

## Original Article

# Protective effect of RIP and c-FLIP in preventing liver cancer cell apoptosis induced by TRAIL

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**Abstract:** TRAIL (TNF-related apoptosis-inducing ligand) is a member of the tumor necrosis factor superfamily that can induce tumor selective death by up-regulating death receptor 4 (DR4) and DR5 expression. The study aimed to explore the role of RIP and c-FLIP genes in TRAIL induced liver cancer cell HepG2 and Hep3B apoptosis and related mechanism. RIP and c-FLIP silenced HepG2 and Hep3B cell model were established through siRNA. Western blot was applied to test c-FLIP, RIP, DR4, DR5, FADD, Caspase-3/8/9, ERK1/2, and DFF45 protein expression. Caspase-8 kit was used to detect Caspase-8 expression. Flow cytometry was performed to measure cell apoptosis rate. Acid phosphatase method was applied to determine cell cycle. TRAIL had no significant effect on Caspase-3/8/9, DR4, DR5, ERK1/2, and DFF45 protein expression, but up-regulated c-FLIP and RIP protein expression and reduced FADD expression level. After treated by the chemotherapy drug mitomycin and adriamycin, c-FLIP and RIP expression decreased significantly, while FADD increased. After knockout c-FLIP and RIP gene, HepG2 and Hep3B cell apoptosis rate induced by TRAIL increased obviously. Meanwhile, cell subG1 percentage increased markedly and exhibited G1 phase growth retardation. In addition, after two kinds of gene knockout, Caspase-8 was activated and produce Caspase-3 P20 and P24, leading DFF45 appeared DNA fragment P17 and P25. c-FLIP and RIP can inhibit Caspase-8 activation and prompting HepG2 and Hep3B resistant to cell apoptosis induced by TRAIL.

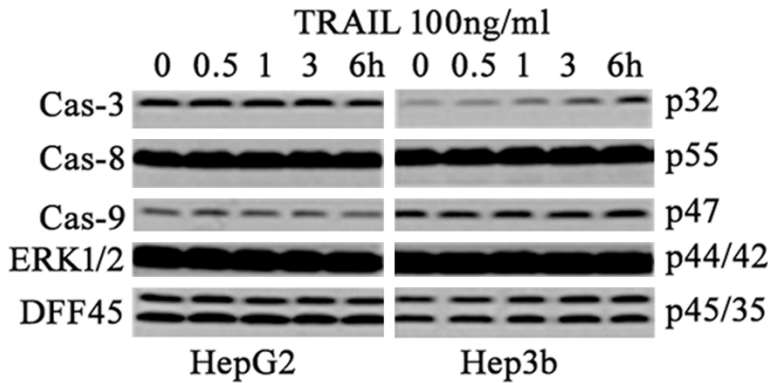
**Keywords:** TRAIL, liver cancer, RIP, c-FLIP, apoptosis

## Introduction

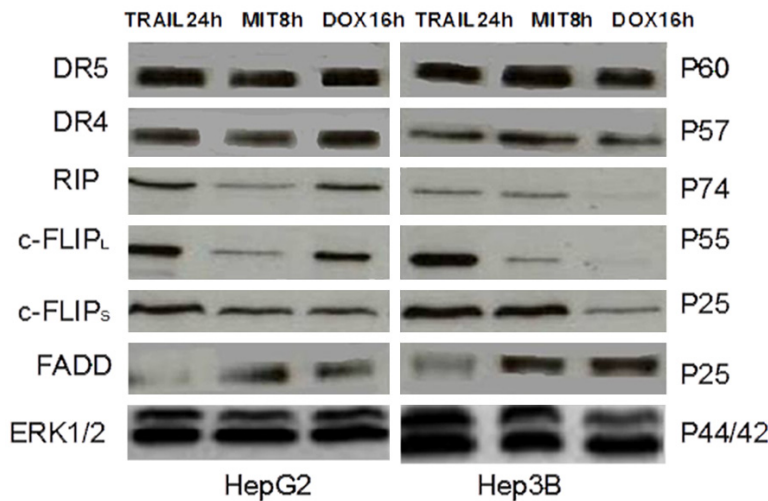
Liver cancer is a malignant gastrointestinal tumor with high incidence in China. Its incidence and death rates are high, and there is still lack of effective treatment method [1]. Therefore, it has important clinical significance to explore the molecular mechanism of liver cancer development, invasion and metastasis, and search effective drug treatment.

Tumor necrosis factor related apoptosis inducing ligand (TRAIL) is a member of the tumor necrosis factor family. It attracts much attention because of its selective cell death role on cancer cells such as liver cancer [2, 3]. TRAIL highly expressed in spleen, lymph nodes, small intestine, prostate, and placenta, and also conditional expressed in immune cells such as natural killer cells, monocytes, B cells and dendritic cells [4-6]. TRAIL can bind with death

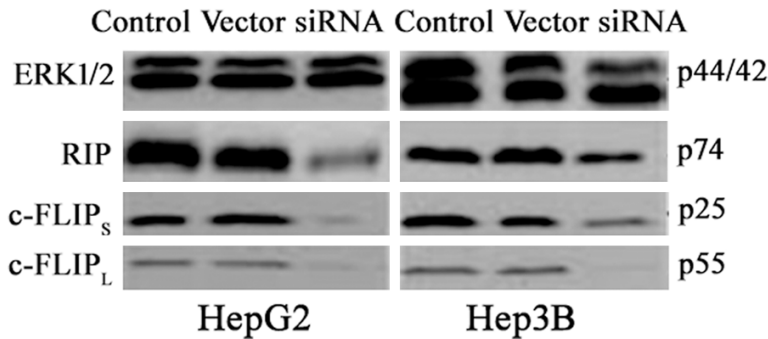
receptor 4 (DR4) and DR5. It can also be recruited to Fas-associated death domain (FADD) and form death inducing signaling complex (DISC) with Caspase-8 precursor [7-9]. Receptor interacting protein (RIP) plays an important role in the tumor necrosis factor signaling pathway, and also is an essential factor in TNF inducing NF- $\kappa$ B activation. RIP can inhibit cell apoptosis and promote proliferation through activating NF- $\kappa$ B, resulting to cancer formation [10, 11]. Cellular caspase 8 (FLICE)-like inhibitory protein (c-FLIP) plays an important role in the process of in regulating death receptor mediated apoptosis and NF- $\kappa$ B pathway activation [12-14]. c-FLIP overexpresses in a variety of cancers such as gastric cancer, liver cancer, colorectal cancer, and prostate cancer. Tumor with higher c-FLIP level presents stronger invasive ability [15]. The most outstanding feature of the c-FLIP is to confront TRAIL induced effect. Cycloheximide, adriamycin, and bortezomib can



**Figure 1.** The impact of TRAIL on apoptosis transduction protein expression in HepG2 and Hep3B.



**Figure 2.** The impact of TRAIL and chemotherapy drugs on c-FLIP and RIP expression.



**Figure 3.** c-FLIP and RIP siRNA transfection efficacy in HepG2 and Hep3B.

suppress c-FLIP expression to overcome TRAIL resistance [16]. It has been pointed out that the liver cancer resistance is mainly caused by its resistance to TRAIL induced apoptosis [17],

though its mechanism is still unclear. We hypothesized that the c-FLIP and RIP may be closely related to liver cancer cells resistance to TRAIL. Therefore, this study intends to investigate the mechanism of RIP and c-FLIP in liver cancer cells resistant to TRAIL induced apoptosis, thus developing a new strategy for liver cancer treatment.

**Materials and methods**

*Main reagents*

HepG2 cell line was bought from Shanghai Chuanxiang biotechnology co., LTD (Shanghai, China). Hep3B cell line was got from Shanghai Bioleaf biochemical co., LTD (Shanghai, China). TRAIL was purchased from PeporTehc (UK). Caspase-8 detection kit was got from Wuhan boster co., LTD (Wuhan, China). c-FLIP and RIP primary antibodies were bought from Alexis. Caspase-3 primary antibody was from StressGen. DR4, DR5, FADD, Caspas-9, ERK-1/2, and DFF45 primary antibodies were bought from Cell Signaling. HRP labeled secondary antibody was from Southern Biotech.

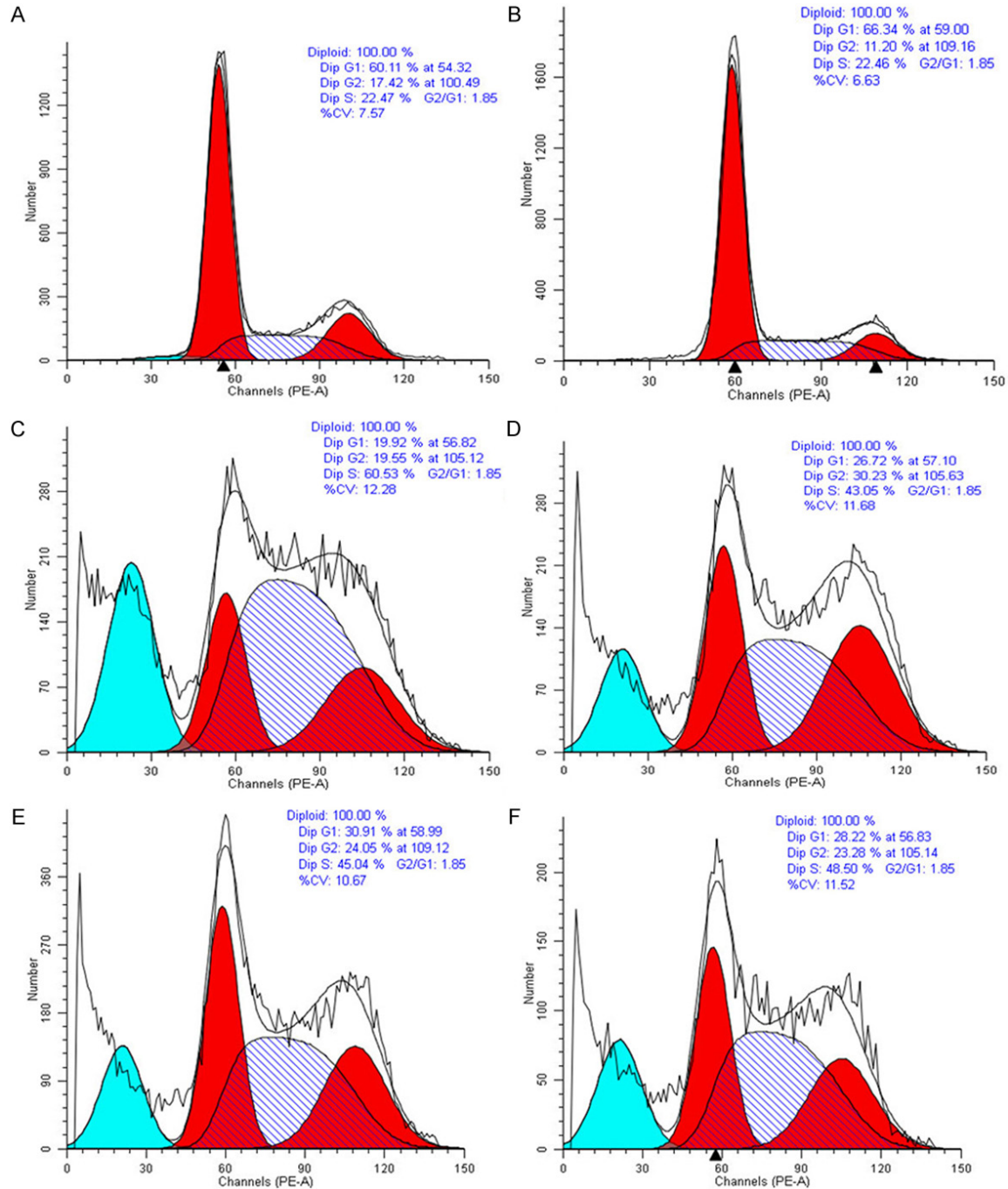
*Cell culture*

HepG2 and Hep3B cells were maintained in RPMI-1640 medium supplemented with 100 ml/L fetal calf serum, 100 kU/L penicillin and 100 mg/L streptomycin in a humid atmosphere containing 5% CO<sub>2</sub> at 37°C.

*c-FLIP and RIP siRNA*

c-FLIP and RIP siRNA were designed according to the genetic sequence in the GenBank and synthesized by QIAGEN. The siRNA sequences were as follows: c-FLIP siRNA: forward primer

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**Figure 4.** c-FLIP and RIP impact on HepG2 and Hep3B cell apoptosis rate. A. HepG2 cell line; B. Hep3B cell line; C. HepG2 transfected with c-FLIP siRNA; D. Hep3B transfected with c-FLIP siRNA; E. HepG2 transfected with RIP siRNA; F. Hep3B transfected with RIP siRNA.

5'-UCGGGGACUUGGCUGAACUUU-3', reverse primer 5'-AGUUCAGCCAAGUCCCCGACA-3'; RIP siRNA: forward primer 5'-CCACUAGUCUGACGG-AUAAUU-3', reverse primer 5'-UUAUCCGUCAG-ACUAUGGUA-3'. To obtain stable siRNA transfection effect, recombinant plasmids pRNAT-U6/Neo/siRNA-c-FLIP and pRNAT-U6/Neo/siR-

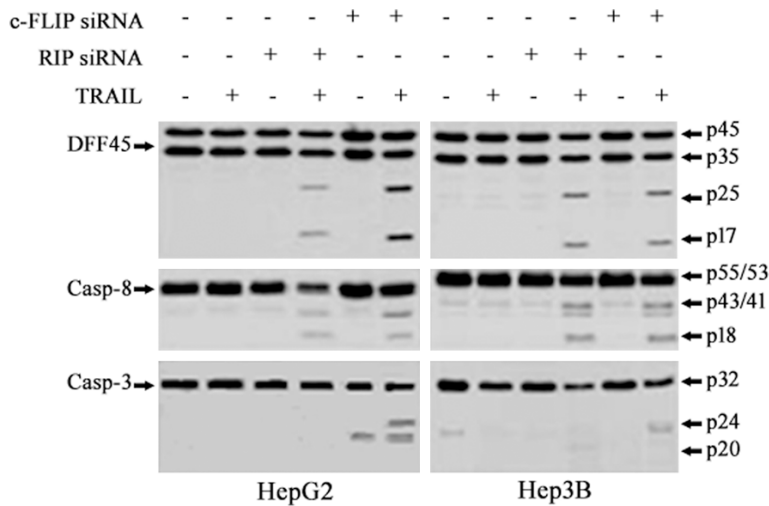
NA-RIP were constructed. Lipofectamine 2000 was applied to transfect pRNAT-U6/Neo, pRNAT-U6/Neo/siRNA-c-FLIP, and pRNAT-U6/Neo/siRNA-RIP to HepG2 and Hep3B cell lines, respectively. After Transfected for 48 h, Western blot was used to confirm transfection effect.

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**Table 1.** c-FLIP and RIP impact on HepG2 and Hep3B cell cycle (n=3,  $\bar{x} \pm s$ ,  $\eta/\%$ )

TRAIL (ng/mL)	Cell cycle					
	HepG2			Hep3B		
	Control 1	c-FLIP siRNA	RIP siRNA	Control 2	c-FLIP siRNA	RIP siRNA
0.00	0.53 ± 0.03	3.27 ± 0.42	3.01 ± 0.21	1.49 ± 0.09	3.65 ± 0.47	3.64 ± 0.29
10.00	0.66 ± 0.04	4.53 ± 0.53	4.01 ± 0.42	2.76 ± 0.18	6.82 ± 0.67	7.04 ± 0.52
30.00	0.91 ± 0.05	12.09 ± 1.89	13.02 ± 1.08	4.41 ± 0.33	12.97 ± 1.57	13.04 ± 2.04
100.00	0.79 ± 0.04	69.31 ± 4.21*	71.98 ± 3.65*	3.78 ± 0.31	78.91 ± 4.97*	77.08 ± 4.65*
300.00	1.87 ± 0.20	87.92 ± 5.09*	90.23 ± 6.01*	7.64 ± 0.59	88.04 ± 5.93*	91.37 ± 5.06*
1000.00	2.93 ± 0.28	90.01 ± 8.75*	95.41 ± 7.01*	9.18 ± 0.81	91.00 ± 5.31*	95.98 ± 6.90*

\*P < 0.01, compared with control.



**Figure 5.** c-FLIP and RIP impact on cell apoptosis transduction protein.

### Cell viability

Acid phosphatase assay (AP) was applied to determine the cell viability.  $1.0 \times 10^4$ /mL HepG2 and Hep3B cells were seeded in 96-well plate. After cultivating for 24 h, the cells were treated with 0, 10, 30, 100, 300, and 1000 ng/mL TRAIL with eight replicates. The cell supernatant was removed after 24 h and 100  $\mu$ L AP detection fluid was added for 2 h. 10  $\mu$ L of 1 mol/L NaOH was added to terminate the reaction. The plate was read at 405 nm wavelength on microplate reader. Cell survival rate = mean value in test group/mean value in control  $\times$  100%. Three independent experiments were performed to get the mean value.

### Cell apoptosis rate

Flow cytometry was used to determine cell apoptosis rate. After treated by 100 ng/mL TRAIL for 24 h, the cells were fixed with 70%

ethanol cell overnight. 0.5 mL cell suspension was incubated with 1 mL PI at room temperature away from light for 30 min. The cells were detected by flow cytometry and the results were analyzed by Cell-Quest software.

### Western blot

48 hours after transfection of pRNAT-U6/Neo/siRNA-RIP or pRNAT-U6/Neo/siRNA-c-FLIP, the cells were harvested and homogenized with lysis buffer. Total protein was separated by denaturing 10% SDS-polyacrylamide gel electrophoresis. Detection was performed with chemiluminescence and analyzed by ImageJ.

### Caspase-8 activity detection

Caspase-8 activity was tested by kit (Boster). The cells was fixed by paraformaldehyde for 15 min, and then dewaxed and hydrated for 30 min, digested by proteinase K for 30 min, and acted with preliminary hybrid liquid for 2-4 h. The cells were further hybridized with digoxin labeled DNA probe fluid at 42°C for 36 h, and then incubated with anti-digoxin antibody at 37°C for 1 h. Finally, DAB was added at 37°C for 3 h. Typical section with hybrid liquid containing no probe was treated as the negative control.

### Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., USA). The data

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were presented as means and standard deviation ( $\pm$  S). Differences between multiple groups were analyzed using one-ANOVA or Duncan's multiple range test.  $P < 0.05$  was considered as significant difference.

### Results

#### *The impact of TRAIL on apoptosis transduction protein expression in HepG2 and Hep3B*

After treated with 100 ng/mL TRAIL for 0, 0.5, 1, 3, and 6 h, Caspase-3/8/9, ERK1/2, and DFF45 protein expression levels were detected. Western blot results showed that TRAIL had no significant effect on Caspase-3/8/9, ERK1/2, or DFF45 protein expression in different time (**Figure 1**).

#### *The impact of TRAIL on c-FLIP and RIP expression in HepG2 and Hep3B*

After HepG2 and Hep3B cells were treated by TRAIL, mitomycin, or doxorubicin, DR4, DR5, ERK1/2, FADD, c-FLIP, and RIP protein expressions were measured. **Figure 2** showed that there was no significant difference of ERK1/2, DR4 and DR5 protein expression between mitomycin and adriamycin treatment, and also in TRAIL treatment group. After chemotherapy drugs treatment, c-FLIP and RIP significantly decreased, while FADD increased. Compared with the chemotherapy drug group, c-FLIP and RIP expression obviously up-regulated in TRAIL group, and FADD expression declined.

#### *Stable c-FLIP and RIP siRNA HepG2 and Hep3B cells construction*

After transfected by pRNAT-U6/Neo/siRNA-c-FLIP and pRNAT-U6/Neo/siRNA-RIP recombinant plasmid for 48 h, Western blot was used to detect c-FLIP and RIP protein expression in HepG2 and Hep3B. **Figure 3** presented that c-FLIP and RIP protein expression significantly reduced after siRNA.

#### *c-FLIP and RIP's role in HepG2 and Hep3B cells apoptosis induced by TRAIL*

Flow cytometry was performed to analyze HepG2 and Hep3B cells apoptosis rate were analyzed. Compared with TRAIL group, cells apoptosis rate increased significantly when the c-FLIP and RIP gene knockout cells treated by TRAIL (**Figure 4**). Cell cycle analysis showed

that compared with TRAIL group, sub G1 percentage increased obviously and G1 phase growth retardation when the c-FLIP and RIP gene knockout cells treated by TRAIL. It further suggested that c-FLIP and RIP play an important role in HepG2 and Hep3B cells resistant to apoptosis induced by TRAIL (**Table 1**).

#### *The mechanism of c-FLIP and RIP mediate TRAIL induced liver cancer cell apoptosis*

HepG2 and Hep3B c-FLIP and RIP siRNA cell model was treated with 100 ng/mL TRAIL. After treated with TRAIL for 3 h, Caspase-8, Caspase-3 and DFF45 expression were detected. **Figure 5** showed that compared with other groups, the cell after the TRAIL processing group, Caspase-8 obviously degraded and new band appeared in the c-FLIP and RIP knockout cells treated by TRAIL. Caspase-3 activation form P20 and P24 appeared, eventually leading to DFF45 DNA fragmentation for P17 and P25. Above results indicated that the c-FLIP and RIP may involve in the cell resistant to apoptosis induced by TRAIL through inhibiting Caspase-8 activation.

### Discussion

Liver cancer progress is complex, and correct treatment selection is particularly important. TRAIL, a new member of the tumor necrosis factor superfamily, can induce tumor cell apoptosis [18]. TRAIL has strong antitumor effect and low toxicity that could be a new anticancer choice. At present, it has entered phase II clinical trial in the United States [19]. Studies had confirmed that liver cancer cell lines present different degrees of resistance to the TRAIL induced apoptosis in vitro. RIP and c-FLIP high expression were speculated to be the key factor this process. This research indicated that RIP and c-FLIP play an important function in TRAIL induced apoptosis through the RIP and c-FLIP gene silencing.

Apoptosis is also called programmed cell death that can keep clearing the cells that cannot be repaired, mutate, aging, or infected by pathogens. It maintains cell development and homeostasis. Cytokines, virus, and antitumor drugs can induce cell apoptosis [20, 21]. TRAIL induced cell apoptosis through death receptor pathway is an important mechanism of natural antitumor. TRAIL and TRAIL receptors combina-

tion could cause FADD effect domain bind with pro-Caspase-8 to form DISC [22]. Pro-Caspase-8 was activated in DISC and activated Caspase-9, further activated Caspase-3 and DFF45, promoting apoptosis occurrence. In this process, some molecules such as c-FLIP, RIP and TRADD were recruited to DISC to participate in regulating apoptosis pathway [23, 24]. Our results indicated that TRAIL had no significant effect on Caspase-3/8/9 and DFF45 expression in HepG2 and Hep3B, suggesting apoptosis signal was inhibited. Thus, liver cancer cell resistant to TRAIL mainly depends on inhibiting Caspase-8 activation in DISC.

c-FLIP is a key anti-apoptosis protein similar to Caspase-8 [25]. Studies confirmed that c-FLIP combining with FADD can inhibit Caspase-8 activation and its cascade reaction [26, 27]. Therefore, c-FLIP overexpression can suppress cancer cell apoptosis. Stanger *et al.* found that RIP plays an important role in the cellular stress response by yeast two-hybrid experiment [28]. Its high expression can degrade I $\kappa$ B $\alpha$  to activate NF- $\kappa$ B signaling pathway. However, whether such effect is related to Caspase-8 is still lack of in-depth study [29, 30]. Recent studies found that c-FLIP and RIP expression level in cancer cells are closely related to Caspase-8 activation. c-FLIP or RIP overexpression resulted in Caspase-8 activation inhibition and apoptosis signal conduction blockade [29, 30]. This study also found that the c-FLIP and RIP overexpressed in liver cancer cells, while chemotherapy drugs and TRAIL both can significantly reduce their expression in DISC. Moreover, c-FLIP and RIP overexpressed in the early stage of liver cancer cell apoptosis induced by TRAIL. After silencing c-FLIP and RIP in HepG2 and Hep3B cells, TRAIL obviously activated Caspase-3 and Caspase-8, leading to DFF45 DNA fragmentation. It confirmed that c-FLIP and RIP participate in the hepatocellular carcinoma cell line HepG2 and Hep3B resistance to TRAIL induced apoptosis.

To sum up, c-FLIP and RIP overexpression in the DISC is the main cause of liver cancer cell line HepG2 and Hep3B resistance to TRAIL. Downregulating c-FLIP and RIP in liver cancer cell removed Caspase-8 inhibition and induced apoptosis signal conduction.

### Disclosure of conflict of interest

None.

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