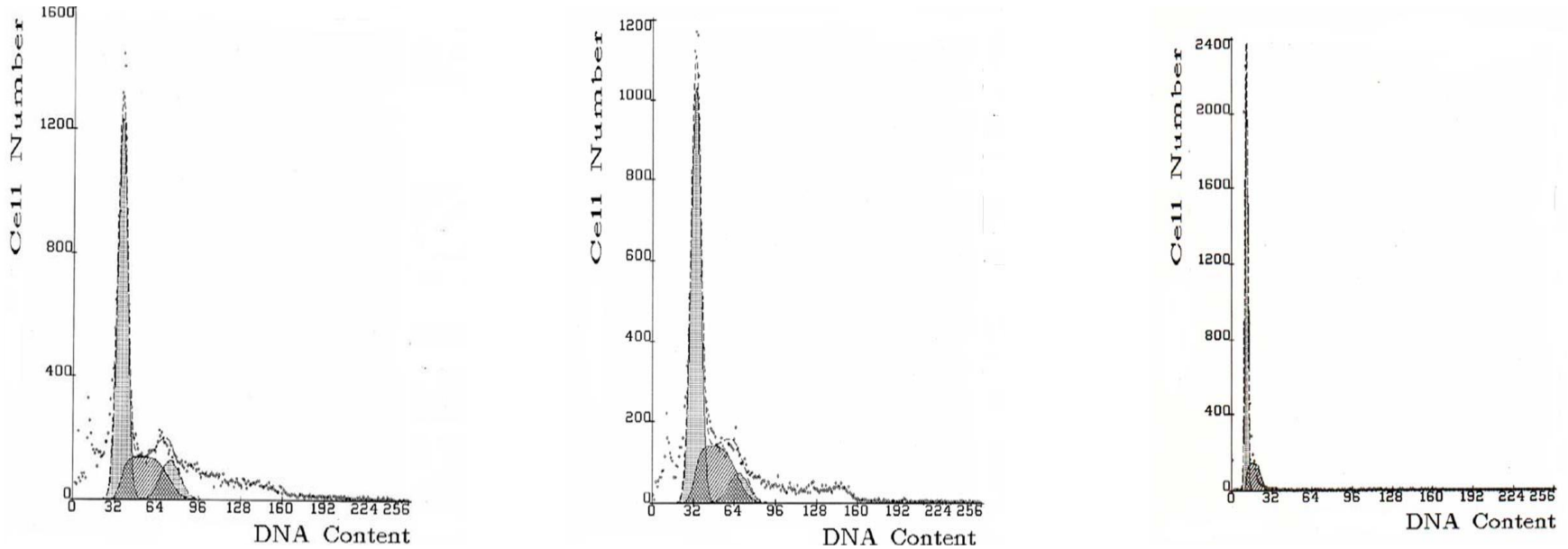


Figure 5. DNA detection result by flow cytometer.

Table 6. Detection result of cell cycle (%, n=5).

Time	Group	G_1 phase	S phase	G_2/M phase
1d	Blank	56.3±3.9	38.0±3.5	5.7±1.2
	Fluorescence	54.9±3.5	40.2±2.7	5.0±1.2
	Silencing	80.4±3.0*▲	16.9±2.7**▲▲	2.7±0.7**▲▲
3d	Blank	58.0±2.7	36.9±3.4	5.1±1.2
	Fluorescence	56.1±3.5	38.7±3.2	5.2±1.4
	Silencing	82.9±2.6*▲	14.6±2.1**▲▲	2.5±1.0**▲▲
6d	Blank	59.5±3.0	35.4±3.0	5.1±1.2
	Fluorescence	56.8±4.1	38.3±4.2	4.9±1.1
	Silencing	84.0±1.9*▲	14.6±2.1**▲▲	1.4±0.7**▲▲

Compared with blank group, *P.<.0.05; ** P.<.0.01; compared with the fluorescence control group,▲,P.<.0.05; ▲▲,P.<.0.01.

Table 7. Proliferation index result in hepatoma carcinoma cells (%, n=5).

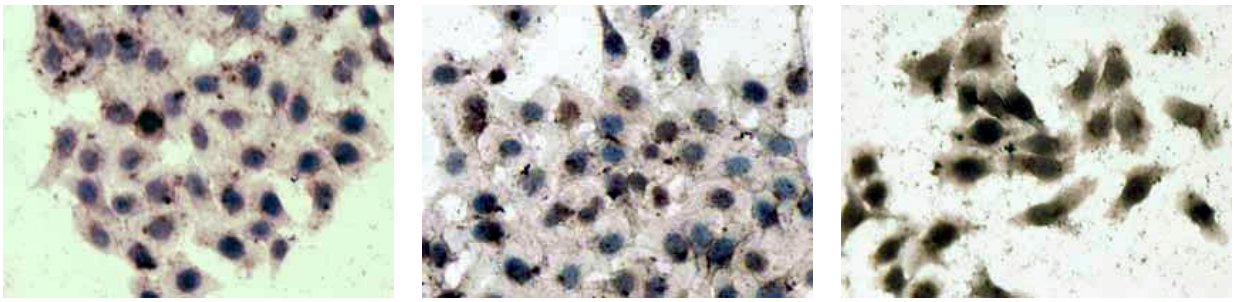
Group	1 d	3 d	6 d
Blank	43.7±3.9	42.0±2.7	40.5±3.0
Fluorescence	45.1±3.5	43.9±3.5	43.2±4.1
Silencing	19.6±3.0**▲▲	17.1±2.6**▲▲	16.0±1.9**▲▲

Compared with blank group,*P < 0.05, ** P < 0.01; compared with fluorescence control group, ▲,P < 0.05; ▲▲,P < 0.01.

Table 8. Apoptotic index of hepatoma carcinoma cells (%, n=5).

Group	1d	3d	6d
Blank	7.1±1.0	7.4±0.9	7.0±0.9
Fluorescence	7.2±1.1	7.2±1.3	7.4±1.1
Silencing	20.8±3.2**▲▲	21.8±2.7**▲▲	22.9±2.5**▲▲

Compared with blank group,*P < 0.05, ** P < 0.01; compared with fluorescence control group,▲.P < 0.05; ▲▲.P < 0.01.



Blank group 3d Fluorescence control group 3d Silencing group 3d

Figure 6. Observations of cells under transwell ventricle filter membrane under light microscope.

Table 9. Detection result of cell invasiveness (n=5).

Group	1d	3d	6d
Blank	70.1±8.4	171.0±16.7	251.8±27.7
Fluorescence	79.1±8.6	172.2±11.8	257.2±25.4
Silencing	35.7±3.8**▲▲	46.6±5.0**▲▲	68.3±6.9**▲▲

Compared with blank group,*P < 0.05; ** P < 0.01; compared with fluorescence control group,▲,P < 0.05;▲▲,P < 0.01.

indicate its possible involvement in the processes of early metastatic spread and its association with poor prognosis. The main function of cathepsin L is to degrade proteins and hydrolyze some precursor proteins (proenzyme and prohormone) to their active form, or activate other proteinase system. Thus, it can participate in a variety of physical activities (Hashimoto et al., 2006). In our study, Cathepsin L mRNA level was detected by RT-PCR, and we found that it was decreased in treansfected hepatoma carcinoma cells significantly. Meanwhile, cathepsin L mRNA level was also decreased by immunofluorescence and western blot. Cathepsin L expression was decreased significantly in siRNA transfected hepatoma carcinoma cells, and it continued to decrease later. The reproductive activity of hepatoma carcinoma cells was significantly inhibited over time, and apoptosis rate was significantly increased, which indicated that cathepsin L expression could be decreased,

and cell proliferation inhibited while the apoptosis could increase by RNAi in hepatoma carcinoma cells. The invasiveness of cathepsin L siRNA transfected hepatoma carcinoma cells decreased significantly. However, there was no significant change in the fluorescence control and blank group, which indicated that cathepsin L might play a great role in the invasion process of hepatoma carcinoma cells. Thus, cathepsin L siRNA technology might significantly inhibit tumor metastasis.

Conclusively, RNAi could effectively silence the cathepsin L expression in hepatoma carcinoma cells, inhibit cell proliferation and induce apoptosis. Tumor metastasis and invasion was an important factor in the death of patients with cancer, and RNAi technology of cathepsin L could significantly inhibit the invasion of tumor cells. Therefore, RNAi technology against cathepsin L could provide a new way for the treatment of tumors.

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