Construction and Functional Identification of Plasmid shRNA Suppressing Human cFLIP Expression*

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ABSTRACT Objective: To construct adenoviral shRNA for inhibiting the cFLIP expression in hepatocellular carcinoma and to investigate the influence of apoptosis on liver cancer cells (HepG2). Methods: According the sequence of the human cFLIP mRNA, four shRNAs targeting to interference cFLIP expression were designed and combined, which were ligased into silencing vectors, were designed. The silencing vectors were transfected into HepG2; RT-PCR and western blot were used to analyze the gene expression. Results: The plasmid down-regulated the cFLIP expression in HepG2 was obtained. The cFLIP expression in HepG2 was significantly suppressed by shRNA transfection. Conclusion: The shRNA plasmid down-regulated the cFLIP expression was successfully constructed, which lay the foundation for studying cFLIP in the proliferation of human hepatocellular carcinoma and clinical application.

Key words:cFLIP; shRNA; Hepatocellular carcinoma; Plasmid

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Introduction

Malignant tumor was hazard to human health. The current surgery, radiotherapy and chemotherapy were often less successful than anticipated, so the fatality was high. New treatments of tumor have been explored continuously. The research- through jointing the treatment of biology and genetic engineering to treat cancer, especially the tumor relative to control gene as a target for cancer therapy -has just begun. That is known as hot spots research and treatment of the etiology of tumor, especially carcinoma. Selecting the appropriate tumor-associated gene as a therapeutic target for the desire to achieve specific treatment for cancer and opening new avenues for the treatment of tumor, has broad application prospects[1-2]. At present, research on RNAi treatment mostly involved in tumor therapy, the use of RNAi technology-related inhibition of gene expression of tumor cell has been an important idea to research into new anti-cancer therapy.

cFLIP is a cell type with the Fas-associated death domain protein-like interleukin-1β converting enzyme inhibitory protein, which is a naturally occurring caspase (caspase) inhibition protein. cFLIP can inhibit the expression of death receptors (Fas, TRAIL, etc.)-mediated apoptosis, and be regulated by a variety of stimulating factor [3]. Blocking death receptor-mediated apoptosis has been considered to be an important step in the development of malignancy tumor cells. It is not only allowing tumor cells survive by evading the anti-immune response, but also enhancing its anti-cancer chemotherapy and other treatment to the tumor.

At present, the constructed plasmid which could specific inhibit the expression of cFLIP has been successfully transfected into tumor cells, and the study confirmed that cFLIP is highly expressed in liver cells, while recent research on the RNAi technique is few overseas and less in China. This research, will observe the effection of proliferation in liver cells by constructing specific and efficient Plasmids to block cFLIP and transfect to HCC cells successfully.

1 Materials and methods

1.1 Sources of material

HepG2 human hematoma cell line and HEK293 human embryonic kidney cell line were was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China; RPMI 1640 medium, fetal calf serum purchased from Hyclone Company; Trizol, Lipofectamine 2000TM transfection reagent was purchased from Invitrogen Corporation; RIPA lysis buffer was purchased from Beyotime Institute of Biotechnology; pGenesil-1 was purchased from buy from Wuhan Genesil Biotechnology Co., Ltd); pAdeasy system was purchased from Stratagene Corporation; Rabbit polyclonal anti-cFLIP antibody (Cat. No. ab8421) and mouse anti-GAPDH monoclonal antibody was purchased from Abcam's U.S. companies; Horseradish peroxides conjugated goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP antibodies purchased from Santa Cruz; ECL chemiluminescence kit was purchased from color Millipore Corporation. All the restriction enzymes, DL5000 Marker, T4 DNA ligase, RT-PCR kit purchased from Takara Bio Inc.; BCA protein concentration assay kit purchased from Pierce; A small amount of gel and plasmid extraction kit, DNA Marker I was purchased from TIANGEN BIOTECH (BEIJING) CO., LTD.; *E. coli* strain DH5α was the hospital cen

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tral laboratory preservation; other reagents were imported or domestic analytical production.

1.2 Cell line total RNA extraction and Real time PCR analysis

Steps in accordance with cell lines cultured cells and total cellular RNA were extracted according to Trizol instructions, frozen saving in -80℃ for use. RNA integrity was detected with Agarose gel imaging system, RNA content and purity were detected by UV spectrophotometer. Reverse transcription-polymerase chain reaction (RT-PCR), the sequence shown in Table 1, and then do the Real time PCR to detect expression different of cFLIP of cell lines.

1.3 Design and synthesis of siRNA for cFLIP mRNA

The sequence NM_172362 of Human cFLIP mRNA was found out in the GeneBank, the and coding sequence was analyzed, then mRNA was designed by using Ambion company online siRNA software. Potential siRNA target sites and genomic sequence databases (people) were compared to exclude other coding sequence of the homologous sequences, and then chemically synthesized four pairs of shRNA validation, while designing a pair of control primers. Specific sequence as shown in Table 1.

Table 1 Primer sequences

Primer No.	Sequence of primers (5'-3')
cFLIP-RT1F	AGAGTGAGGCGATTTGACCTG
cFLIP-RT1R	GTCCGAAACAAGGTGAGGGTT
GAPDH-F	TCATGGGTGTGAACCATGAGAA
GAPDH-R	GGCATGGACTGTGGTCATGAG
cFLIP-1F	GATCCGGCAAGGAGAAGAGTTTCTTTTCAAGACGAAGAAACTCTTCTCCTTGCTTTTTTGTCGACA
cFLIP-1R	AGCTTGTCGACAAAAAAGCAAGGAGAAGAGTTTCTTCGTCTTGAAAAGAAACTCTTCTCCTTGCCG
cFLIP-2F	GATCCGGGAGCAGGGACAAGTTACATTCAAGACGTGTAACTTGTCCCTGCTCCTTTTTTGTCGACA
cFLIP-2R	AGCTTGTCGACAAAAAAGGAGCAGGGACAAGTTACACGTCTTGAATGTAACTTGTCCCTGCTCCCG
cFLIP-3F	GATCCGCCAGATCAACTGGATTTATTTCAAGACGATAAATCCAGTTGATCTGGTTTTTTGTCGACA
cFLIP-3R	AGCTTGTCGACAAAAAACCAGATCAACTGGATTTATCGTCTTGAAATAAATCCAGTTGATCTGGCG
Control-F	GATCCGCTAGGTGTTCTAGTCTGGACTTTCAAGACGAGTCCAGACTAGAACACCTAGTTTTTTA
Control-R	AGCTTAAAAAACTAGGTGTTCTAGTCTGGACTCGTCTTGAAAGTCCAGACTAGAACACCTAGCG

1.4 shRNA vector construction for the cFLIP mRNA

Each of the above single-chain gene fragments was dissolved with 50 μl annealing buffer. 2 μl of each complementary single-stranded oligonucleotides was mixed with 16 μl annealing buffer, we anneal the compound in 94 ℃ water bath then refrigerate it to room temperature. The annealing product was used on ligation reaction in downstream with 1 μl.

Carrying EGFP fluorescence protein pGeneSil-1 vector was enzyme cleavaged by *Sal* Ⅰ. The product was ligated with the annealing products after being electrophoresised and recovered. The ligation reaction product was transformed into *E. coli* DH5α. The positive clones were screened and submitted to the Shanghai Biological Engineering Co. for sequencing. The correct plasmids were named pGeneSil-cFLIP-1, pGeneSil-cFLIP-2, pGeneSil-cFLIP-3, pGeneSil-cFLIP-4, and pGeneSil-control.

1.5 Cell culture and shRNA transfection

HepG2 cells was cultured in RPMI 1640 medium (containing 10% fetal calf serum, 1% penicillin - streptomycin) at 37℃, with 5% CO_2 incubator temperature incubation passaging. The day before transfection the HepG2 cells were inoculated into 6-well plates, cultured for 24 h until the cell density reached between 80% and 90%. The transfection experiment was played according to Lipofectamine™ 2000 liposomal transfection reagent manual. HepG2 cells were divided into 5 groups marked A, B, C, D, and E. Group A, B, C, D were named cFLIP shRNA section, group E as the control and group F was named negative control one without any transfected plasmid.

1.6 Expression of the target gene was detected by Western blotting after recombinant plasmid transfection

48 h later after transfection, cells were washed with cold PBS solution. Appropriate amount of RIPA lysis buffer 4 ℃ was added to each culture dish and incubated on ice for 40 min. The cells was scraped off with scraping rod, lysate was collected and blended in ultrasonic homogenizer, then centrifuged in 4℃, 12 000 × g for 25 min, the supernatant was frozen saving at - 80 ℃. According to the sample protein concentration, the volume was calculated in 30μg of total protein. Vertical electrophoresis in SDS-PAGE, product was transferred to NC membrane. The product was closed with 5% skim milk powder (dissolved in TBS-T buffer) at room temperature 1 h, then rabbit anti-cFLIP antibody (1:500) and mouse anti-β-actin antibody (1:2 000) were added, 4℃ overnight incubation. The product was oscillating washed with TBS-T for 5 min for 3

times. The appropriate goat anti-rabbit IgG antibody (1:10,000) or goat anti-mouse IgG antibody (1:8,000) were added in room temperature incubating for 1 h. Membrane was washing by the former method three times, color development with the ECL chemiluminescence, X-ray was developed in lithography .

2 Results

2.1 cFLIP expression in tumor cells

Real time PCR was ued to detect the expression of cFLIP in various tumor cells, the results showed that cFLIP in liver cancer cells present a high level of expression (Fig.).

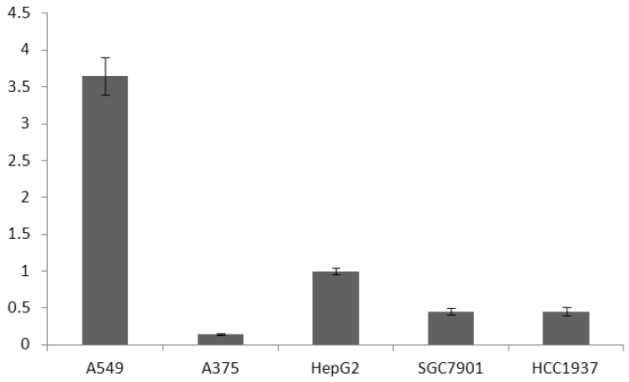

Fig. **I** Detection of cFLIP expression in the tumor cells by real-time PCR

2.2 Results of recombinant plasmid restriction enzyme digestion and sequencing identification

After linearized by *Hind* **III** and *BamH* **I** , plasmid pGenesil-1 was ligated with fragments synthesis of shRNA. The product was transformed and cultured in LB medium. After plasmid extraction, new plasmid was got. The new plasmid was identified by restriction enzyme SalI restriction enzyme digestion and detected by electrophoresis in 1% agarose gel. Positive plasmids were obtained which we re named pGeneSil-cFLIP-1, pGeneSil- c FLIP-2, pGeneS ilcFLIP-3, pGeneSil-cFLIP-4, pGeneSil-control (Fig. **II**). Positive plasmids were sequenced to the sequencing of the company and the results fully meet the design requirements.

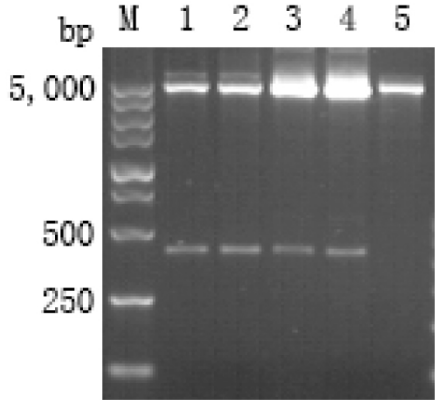

Figure. **II** plasmid DNA identified by restriction enzyme digestion with Sal **I** :M :DNA marker DL5000 ;1 :pGeneSil-cFLIP-1 ;2 :pGeneSil-cFLIP-2 ;3 :pGeneSil-cFLIP-3 ;4 :pGeneSil-control ;5 :PGeneSil

2.3 Detection of cell transfection efficiency

Gene expression of green fluorescent protein reporter of HepG2 cells was surveyed with inverted fluorescence microscope after transfection by the Lipofectamine™ 2000 for 48 h to detect the transfection efficiency. Fluorescence showed strong fluorescence expressing at 48 h, the transfection rate was about 70% (Fig.**III**).

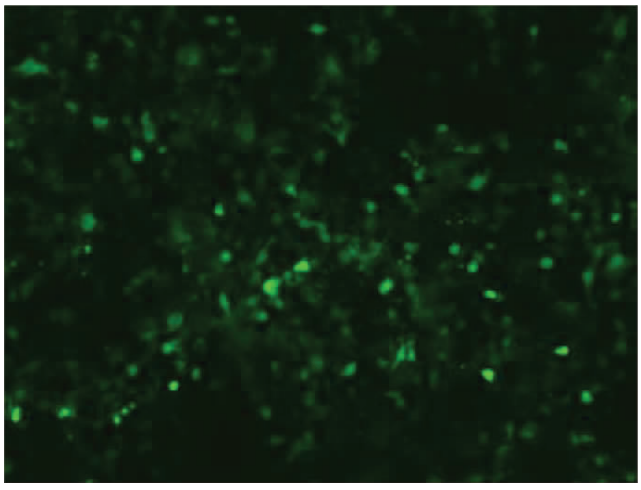

Fig. **III** The pictures of plasmid DNA transfecting cell lines

2.4 Detection of cFLIP protein expression of the cells after shRNA transfected

HepG2 cells (including shRNA1, shRNA2, shRNA3, shRNA4, shRNAC) were transfected with the same quality of shRNA vector applicating the liposome 2000TM Western blotting analysis the protein expression 48h later. The results showed that four pairs of shRNA could inhibit the expression of cFLIP on varying degrees, that the strongest inhibition is pGeneSil-cFLIP-1(Fig.**IV**)

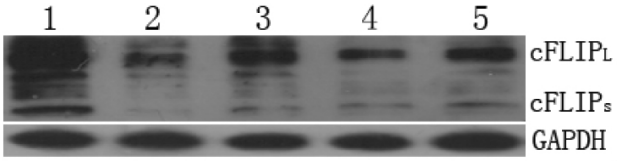

Fig. **IV** cFLIP gene silencing detection by Western blot:1 :Blank cell ;2 : pGeneSil-cFLIP-1 ;3 :pGeneSil-cFLIP-2 ;4 :pGeneSil-cFLIP-3 ;5 : PGeneSil-control

3 Discussions

Hepatocellular carcinoma (hepatocellular carcinoma, HCC) is a common malignant tumor with a very high degree, which is rapidly developing and in high mortality, at the same time, usually late found, however, its mechanisms of occurrence and development are not quite clear. Recent years, with the new understandings of the research and ideas on a new expansion were conformed on the molecular mechanisms of HCC development. Among them, the relationship between cell proliferation and apoptosis has become one of the hot spots of the mechanisms of

liver cancer.

Tumor cells can evade apoptosis though several mechanisms that affect both intrinsic and extrinsic apoptotic pathways, and resistance to chemotherapeutic treatment is frequently related to failure of programmed cell death. Increased c-FLIP expression has been observed in several human malignancies, such as ovarian, colon, breast and prostate cancer and glioblastoma, and it was found to be involved in resistance to both CD95/Fas- and TRAIL receptor-induced apoptosis in most of them. Resistance was found to directly occur at the level of the DISC formation where the recruitment and activation [4-5].

A large body of evidence indicates the major role of c-$FLIP_L$ expression in the negative regulation of apoptotic signaling in tumor cells in vitro. It has been reported that the anti-apoptotic protein, FLIP, is regulated by heterogeneous nuclear ribonucleoprotein K and correlates with poor overall survival of nasopharyngeal carcinoma patients [6]. Paxilline enhances TRAIL-mediated apoptosis of gloma cells via modulation of c-FLIP [7]. Enhanced death ligand-indeced apoptosis in cutaneous SCC cells by treatment with Diclofenac/Hyaluronic Acid correlates with down-regulation of c-FLIP [8]. Resistance of cutaneous anaplastic large-cell lymphoma cells to apoptosis by death ligands is enhanced by CD30-mediated over-expression of c-FLIP [9]. c-FLIP degradation mediates sensitization of pancreatic cancer cells to TRAIL-induced apoptosis by the Histone Deacetylase inhibitor LBH589 [10]. β-Elemene Piperazine Derivatives Induce Apoptosis in Human Leukemia Cells though Down-regulation of c-$FLIP_L$ and Generation of ROS [11]. For example, small interfering RN As (siRNAs) that specifically knocked down expression of c-FLIPL in diverse human cancer cell lines, e.g., lung and cervical cancer cells, augmented TRAIL-induced DISC recruitment, and thereby enhanced effector caspase stimulation and apoptosis. Therefore, the outlook for the therapeutic index of c-FLIP-targeted drugs appears excellent, not only from the efficacy observed in experimental models of cancer therapy, but also because the current understanding of dual c-FLIP action in normal tissues supports the notion that c-FLIP-targeted cancer therapy will be well tolerated [12]. The incidence of liver cancer has been shown many general characters including: multi-factors, multi-steps, multi-gene mutations [13], so gene therapy may be the fundamental therapeutic approach. Among the gene therapy strategies, RNAi has been concerned gradually, and has become the new technology to silent the function of specific gene. It has been shown that the important factors of stable genetic and biological evolution in biosphere are the genetic stability and the invasions of the outside world decrease, RNAi included[14]. Mechanism of RNAi is the degradation of homologous mRNA induced by the siRNA, which prevents the translation of the mRNA, leading to gene silencing. As silence takes place in post-transcriptional level, it is known as transcriptional gene silencing (Post-transcriptional gene silencing, PTGC) [15]. Main process of RNAi is the si RNA antisense strand forming RNA-induced silencing complex (RNA-induced silencing complex, RISC) with endonuclease, exonuclease, helicase enzymes together, which-mediated cutting target gene mRNA molecules that can complement with siRNA antisense strand to interfere gene expression. Because RNAi has highly sequence-specific and energy efficient interference and silence specific genes, the technology has been used in gene function and gene therapy widely [16]. At present, duing to inducing mammalian gene silencing efficiently, RNAi has become a powerful tool in many study aspects of gene function. Expression of cFLIP can be inhibited by siRNA interference, the performance reduction of cFLIP mRNA and cFLIP protein.

Many studies reported that siRNA-mediated silencing of cFLIP induced spontaneous apoptosis in the panel of P53 wild-type, mutant and null colorectal cancer cell lines. Moreover, the anti-apoptosis role of cFLIP in the regulation of TRAIL-mediated apoptosis colon cancer cell was clearly shown using siRNA methodology [17]. Furthermore, cFLIP down-regulation sensitizes colorectal cancer cells to chemotherapy [18]. Moreover, the specificity silencing of c-$FLIP_L$ was sufficient to sensitize MDA435 cells to doxorubicin. Some studies have shown that cFLIP gene silencing enhanced the doxorubicin-induced HCC cells apoptosis. These results indicate that cFLIP may be an important regulator of chemotherapy-induced cell death in human HCC cells [19].

At the present time, the development of small molecules inhibiting c-$FLIP_L$ recruitment to the DISC is still hampered by the high homology among DED-containing proteins. On the contrary, the silencing of c-$FLIP_L$ and c-$FLIP_S$ using c-FLIP-specific antisense oligonucleotides or siRNA lipocomplexes efficiently induced spontaneous apoptosis in various cancer cell types and in tumor xenografts [20]. However, the possible therapeutic application of c-$FLIP_L$ stable silencing strategies awaits further optimization and validation in ad hoc preclinical studies. This study, firstly by siRNA expression vector method, synthesized shRNA and plasmid pGenesil-1, which were linearized by *Hind* Ⅲ and *BamH* Ⅰ, then inserted the DNA oligonucleotides with the shRNA hairpin into pGenesil-1 plasmid vector. The vector contained U16 promoter which could transcript and generate shRNA in intracellular. shRNA was cut into siRNA and played a role of interference by Rnase Ⅲ in intracellular. Synthetic plasmids were restriction enzyme cut by restriction endonuclease *Sal* Ⅰ and identified by gel electrophoresis to obtain positive plasmid. The 5 positive groups were completely corresponding to design of experiment after sequencing. The recombinant plasmid vector were transfected transiently into HepG2 cells apply Lipofectamine™ 2000 lipofection method, then the transfection efficiency was detected though gene expression of green fluorescent protein reporter with an inverted fluorescence microscope in HepG2 cells. Fluorescence showed that strong fluorescence was expressed to the highest level at 48 h. The transfection rate was about 70% and met the test requirement. The efficien-

cy of gene interfere of cFLIP was detected by Western blot .Results show that the same quality of shRNA vector transfected HepG2 cells (including shRNA1, shRNA2, shRNA3, shRNA4, shRNAC) with Lipofectamine 2000 method for 48 h then analysised gene expression by Western blotting: the results showed that four pairs of shRNA have different degrees of inhibition on cFLIP expression, the strongest inhibition is shRNA2, which could be used as the preferred carrier for further experimental analysis.

More evidence showed that, major role of c-$FLIP_L$ was the negative control to the expression of apoptosis signal. Consistent with its function, c-$FLIP_L$ prognostic relevance had come into focus. Indeed, c-FLIPL has been identified as an independent adverse prognostic factor in various cancer types (colon, endometrial, Burkitt's lymphoma and ovary), suggesting that it might significantly contribute to the identification of patients at higher risk of cancerrelated death who could benefit from alternative therapeutic modalities. For all these reasons, c-$FLIP_L$ may represent a critical target for a therapeutic intervention [21-22]. Therefore cFLIPL regulation could significantly increase the ability of c-$FLIP_L$ of anti-apoptotic mechanism, and the possibility of using RNAi technology to specifically inhibit the expression of cFLIPL or c-$FLIP_L$ signaling pathways to regulate its expression has not been reported by now.

In this study, the purpose of cFLIP-targeted siRNA vector constriction was specific to silence cFLIP, then build plasmid vector with or without cFLIP targeted shRNA vector, and transfect the vector into HepG2 cells. The results showed that cells with different shRNA vector expression of cFLIP mRNA and protein levels were significantly different (Figure **Ⅳ**). Selected from the positive clones with low expression of cFLIP showed that cFLIP-targeted siRNA could specifically inhibit the expression of cFLIP.

The experiments fully confirmed high expression of the apoptotic gene cFLIP in liver cancer cells, which indicated that cFLIP gene was closely related to liver cancer occurrence and development. shRNA plasmid was successfully constructed and transfected into HepG2 cells. Specific silencing cFLIP could inhibit its mRNA and protein expression obviously. Inhibition of cFLIP expression may down-regulate vitality and up-regulate drug-induced apoptosis in HCC cells. Target-cFLIP combination with anti-tumor therapy could play a therapeutic potential through enhance apoptosis and laid the theoretic foundation for the conduct of hepatocellular carcinoma and other malignant targeted therapy and tumor-specific anticancer drugs targeted therapy. Some scholars have suggested that low level expression of c-FLIP is an inducing factor to apoptosis, high level expression could specifically inhibit apoptosis, but the exact mechanism and the boundaries of expression are not clear[23]. Recently, some scholars demonstrated a pivotal role for FLIP in protecting cells from the apoptosis and attenuating cytokine responses induced by cytoplasmic dsRNA. They have identified FLIP as a crucial molecule that regulates the response in MEFs: FLIP suppresses both inflammatory and apoptotic pathways. Strategies to increase expression of FLIP would be expected to dampen the excessive inflammation as well as apoptosis [1]. Above characteristics may reduce the incidence of infection to the patients with tumor after chemotherapy treatments. Therefore, based on the above theory, this study do the research of new anticancer drugs direct at the different expression of c-FLIP, which has definite instruct meaning to carry out clinical tertiary prevention and treatment to cancers such as HCC.

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抑制人 cFLIP 基因表达的 shRNA 质粒的构建与功能验证 *

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摘要 目的：构建特异性抑制 cFLIP 基因表达的质粒并检测其对肝癌细胞的影响。方法：根据人 cFLIP mRNA 的序列，设计合成 3 对 cFLIP 基因的 shRNA，将其连入干扰载体，转染 HepG2，蛋白印迹法检测基因表达情况，以检测其对肝癌细胞的影响。结果：构建了特异性抑制肝癌细胞中 cFLIP 表达的质粒。结论：成功构建能特异且高效阻断 cFLIP 表达的 shRNA 表达质粒，为进一步研究 cFLIP 基因对肝癌增殖的影响及其临床应用奠定了基础。

关键词：cFLIP；shRNA；质粒；肝癌
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