

Adriamycin increased apoptosis sensitivity study of TRAIL gene on human hepatocellular carcinoma SMMC-7721 cells

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Abstract: To explore apoptosis sensitivity effect of the different concentration of adriamycin (ADM) combined with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene on human hepatocellular carcinoma (HCC) SMMC-7721 cells, and further explore the mechanism of apoptosis of SMMC-7721 cells. TRAIL lentivirus infected SMMC-7721 cells, and the efficiency of infection was observed by fluorescence microscopy. Expression of TRAIL gene was detected by qRT-PCR. The effect of different concentration of ADM combined with TRAIL gene on the proliferation of SMMC-7721 cells was detected by CCK8. The apoptosis of cell was detected by Hoechst 33258 and Western blot. The results of qRT-PCR showed the expression of SMMC-7721-TRAIL increased significantly ($P < 0.001$), and the expression of TRAIL gene in the TRAIL-NC was basically no difference ($P > 0.05$). The results of CCK8 showed that inhibition rates of cell treated with ADM combined with TRAIL gene for 24h and 48h, compared with the control group, $F=24.16$, $p=0.01$ and $F=89.43$, $P < 0.001$. Hoechst 33258 showed that ADM combined with TRAIL gene could cause chromatin condensation and nuclear fragmentation. Further studies show that the expression of DR4 and DR5 protein in cells detected by western blot was up-expression, compared with the control group, $F=43.72$, $P=0.0144$ and $F=12.12$, $P=0.0024$. ADM could increase the sensitivity of TRAIL gene and promote the apoptosis of SMMC-7721 cells, which indicates that chemotherapy drugs have broad application prospects in tumor gene therapy.

Keywords: Hepatocellular carcinoma; TRAIL; Adriamycin; Apoptosis

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1. Introduction

At present, hepatocellular carcinoma (HCC) is one of the ten most malignant tumors in the world[1]. The overall incidence of HCC is high, and the application effect of chemotherapy is poor, leading to HCC in the forefront of various human diseases of death[2]. Nowadays, the treatment of HCC is mainly based on surgical resection, but the mortality and recurrence rate are high, and the possibility of resection is less.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family (Regulation of TRAIL-receptor expression by the ubiquitin-proteasome system). Meanwhile, the apoptosis of tumor cells induced by it happens in a multi-level and multi way[3,4]. The study found that TRAIL gene can specifically induce apoptosis of tumor cells and no effect on normal cell[5-7]. This has given rise to new ideas for the treatment of tumor. As a new treatment modality, TRAIL gene has been put forward in the study of in vitro. It can express in many tissues and organs, and combine with TRAIL gene specific death receptor. Finally, it induced apoptosis of tumor cell[8,9]. We can improve the patient's quality of life as a new treatment for HCC according to this biological characteristic. In the previous study, we have successfully constructed the recombinant lentiviral expression vector of TRAIL gene PCDH-CMV-TRAIL-EF1-GFP-T2A-Puro and the recombinant lentiviral TRAIL gene[10]. The above research results of theory and based on the previous, we applied different concentrations of ADM and

TRAIL gene to SMMC-7721 cells cultured in vitro and investigated the influence on apoptosis sensitivity, meanwhile, the basis for the development of a new method of treating HCC.

2. Materials and Methods

2.1. Materials

Sijiqing fetal bovine serum purchased from Tianhang Biological Technology Co. Ltd. (Zhejiang, China). 0.25% pancreatin and DMEM were purchased from Hyclone (Logan, USA). Lipofectamine2000 Kit purchased from Invitrogen (Carlsbad, CA, USA). Doxorubicin hydrochloride (ADM) was purchased from sigma-Aldrich (St. Louis, MO, USA). Cell Counting Kit8 (CCK-8) apoptosis detection kit was purchased from Beyotime Biotechnology (Shanghai, China). Hoechst 33258 staining kit, RIPA Lysis Buffer and BCA protein quantitative Kit was purchased from Beyotime Biotechnology (Shanghai, China). Trizol, PrimeScript™ RT Reagent Kit (RR037A) and SYBR® Premix Ex Taq™ II (RR820A) was purchased Takara (Otsu, Japan). Antibodies against GAPDH (ab8245), DR4 (ab8414), DR5 (ab8416), Goat Anti-Rabbit IgG, H & L HRP (ab6721) and Goat Anti-Mouse IgG, H & L HRP (ab6789) were purchased Abcam (Cambridge, MA, USA).

2.2. Methods

2.2.1. Cell lines and cell culture

The HCC cell line SMMC-7721 and human

embryonic kidney cell line 293T were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS at 37 °C with 5% CO₂ in a humidified incubator.

2.2.3. Infected cells with TRAIL lentivirus

In a previous study, we have successfully constructed the lentiviral expression vector pCDH-CMV-TRAIL-EF1-GFP-T2A-Puro. Then, we will plasmid after positive identification transfected into 293T cells by Lipofectamine 2000. After 48 hours, we obtained the TRAIL lentivirus and determined that infection rate was higher when the virus titer MOI=20. Cell was inoculated into 48-well plates, divided into control, negative control lentivirus LV-control-GFP-Puro (SMMC-7721-NC) and TRAIL lentivirus LV-TRAIL-GFP-Puro (SMMC-7721-TRAIL). After the cell wall adhered, the same amount of empty lentivirus and TRAIL lentivirus (MOI=20) were added to SMMC-7721-NC and SMMC-7721-TRAIL. The control group didn't do the treatment, and put them into the incubator to culture 24h and 48h. We used fluorescence microscopy to observe the number of cells expressing green fluorescent protein (GFP), and then according to the expression efficiency of GFP judgment TRAIL lentivirus infected SMMC-7721 cells.

2.2.4. qRT-PCR assay

TRAIL lentivirus infected cells after 48h, and we collected cells from control, SMMC-7721-NC and SMMC-7721-TRAIL. Total cellular RNA was extracted with a reagent trizol according to the manufacturer's protocol. cDNA was synthesized using a PrimeScript™ RT Reagent Kit. Then, qRT-PCR was performed using the SYBR green system on FTC-3000 PCR instrument to determine the Mrna expression of targeted genes. The sequences of primer were as follows: GAPDH forward, 5'-TCATGGGTGTGAACCATGAGAA-3', and reverse, 5'-GGCATGGACTGTGGTCATGAG; TRAIL forward, 5'-CTTGACCTGACCCCGAGATA-3', and reverse, 5'-CCCACAGAGAAAGGAAGCAG. All the information collected by the qRT-PCR instrument and the 2^{-ΔΔct} method for the analysis of the data.

2.2.5. Cell proliferation assay

Cell viability was determined by the CCK8 assay. After cell overnight, each group was infected with TRAIL lentivirus for 48h, then added different concentrations of ADM (0umol/L, 0.1umol/L, 1umol/L, 10umol/L, 100umol/L) and control treatment with 24h and 48h. Finally, 10ul of CCK8 reagent were added to each well and incubated at 37°C for 2h. The OD value of each hole in 450nm was detected by microplate

reader (Thermo Fisher Scientific Oy, USA).

2.2.6. Hoechst 33258 assay for apoptosis

After TRAIL lentivirus infected cell for 48h, cell were treated with different concentrations of ADM (1umol/L, 10umol/L, 100umol/L) and control treatment with 24h. Then, apoptosis was detected by Apoptosis-Hoechst 33258 Staining Kit, according to the manufacture's instruction. Cell were first fixed with methanol and then stained with Hoechst 33258. Last, the stained cells were visualized and photographed under a fluorescence microscope (Olympus IX50, Tokyo, Japan) at visual field at ×200.

2.2.7. Western blot assay

After TRAIL lentivirus infected cell for 48h, cell were treated with different concentrations of ADM (1umol/L, 10umol/L, 100umol/L) and control treatment with 48h. Total protein was extracted from cells using RIPA lysis buffer then, concentration was determined by BCA protein quantitative Kit. Equal amounts of protein samples was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto 0.45um PVDF membrane. At room temperature, the membranes were blocked in 5% evaporated milk for 60min. The membranes were placed in the antibody with different appropriate dilution overnight at 4°C. The next day, the membrane was incubated for 60 min of second antibody. The bands were detected using an enhanced chemiluminescence reagent and Vilber Fusion FX7 Sestem (Vilber Lourmat, France). Image software was use to calculate the gray value of each protein band to obtain the protein expression. GAPDH was used as a control.

2.3. Statistical analysis

Statistical analysis used SPSS18.0 software. Data are presented as means ± SD for three independent experiments. In the experiment, Student's t-test were used to determine the differences between two groups; comparisons between groups were one-way analysis of variance (ANOVA) and the level of P<0.05 was considered statistically significant.

3. Results

3.1. The efficiency of cell infection

We observed the efficiency of the cells by fluorescence microscopy. The results show that after the trail lentivirus infected cells, we observed more and more green fluorescent particles for 24h and 48h in fluorescence microscopy (Figure1A). Then, the expression of TRAIL in mRNA was detected by qRT-PCR. We found that the expression of SMMC-7721-TRAIL increased significantly, compared with the control group (P<0.001). Compared

with the control group, the expression of TRAIL gene in the TRAIL-NC was basically no difference (P>0.05, Figure1B).

Table 1. Inhibition of SMMC-7721 cells proliferation by various concentrations of ADM combined with TRAIL ($\bar{x} \pm s, n=3$)

Group	ADM (umol/L)	Inhibition rate (100%)	
		24h	48h
Control	-	-	-
TRAIL	-	0.110±0.017	0.150±0.030
TRAIL+ADM	0.1	0.130±0.050	0.164±0.046
	1	0.173±0.068	0.241±0.040*
	10	0.293±0.069*	0.418±0.067**
	100	0.461±0.085**	0.738±0.057***

Note: *P < 0.05, **P < 0.01, ***P < 0.001, compared with the TRAIL group.

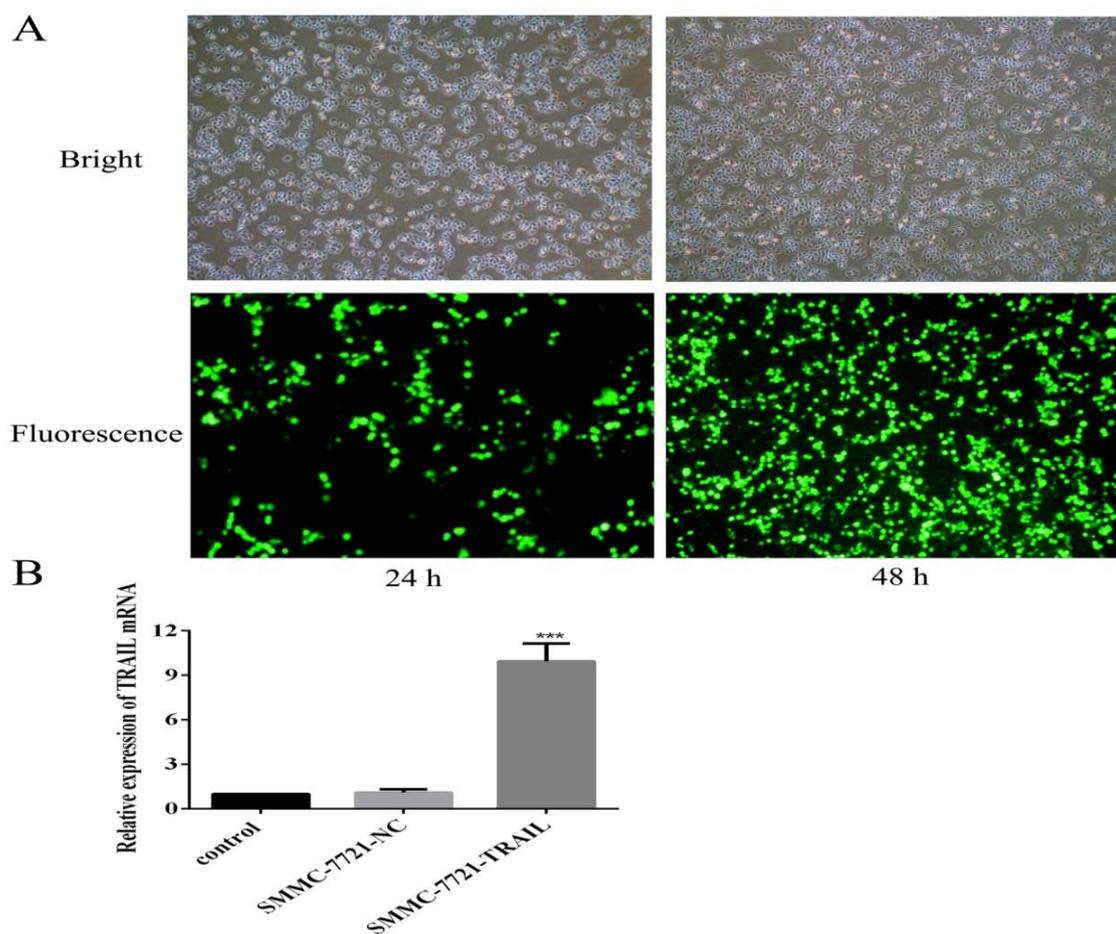


Figure 1. (A) GFP expression of TRAIL lentivirus infected SMMC-7721 cells under fluorescence microscopy. (B) After lentivirus infection, the relative expression of the TRAIL was compared with control group,*P < 0.001.**

3.2. Inhibitor of cell proliferation by ADM combined with TRAIL

ADM combined with TRAIL was assessed by a cell proliferation assay using the HCC SMMC-7721. The proliferation of SMMC-7721 cells in the presence of

various concentration of ADM combined with TRAIL was examined. Under the experimental conditions used (24h and 48h), ADM combined with TRAIL exhibited a marked inhibitory proliferation of SMMC-7721 cells (Table1). The result show that SMMC-7721 cells treated by ADM combined with TRAIL were reduced proliferation capacity in a dose and time dependent manner. Inhibition rates of cell treated with ADM combined with TRAIL gene for 24h and 48h, compared with the control group, $F=24.16$, $P=0.01$ and $F=89.43$, $P<0.001$.

3.3. Promotion of cell apoptosis by ADM combined with TRAIL

We assessed whether ADM combined with TRAIL causes apoptotic cell death in SMMC-7721 by Hoechst 33258 assay. As shown in Figure2A, significant morphological change associated with apoptosis, formation of condensed and fragmented nuclei, were induced by ADM combined with TRAIL. In order to further clarify the cause of apoptosis, we detected the expression of death receptor DR4 and DR5 protein by western blot. The results show that DR4 and DR5 protein were up-regulated (Figure 2B), compared with the control group, $F=43.72$, $P=0.0144$ and $F=12.12$, $P=0.0024$.

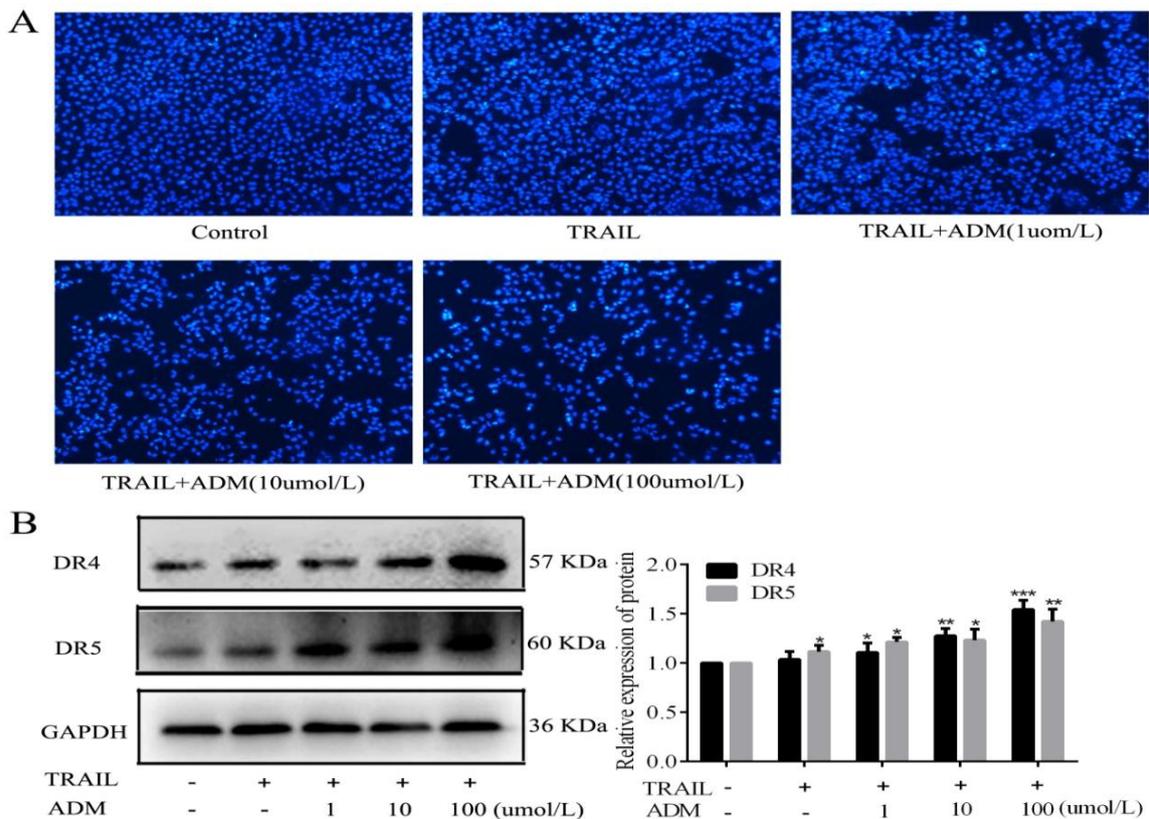


Figure 2. (A) The morphological changes of SMMC-7721 cells were induced in each group. The bright point indicates the concentration of chromatin in the cell and the fragmentation of the nucleus ($\times 200$). (B) The protein expression of DR4 and DR5 in SMMC-7721 cells, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

4. Discussion

TRAIL gene exists widely in human tissue, and the expression of this gene can induce the apoptosis of cell. It can be expressed over the surface to induce apoptosis of tissue cells in monocytes, lymphocytes, neutrophils and other immune cell[11,12]. Over expression of the TRAIL gene has no effect on the normal cell cycle, but could induce apoptosis of tumor cell[13]. Up-regulation of TRAIL can significantly inhibit tumor cell proliferation and promote apoptosis in many tumor cells[14,15].

In this study, Hoechst 33258 staining experiments showed that ADM combined with TRAIL could cause cell morphological changes, cell chromatin condensation and nuclear fragmentation. Death receptor DR4 and DR5 are the early discovered TRAIL receptors. It combined with death receptor may initiate the transmission of the death signaling pathway, leading to apoptosis of tumor cells[16]. To further investigate the cause of apoptosis, we detected the expression of DR4 and DR5 at the protein level by Western blot. We found that up-regulate the expression of DR4 and DR5 by ADM combined with TRAIL, and

promoted apoptosis of the cells. TRAIL were involved in mitochondrial apoptosis pathway in death receptor, and promoted cell apoptosis[17]. Büneker[18] the study found that different ratios of TRAIL receptors can produce different results for cell apoptosis. We found that the ADM can increase the sensitivity of HCC SMMC-7721 to TRAIL and increase the expression of DR4 and DR5 in the tumor cell membrane. This may be due to the activation of mitochondrial pathways by chemotherapeutic agents, then, increasing the TRAIL mediated death receptor pathway, and inducing apoptosis.

5. Conclusion

In summary, our results indicate that ADM combined with TRAIL were promoted the apoptosis of HCC SMMC-7721, and the mechanism may be achieved by up-regulated the expression of DR4 and DR5. Therefore, our will further explore the killings effects of other chemotherapeutic drugs combined with TRAIL on tumor cells, so to achieve the maximum benefit of tumor treatment.

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