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Down-regulation of c-FLIP Gene by siRNA Interference Enhances Breast Cancer Cells to TRAIL-Induced Apoptosis *

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ABSTRACT Objective: To investigate the effect of c-Flip gene suppression on enhancing breast cancer cell MCF-7 to TRAIL-induced apoptosis. **Methods:** MCF-7 cells were infected with recombinant adenoviruses Ad-cFLIP-siRNA and Ad-sTRAIL, alone or combined respectively. Real-time quantitative polymerase chain reation (Real time PCR) was used to detect the expression of c-FLIP and TRAIL in MCF-7 cells. Methythiazol tetrazolium (MTT) assay and Crystal violet staining were applied to detect the cell proliferation. And Hoechst 33258 staining was used to detect the apoptosis of MCF-7 cells. **Results:** Compared with that in the negative control group, the relative expressions of c-FLIP in the c-FLIP-siRNA and the c-FLIP-siRNA+TRAIL groups were (0.32± 0.16) and (0.39± 0.48) times respectively; The relative expressions of TRAIL in the TRAIL and the c-FLIP-siRNA+TRAIL groups were (96.21± 1.54) and (87.33± 1.66) times respectively. The inhibition rate (%) of cells in TRAIL, c-FLIP-siRNA, and TRAIL+c-FLIP-siRNA group were (60.27± 1.25), (11.34± 1.74) and (74.91± 2.12), respectively. Compared with that in the negative control group(3.12± 1.54), the apoptosis rates of cells in TRAIL (12.79± 2.46) and c-FLIP-siRNA+TRAIL group (25.50± 3.17) increased significantly ($P < 0.05$). However, the apoptosis rates of cells in c-FLIP-siRNA group (6.85± 2.82) had no statistical significance with that in negative control group ($P > 0.05$). **Conclusion:** Down-regulation of c-FLIP by siRNA interference could enhance the breast cancer cells MCF-7 to TRAIL-induced apoptosis.

Key words: TRAIL; c-FLIP; Breast cancer; Adenovirus; Apoptosis

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Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF super family. It appears to be a promising anticancer agent owing to it can selectively induce apoptosis in a wide variety of cancer cells, while has no significant side effects on normal tissue[1,2]. However, not all cell lines are sensitive to TRAIL. Keane MM found that some of breast cancer cell lines (including MCF-7) are not sensitive to TRAIL, which remains a major obstacle for successful treatment of cancer using TRAIL-based therapy [3]. Cellular FLICE-like inhibitory protein (c-FLIP), has been confirmed as an important factor in TRAIL-induced apoptosis, but the mechanism is still not clear in breast cancer[4,5]. Therefore, it is very important to find out the mechanism of the resistance. This research will explore how c-FLIP efficiently enhances TRAIL-induced apoptosis in MCF-7 cells using recombinant adenoviruses Ad-c-FLIP-siRNA and Ad-sTRAIL.

1 Materials and methods

1.1 Source of materials

Human breast cancer cell line MCF-7 was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. RPMI 1640 medium, fetal calf serum was purchased from Hyclone Company. Recombinant adenoviruses Ad-c-FLIP-siRNA and Ad-sTRAIL were provided by the Medical Research Center, affiliated hospital of Qingdao University. Trizol, RT and RT-PCR kits were purchased from Takara Bio Inc.. MTT reagent and Hochest33258 assay kit were purchased from Sigma and Beyotime Institute of Biotechnology respectively.

1.2 Infection with recombinant adenoviruses

MCF-7 cells were grown in RPMI1640 medium, supplemented with 10% fetal calf serum (FCS) and maintained in the presence of 5% CO_2 at 37℃ . The day before transfection the MCF-7 cells were inoculated into 6-well plates, and allowed the cell density to reach 80% for 24 h. Before treatment, the medium was replaced using the serum-free RPMI1640 and the MCF-7 cells were infected with Ad-c-FLIP-siRNA and Ad-sTRAIL, alone or combined for 2 h. The infection performed at multiplicity of infec-

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tion (MOI) of 10. Every 20 min, the plates were shaked gently. The virus solution was then decanted after cultured 2 h and complete culture solution was added. After incubation for 48 h, the expression of green fluorescent proteins (GFP) was observed and the cells were collected for the following biological activity research and mRNA levels detection. So, there are four groups in this research, TRAIL group, c-FLIP-siRNA group, c-FLIP-siRNA+TRAIL group and negative control group.

1.3 Total RNA extraction and Real time PCR analysis

Total mRNA was extracted from the cells of the four groups infected with different viruses for 48 h by using Trizol reagent and then quantitated in ultra violet spectrophotometer (Beckman Company) by measuring A260 and A280. Then 0.5 mg mRNA of each sample was reversely transcribed into cDNA using the PrimeScript reverse transcriptase kit. To assess the levels of mRNA of target gene, we used real-time fluorescence quantitative PCR analysis based on the SYBR Green method in A480 real time thermal cycler (Roche). The PCR reactions in duplicate were subjected to an initial denaturation at 95℃ for 10 seconds, followed by 40 cycles of denaturation at 95℃ for 5 seconds, annealing and extension at 60℃ for 45 seconds. The value of threshold cycle (CT) for each reaction was recorded. The levels of target mRNA transcripts relative to GAPDH were expressed as the (CT (CTTarget CTGAPDH) and further quantified using the 2-((CT method, where ((CT= (CT-Target CTGAPDH) experiment group - (CTTarget CTGAPDH) control group [6]. The PCR primers used were as follows: c-FLIP (+). 5'-AGAGTGAGGCGATTTGACCTG, (-) GTCCGAAACAAGGTGAGGGTT-3', TRAIL(+)5'-AGTGAGAGAAAGAGGTCCTCAG, (-) CCAGAGCCTTTTCATTCTTGGA-3', and GAPDH(+) 5'-TCATGGGTGTGAACCATGAGAA, (-) GGCAT GGACTGTGGTCATGAG-3'.

1.4 MTT assay for cellular proliferation inhibition rate

The effect of Ad-cFLIP-siRNA and Ad-sTRAIL on MCF-7 cell viability was measured by using the 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were treated with Ad-cFLIP-siRNA (MOI=10) and Ad-sTRAIL (MOI=10), alone or in combination for 48 h, and seeded in each well of a 96-well plate (8×10^4 cells/well in 100 μL medium). Then MTT assay was carried out to analyze the cell viability after incubation for 48 h. Thereafter, 10 μL of MTT (5 mg/ml) was added into each well and incubated under 5% CO_2 and at 37℃ for another 4 h. After the removal of the culture medium, the cells were lysed in 150 μL of dimethylsulfoxide (DMSO). The plate was shaken until the crystals were dissolved. Afterwards, the absorption at 490 nm (A490) was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). The inhibitory rates of different groups were calculated using the formula: cell inhibitory rate (%) = (1-A490 of the experimental group/ A490 of the control group) × 100%.

1.5 Crystal violet staining assay for cell viability

MCF-7 cells were seeded in each well of a 24-well plate with a density of 2×10^5/well and were incubated for 24 h to adhere. The cells were infected with Ad-c-FLIP-siRNA and Ad-sTRAIL at different MOIs (0,1,5,10,50,100), alone or combined. Then placed them in the incubator until the confluence of cells in control group reached 100%. The culture medium was carefully discarded and , washed with PBS and fixed for15 min with 95% ethanol.The cells were exposed to 2% crystal violet in 20% methanol for 15 min. Then plates were washed with deionized water until the dye was washed off and finally documented as photographs. The experiment was repeated 3 times.

1.6 Hoechst 33258 staining

MCF-7 cells were infected with Ad-cFLIP-siRNA (MOI=10) and Ad-sTRAIL (MOI=10), alone or combined for 48 h. The cells in each group were fixed in 4% paraformaldehyde for 20 min and stained with Hoechst 33258 following the manufacture's protocol. The cells were then observed under a fluorescence microscope. The apoptosis rates = (apoptotic cells/total cells) × 100%. The experiment was repeated 3 times.

1.7 Statistical Methods

The data were analyzed by SPSS 16.0 statistical software, and data denoted by ± S, the comparison between groups were done with single factor analysis of variance. $P<0.05$ indicated significant difference.

2 Results

2.1 Infection of MCF-7 with adenovirus

Green fluorescent protein (GFP) was observed under the fluorescence microscope 48 h after infection with adenovirus (Fig. 1). The results showed that the MCF-7 cells exhibited high infection efficiency, because more than 90% cells were infected with Ad-GFP when the MOI was 10.

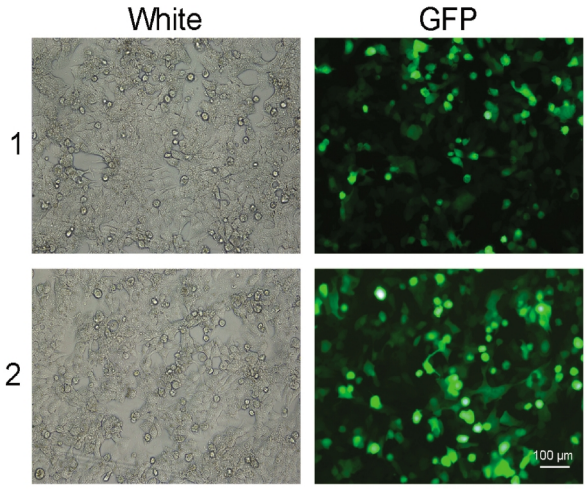

Fig. 1 Analysis on the expression of GFP in MCF-7 cells under fluorescence microscope: 1:c-FLIP-siRNA group 2:TRAIL group

2.2 mRNA levels of c-FLIP and TRAIL

Used $2^{-\triangle\triangle\alpha}$ method to calculate the results of Real-time PCR. .The relative expression of c-FLIP mRNA and TRAIL mRNA as shown in Figure 1. Compared with the negative control, the mRNA relative expression of c-FLIP was (0.32± 0.16) and (0.39± 0.48) times in c-FLIP group and c-FLIP-siRNA+TRAIL group, respectively; The mRNA relative expression of TRAIL were (96.21± 1.54)and (87.33± 1.66)times in TRAIL group and the c-FLIP-siRNA+TRAIL group, respectively. The mRNA relative expression between the two groups had statistically significant differences($P < 0.05$).

2.3 Detection the inhibition rates by MTT assay

Results of the MTT assay showed that compared with that in the negative control group, the inhibition rates of cells infected with Ad-c-FLIP-siRNA and Ad-sTRAIL alone were 11.34 ± 1.74% and 60.27± 1.25%, respectively. While it reached 74.91± 2.12% when infected with Ad-c-FLIP-siRNA and Ad-sTRAIL together. And the inhibition rate had statistic significance between the c-FLIP-siRNA+TRAIL group and any other groups ($P<0.05$, Fig. 2). These data suggested that down-regulation of c-FLIP had no significant influence on the proliferation of MCF-7 cells ($P>0.05$), but could enhance the inhibition of TRAIL on MCF-7 cells.

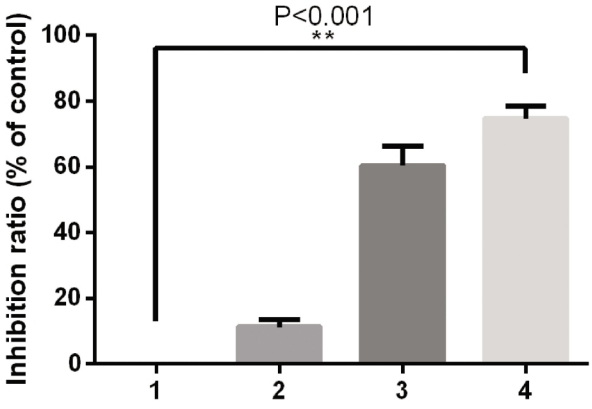

Fig. 2 Inhibition ratio histogram(*On behalf of the other two groups compared $P < 0.05$)
1:control group 2:c-FLIP-siRNA group 3:TRAIL group;
4:TRAIL+c-FLIP-siRNA group

2.4 Cell viability assay by crystal violet staining

The differences of each group in cell viability were determined by using crystal violet staining. With the virus MOI increased gradually, the number of cells infected by the virus increased gradually, the cell viability was also gradually reduced with the increase of the amount of virus in TRAIL and c-FLIP-siRNA +TRAIL groups, which was dose-dependent. But the cell viability was relatively lower in c-FLIP-siRNA +TRAIL group than that in TRAIL group at the same dosage. However, the cell viability changed little in c-FLIP-siRNA group although increasing the the amount of Ad-c-FLIP-siRNA($P>0.05$, Fig. 3).

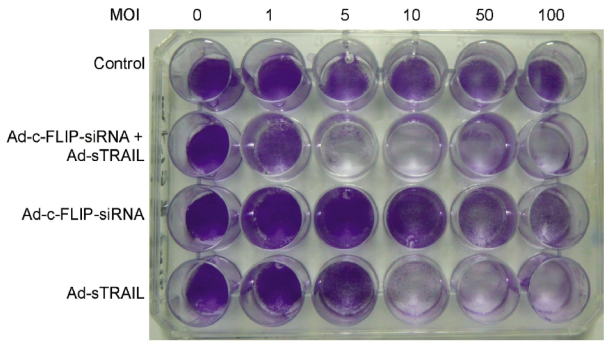

Fig. 3 Cell viability assay by crystal violet staining

2.5 Cell apoptosis detection with Hoechst 33258

Hoechst 33258 staining could distinguish the structure differences of cell nucleus between normal and apoptotic cells. The typical apoptotic morphological changes including chromatin condensation and nuclear shrinkage, fragmentation were observed after the cells were infected by Ad-sTRAIL alone or Ad-c-FLIP-siRNA and Ad-sTRAIL combined. Compared that with negative control group (3.12± 1.54), the apoptosis rates in TRAIL group(12.79± 2.46) and c-FLIP-siRNA+TRAIL group (25.50± 3.17) increased

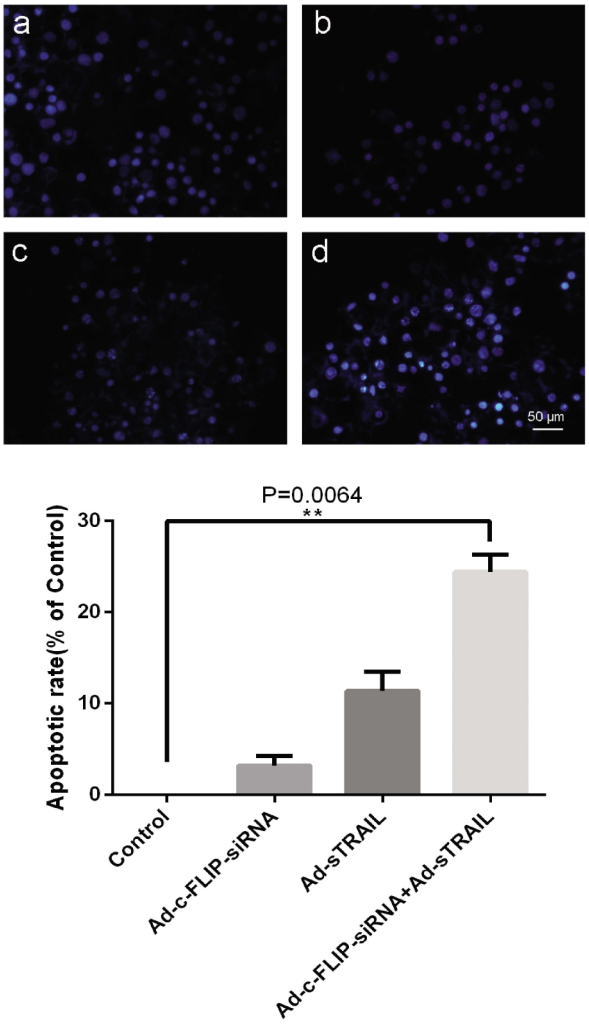

Fig. 4 Cell apoptosis detection with Hoechst 33258 (× 400)
a: control group b: c-FLIP-siRNA group c: TRAIL group;
d:TRAIL+c-FLIP-siRNA group

significantly ($P < 0.05$), while it was in (6.85 ± 2.82) in c-FLIP-siRNA group, and there was no statistical significance($P > 0.05$). These results comfirmed that c-FLIP could enhance the breast cancer cells MCF-7 to TRAIL-induced apoptosis.

3 Discussions

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths [7]. With the research on gene therapy in recent years, gene therapy has become a very effective way of tumor treatment[8]. Lin T found that TRAIL gene therapy can enhance chemotherapy killing effect, restrain the growth and metastasis of the sensitive and resistant breast cancer cell lines[9]. Although TRAIL showed a promising chemotherapeutic target for cancer, resistance to TRAIL-induced apoptosis has been reported in several tumor cells, including breast cancer cell lines [10]. Study found that some over-expression of apoptosis inhibiting gene was one of the important factors causing apoptosis signal termination in a variety of TRAIL-resistant cell lines [11].

c-FLIP, a highly conservative anti-apoptosis protein, could inhibit TRAIL-induced apoptosis and chemotherapy-triggered apoptosis in malignant cells. c-FLIP was firstly found in virus by THOME in 1997 and cloned from the activation of leukocytes on the basis of the virus type FLIP (vFLIP) by Irmle [12]. Still now, 13 genetypes of c-FLIP have been reported, but only three protein have been transcribed, called c-FLIPL, c-FLIPS, c-FLIPR, respectively. c-FLIPS and c-FLIPR have similary structure and lack a 20 amino acid fragments' extension on C-terminal compared with c-FLIPL. Chang found that c-FLIPL could regulate the activity of caspase-8 and regulate apoptosis in two-way[13]. c-FLIPL could inhibit the activation of caspase-8 when over-expression in cells, but it also could promote activation of caspase-8 when c- FLIPL low expression .

As an anti-apoptosis protein, c-FLIP had a close relationship with the growth of tumor, and its over-expression had been observed in several human malignancies, such as colon [14], liver[15], stomach[16]and melanoma and so on [17]. c-FLIP showed a positive correlation with tumor's grade and clinical stages. Recent studies found th at downregulation of c-FLIP could enhance TRAIL-induced apoptosis in renal cancer[18], leukemia/lymphoma[19], ovarian cancer[20], pancreatic cancer[21], liver cancer[22] and breast cancer[23].

In this research, recombinant adenoviruses Ad-cFLIP-siRNA and Ad-sTRAIL were used to infect the breast cancer cell line MCF-7, alone or combined, and the results showed downregulation of c-FLIP protein in MCF-7 cells could be significantly contributed to the growth inhibition and apoptosis in TRAIL-induced MCF-7 cells. Therefore, the over-expression of c-FLIP might be one of the main reasons resulting in the resistance of breast cells to TRAIL-inducing apoptosis. Study on the the molecular mechanism and functional role of c-FLIP will benefit to the breast cancer gene therapy, and help to improve TRAIL in the clinical application.

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抑制 c-FLIP 基因促进 TRAIL 诱导的乳腺癌细胞凋亡*

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摘要 目的：探讨抑制 c-FLIP 的表达对 TRAIL 诱导乳腺癌细胞 MCF-7 凋亡的影响。**方法：**重组腺病毒 Ad-c-FLIP-siRNA 和 Ad-sTRAIL 单独及联合感染对 TRAIL 耐药的乳腺癌细胞 MCF-7，应用实时荧光定量聚合酶链反应（Real-time PCR）检测病毒感染后各组细胞内 c-FLIP 和 TRAIL 的 mRNA 表达变化；MTT 法和结晶紫染色法检测 MCF-7 细胞活性，Hoechst 33258 荧光染色检测各组细胞的凋亡情况。**结果：**与阴性对照组比较，c-FLIP-siRNA 组和 c-FLIP-siRNA+TRAIL 组 c-FLIP 的 mRNA 相对表达量分别是（0.32± 0.16）和（0.39± 0.48）倍；TRAIL 组和 c-FLIP-siRNA+TRAIL 组 TRAIL 的 mRNA 相对表达量分别是（96.21± 1.54）和（87.33± 1.66）倍；TRAIL 组、c-FLIP-siRNA 组及 c-FLIP-siRNA+TRAIL 组的抑制率（%）分别为（60.27± 1.25）、（11.34± 1.74）及（74.91± 2.12）。对比阴性对照组的凋亡率（3.12± 1.54），TRAIL 组（12.79± 2.46）和 c-FLIP-siRNA+TRAIL 组 (25.50± 3.17)组的凋亡率明显增高（P＜0.05），c-FLIP-siRNA 组（6.85± 2.82）的凋亡率变化不明显，差异无统计学意义（P＞0.05）。**结论：**siRNA 抑制 c-FLIP 基因的表达能显著促进 TRAIL 对乳腺癌细胞 MCF-7 凋亡的诱导作用。

关键词：TRAIL；c-FLIP；乳腺癌；腺病毒；凋亡

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