Construction of Lentiviral Vector Carrying TRAIL Gene and Its Expression in Hepatocarcinoma Cells*

LUAN Kun-ye[1], YU Jian[1], YAO Ru-yong[2], ZHANG Bing-yuan[1], SUN Chuan-dong[1△]

(1 Department of general surgery, Affiliated Hospital of Qingdao University, Qingdao 266003;

2 Center laboratory, Affiliated Hospital of Qingdao University, Qingdao 266003)

ABSTRACT Objective: To construct lentiviral expression vector carrying TRAIL gene and realize its stable high expression in hepatocellular carcinoma cell line HepG2. Methods: PCDH-CMV-TRAIL-EF1-GFP-T2A-Puro recombinant lentiviral vector was constructed. The 293T cells were contransfected with the recombinant lentiviral vector and lentivirus package plasmid to produce lentiviral particles by lipofectamine method. The lentivirus was purified and virus titer was measured. Western blot was used to detect the expressions of TRAIL protein. Results: The recombinant lentiviral vector carrying TRAIL was confirmed by restriction end nuclease analysis and DNA sequencing. Virus titer reached to 1.02× 10^4 ifμ/μL. After puromycin selection, the stable high expressions of TRAIL protein were confirmed by Western blotting. Conclusion: Lentiviral vector carrying TRAIL gene has been successfully constructed realizes its stable high expression in HepG2 cells.

Key words: TRAIL; Lentiviral vector; HepG2

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Introduction

HCC (Hepatocellular carcinoma, HCC) is a global human malignant tumor, with high morbidity and high mortality characteristics of a serious threat to human health [1]. However, duing to the sophisticated formation, there is no ideal treatment for liver cancer. TRAIL that tumor necrosis factor-related apoptosis-inducing ligand (TNF-related apoptosis inducing ligand, TRAIL), also known as apoptosis-2 ligand (Apo2 ligand, Apo2L), first by Wiley from the human heart cDNA library cloned [2]. In recent years, TRAIL biological function of the role in the treatment of liver cancer continue to be interpreted, in vivo studies have confirmed, TRAIL can selectively induce apoptosis in various tumor cells, but had little effect on normal cells [3]. However, not all cell lines were sensitive to TRAIL, such as A875, FADU, J82 and SKOV3. They showed resistance to TRAIL, and HCT116, H460, PA1, SKBr3, etc. were sensitive to TRAIL [4]; Therefore, it is very important to find out the mechanism of resistance. This study was to insert TRAIL coding sequence into downstream of the CMV promoter of the expression vector, construct TRAIL lentiviral expression vector, and other packaging plasmid vector in the aid package into lentiviral particles, HepG2 cells infected in vitro, obtained stable TRAIL expression after puromycin screen.

1 Materials and methods

1.1 Materials

Human embryonic kidney 293T, HepG2 cells were purchased from the Shanghai Institute of Life Sciences, Cell Resource Center. Animal experiments with BalB/C nude mice were purchased from the Military Academy of Medical Sciences. Lentiviral packaging system (System Biosciences, Cat # CD513B-1), PrimeSTAR high-fidelity DNA polymerase, T4 DNA ligate, restriction enzymes, DNA marker, DNA gel extraction kit were all purchased from Takara Bio (TaKaRa) Engineering Co., Ltd.; Plasmid extraction kit were purchased from Qiagen Company; DMEM culture base, FBS (fetal bovine serum, FBS) were purchased from Gbico company; TR Izo l Reagent, liposomes Lipofectamine TM 2000 were purchase from Invitrogen Corporation; E. coli DH5α cells by the laboratory; RIPA lysis buffer were purchased from Pik-day company; ECL chemiluminescence kit were purchased from Millipore Corporation; TRAIL monoclonal antibody and HRP-labeled anti-rabbit secondary antibody were purchased from Abcam company; GAPDH monoclonal antibody and HRP-labeled goat anti-mouse secondary antibody were purchased from Cell Signaling Technology, Inc.; other reagents were domestic analytical production.

1.2 Methods

1.2.1 TRAIL recombinant expression vector According to GeneBank accession number U37518, primers: (upstream) 5'-CTAGCTAGCGTGAGAGAAAGAGGTCCTCAGA-3 ' (NheI restriction site is underlined points); (downstream) 5'-CGGAATTCTTAGCCAACTAAAAAGGCCCCGA-3' (EcoRI restriction site is underlined points). According to TRAIL cDNA that the laboratory had received as the template for PCR amplifica-

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Author introduction: Luan Kun-ye, (1984), male, master, Mainly engaged in therapy of hepatocellular carcinoma;

E-mail :luankunye-qd@163.com

△Corresponding author: SUN Chuan-dong, E-mail :sunchuandong@hotmail. com.

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tion. Cycle parameters for the 98 ℃ 10 s, 58 ℃ 15 s, 72 ℃ 45 s, 38 cycles. The recovered PCR product and pCDH-CMV-MCS-EF1-GFP-T2A-Puro vector used NheI and EcoRI to double digestion respectively, digestion products connected 2 h at 22 ℃. Connected the products into DH5α competent cells, identified by PCR, plasmid clones, double digested and sequenced. Identification of the correct plasmid was named pCDH-CMV-TRAIL-EF1-GFP-T2A-Puro.

1.2.2 Packaging and production of lentiviral particles The logarithmic phase of 293T cells were digested with pancreatic digestive enzymes, and plated at 1.5× 10^6 cells per dish, used fresh DMEM medium containing 10% FBS medium, cultured under the conditions of 37 ℃ and 5% CO_2. Cells were seeded after 24 h, the adherent cells' density was 80% by microscopy, and they were spindle, central-bright, full form, suitable for transfection experiments. Used five 10 cm dishes, each dish used the lentiviral packaging plasmid mixture (500 ng/μL) 22 μL and TRAIL recombinant plasmid pCDH-CMV-TRAIL-EF1-GFP-T2A-Puro (500 ng/μL) 4 μL co-transfected 293T cells, medium was changed before the cells transfected by using fresh serum-free DMEM medium; transfection reagent dose was 50 μL per dish.4 h after transfection, cells liquid was changed by used freshly DMEM culture medium containing 10% FBS. 48 h after the transfection, yellow cells liquid can be seen, density of adherent cells were 90%, flat and adherent state better. Collected Supernatant, centrifuged it at 2,000 × g for 5 min, removed cell debris sedimentation, used PVDF membrane filter supernatant, and then placed in 40 ml ultracentrifugation tube, centrifuged it at 25,000 r / min for 120 min at 4 ℃; Resuspended virus precipitation with ice-cold sterile PBS, dissolved overnight at 4 ℃ , then obtained virus LV-TRAIL-GFP-Puro. Negative control viruses LV-control-GFP-Puro were obtained in kind. Next day, packed 10 μL virus solution per tube, and placed in -70 ℃ refrigerator.

1.2.3 Determination of virus titer To proceed passage of 293T cells before titer detected, seeded into 96-well plates 1.5 × 10^3 per well cells, the cell suspension volume was 100 μL; 24 h later, each tube was added 90 μL fresh medium to 10 sterile centrifuge tubes (without antibiotics, added 10% FBS); 10 μL virus stock solution was added to the first one, after mixed and 10 μL virus stock solution was added. Cells liquid was changed before infection, added fresh medium containing the virus solution, cultivated under the conditions of 37 ℃ and 5% CO_2. After 24 h, 100 μL fresh medium (without antibiotics, added 10% FBS) was added to change the infected cells liquid. 72 h later, it was clear that the number of fluorescent cells increased with the dilution factor decreased under the microscope. The total number of cells and the number of cells expressing fluorescent were counted with the microscope, the virus titer were obtained by dividing the appropriate dilution.

1.2.4 Transfected HepG2 cells with lentivirus Logarithmic growth phase HepG2 cells were used to prepare cell suspensions by trypsin digestion, cells were inoculated into 6-well plates, 1 × 10^5 cells per well, divided into control group BLANK, negative control lentivirus LV-control-GFP-Puro (named HepG2-con) and TRAIL lentivirus LV-TRAIL-GFP-Puro (named HepG2-TRAIL), each group with 2-hole cells. After cultured 3 d, according to the virus titer, the appropriate volume of virus stock solution were taken, multiplicity of infection (multiplicity of infection, MOI) of the virus was 5, the virus stock solution and culture medium were mixed and added to the cells, 1.5 mL each well, meanwhile 10 μg/mL final concentration of Polybrene were added and gently shaked the plate. The virus solution were decanted after cultured 24 h at 37 ℃ and 5% CO_2, complete culture solution were renewed and cultured, then the condition of cells expressing fluorescent were observed after 48 h. At the same time puromycin were added to the complete culture medium for screening and liquor were changed every 5 days, about 25 d later, positive clones were selected for monoclonal 2-3 times by limited dilution, the cloning cells were amplified and cultured, part of the cells were frozen in liquid nitrogen, part of the collection were used for Western blotting analysis.

1.2.5 Western blotting analysis TRAIL protein expression after lentiviral infection in HepG2 HepG2 cells were divided into control group, empty virus control group. TRAIL lentiviral infection, infected cells were collected and lysis buffer was added to extract protein. Protein concentration was determined by the BCA, 10% SDS-PAGE gel was prepared , electrophoresis was proceeded at 80 V constant voltage, when the sample into the separation gel, changed to 200 V constant voltage. Then transferred the protein to PVDF membrane at 400 mA and 60 min. The membrane was blocked for 2 h in PBST buffer with 5% skimmed milk at room temperature, washed it with PBST. The working concentration of an anti-TRAIL was 1:1,000, GAPDH antibody working concentration was 1:2,000; 4 ℃ overnight, washed membrane 10 min × 3 times with PBST; Secondary antibody was added, its working concentration was 1:15,000, GAPDH secondary antibody working concentration was 1:10,000，incubated 1 h at room temperature, washed membrane 10 min × 3 times with PBST. Added chemiluminescent substrate, pressed the X-ray film in the chamber, developed and fixed after exposed。

1.3 Statistical Methods

The data was analyzed by spss 16.0 statistical software, measurement data denoted by $\bar{x} \pm s$, the comparison among groups were compared with single factor analysis of variance. $P < 0.05$ indicated significant difference. Mapping software adopted GraphPad Prism5.

2 Results

2.1 PCR amplification of TRAIL

DNA fragments of TRAIL sequence were amplified successfully by PCR, Electrophoresis showed the specific band of about 524 bp (Figure 1A). TRAIL gene connected to pCDH-CMV-MCS-EF1-GFP-T2A-Puro lentiviral shuttle vector, the digestion products were analyzed by electrophoresis, as shown in Figure 1B.

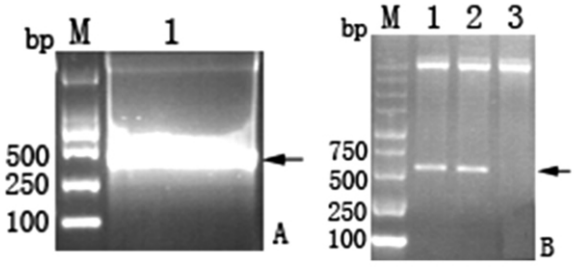

Fig.1 Gel electrophoresis of the pCDH-CMV-TRAIL-EF1-GFP-T2A-Puro vector: A: PCR amplification of TRAIL gene, M: DL2000 marker; 1: Target gene (Arrowhead shows);B: Double digestion of pCDH-CMV-TRAIL-EF1-GFP-T2A-Puro vector by NheI and EcoRI, M: DL5000 marker; 1-2: Double digestion of pCDH-CMV-TRAIL-EF1-GFP-T2A-Puro vector by NheI and EcoRI (Arrowhead shows); 3: Double digestion of pCDH-CMV-MCS-EF1-GFP-T2A-Puro vector by NheI and EcoRI

2.2 Lentiviral packaging and titer determination

The three plasmids were transfected into the 293T package cells by lipofectamine method, the expression of green fluorescence were seen 24 h later, supernatant were collected at 48 h and purified. 293T cells were transfected by lentiviral vector that had been purified after gradient dilution, the GFP expression were observed under an inverted fluorescence microscope (Figure 2). The 293T-positive cells were counted in each hole, the number of fluorescent cells were decreased with the dilution factor increased; 10 μL stock solution were diluted to 10 times, then 102 GFP-positive cells were observed in the hole, the virus titer calculated by the formula: titer = (102/10) × 10^3=1.02× 10^4 ifμ/μL.

2.3 Optimum MOI lentivirus infect HepG2

Different volumes of virus stock solution were added, living cells were analysed and fluorescence count after 72 h of lentivirus infection. The results showed that the minimum liquid volume of the virus making cells reach 100% was 40 μL, so the optimum MOI = 40 μL× 10^4 ifμ/μL÷ [(121+96+113)÷ 3× 10^4× 0.05]≈8 (5.42). The inverted fluorescence microscope showed that HepG2 fluorescence-positive rate was above 90% and cells growth in good condition, when infected HepG2 with stock solution (MOI =8) 72 h later. This result illustrated that the recombinant lentiviral transfected efficient in vitro when MOI =8. In order to achieve the optimum dose-effect ratio, selected MOI =8 for the further experiments.

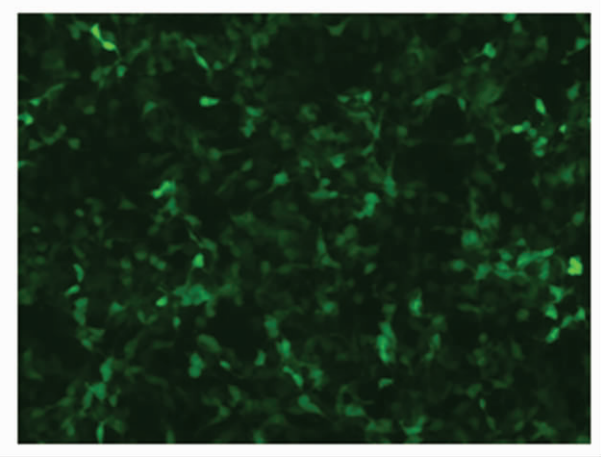

Fig.2 Analysis the expression of GFP in 293T cells under fluorescencemicroscope

2.4 The expression of TRAIL protein in HepG2 cells

Three stable expression of TRAIL-positive cells were obtained by screened by puromycin and monocloned. The expression of TRAIL protein was detected by Western blotting. The results showed that TRAIL protein had high expression in LV-TRAIL-GFP-Puro compared with that in the blank and LV-control-GFP-Puro, and its expression differences were statistically significant ($P < 0.01$); While the differences between the blank and LV-control-GFP-Puro were not statistically significant ($P > 0.05$) (Figure 3).

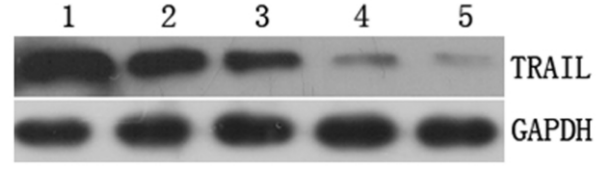

Fig 3 Expressions of TRAIL protein in each group:1-3: LV-TRAIL-GFP-Puro group; 4: LV-control-GFP-Puro group; 5: Normal HepG2 cell

3 Discussions

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, malignant high, life span short, 5-year survival rate is about 3% -5%[5], the third largest cause of death in malignant tumor. Over the past decades the incidence of HCC has observably increased [6]. HCC is clinically characterized by its invasiveness, poor prognosis and limited therapeutic opportunities, mostly due to the high resistance of HCC cells towards chemotherapeutic agents. Today, surgery is considered to be the only curative treatment procedure for most HCC patients [7]. However, in many patients, HCC is diagnosed at an advanced or metastasized stage, which lead to low surgical resection rate, and chemotherapy and biological therapy is not ideal. So search for new methods of clinical treatment is necessary. Now, combine the new treatment

with existing treatments efficiently has been research focus of gene therapy and biological therapy for HCC.

Now, it has been found that many anti-cancer therapies are in relation to induced tumor apoptosis, but a number of apoptosis-inducing substances can cause cytotoxicity or other non-selective side effects, so it's difficult to enter the clinical, such as severe inflammation start by TNF or Fas cause severe liver damage. TRAIL is a new member of TNF family. Studies have shown that TRAIL expressed in a variety of normal human tissues, induced apoptosis of transformant, tumor cells and virus infected cells, while had no significant side effects to normal tissue [8]. Preclinical studies suggest that TRAIL induces apoptosis of tumor cells in vivo without lethal toxicities [9]. Therefore, TRAIL in tumorous therapy has a promising prospect, may provide a new therapeutic path for liver cancer. TRAIL as a promising apoptosis gene induced apoptosis through receptor-mediated [10,11]. Currently, there are five TRAIL receptors that: death receptors DR4, DR5, decoy receptors DcR1, DcR2 and the soluble receptor OPG. The death receptors (DR4, DR5) have similar cytoplasmic death domain with other members of the TNF receptor superfamily, specific binding with TRAIL can stimulate and transmit apoptosis signal, activate Caspase proteolysis cascade reaction, and result in cell death [12]. A major obstacle for the clinical use of TRAIL is its limited efficacy in monotherapeutic approaches in different tumor entities. Researches show that malignant cells often carry mutations in the proteins that control intrinsic and extrinsic apoptotic signaling pathways, resulted in a decreased susceptibility to cell death. For example, the decreasing expression of death receptors (DR4, DR5) make levels of caspase-8 descend, induce resistance to TRAIL[13]. Thereby, it appears worthwhile to enhance TRAIL's susceptibility for apoptosis induction [14,15]. Most recently, it was proposed that the ratio of TRAIL-R1 to TRAIL-R3 and TRAIL-R4 predicted the sensitivity of tumor cells to TRAIL-mediated apoptosis [16]. It was recently shown that pancreatic cancer cells were susceptible to TRAIL gene therapy [17], and TRAIL gene therapy in combination with gemcitabine might be a useful therapeutic approach for treating metastatic pancreatic cancers [18]. The large majority of primary hematologic tumors are resistant to TRAIL-mediated apoptosis, while used chemotherapeutics in combination with TRAIL can enhance TRAIL-R1/-R2 expression, reinforce Recent findings demonstrated that Salmonella-mediated tumor targeted therapy with TRAIL could reduce tumor growth and extend host survival on melanoma mice model [19]. A recent report examining the combined effects of bortezomib and TRAIL on 15 different squamous cell carcinoma lines found that bortezomib also enhanced TRAIL efficacy through increased recruitment of caspase 8 and FADD in to the DISC, and augmentation of the intrinsic apoptotic pathway [20]. So the combination of TRAIL and bortezomib has shown promise as a therapeutic for TRAIL-resistant breast, colon and kidney tumors [21]. It is know that Hep-G2 show a resistance to TRAIL-induced cell death, but chemotherapy with Doxo or 5-FU can increase TRAIL susceptibility in Hep-G2 Cells, then opening the possibility of a treatment regime including reduce doses of chemotherapeutic drugs in combination with TRAIL [22]. All these results demonstrate that TRAIL play an important role in inducing apoptosis of tumor cells, and have high clinical value combination with chemotherapy.

This research constructed lentiviral vector carrying TRAIL, and realized its expression in Hepatocarcinoma cells. The key point was to construct an effective gene delivery system. It was reported that adenovirus vectors in mouse model of hepatitis, virus infection caused TRAIL and DR5 overexpression, resulted in infected liver cells apoptosis [23], and in vitro studies had found that adenovirus transduction mTRAIL easily lead to human normal liver cell apoptosis seriously [24], damaged to liver function. These need a more secure carrier. There aren't researches use lentiviral vector for TRAIL gene therapy at home and abroad. Lentiviral vector is based on the transformation of HIV-l production, compare with the previous vectors such as adenovirus, adeno-associated virus, retrovirus, etc., have the capacity of large exogenous gene fragments, stable expression, immune response small and safety [25, 26], and can infect non-dividing cells or dividing cells, transfect comprehensive organizations and can be condensed into a high-titer, etc. [27, 28], so it's an effective tool for gene delivery. This study successfully construct TRAIL lentiviral expression vector, provides an ideal viral vector for TRAIL gene therapy and settle a foundation for efficient and stable infection.

In this study, TRAIL cDNA was obtained and used as template, which amplified PCR from TRAIL and constructed recombinant plasmid pCDH-CMV-TRAIL-EF1-GFP-T2A-Puro, and then were identified successfully by restriction enzyme analysis and PCR. Produced activated lentivirol particles by lipofectamine method, transfected HepG2 cell by utilizing the trait of ideal genetic carrier, and HepG2 was detected from LV-TRAIL-GFP-Puro by Western blotting, the results showed that TRAIL protein were high expression, and there were significant difference between these groups ($P<0.01$), which confirmed the lentiviral vector was an ideal gene vector for TRAIL, and had better bio-compatibility, effectiveness, System Stability. The experiment can supply important foundation and data for the study of the mechanisms of TRAIL inducing hepatocarcinoma cells apoptosis and its clinical application will be continued to explore in the next study.

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TRAIL 慢病毒载体构建及其在肝癌细胞中的表达 *

栾坤业[1] 于 健[1] 姚如永[2] 张炳远[1] 孙传东[1△]

(1 青岛大学医学院附属医院普外二科 山东 青岛 266003;2 青岛大学医学院附属医院中心实验室 山东 青岛 266003)

摘要 目的:构建携带 TRAIL 基因的慢病毒表达载体并实现其在肝癌细胞株 HepG2 中的稳定高表达。方法:构建 TRAIL 重组慢病毒表达载体 pCDH-CMV-TRAIL-EF1-GFP-T2A-Puro,脂质体法将重组慢病毒载体和包装质粒混合物共转染 293T 细胞,包装产生慢病毒颗粒,纯化并测定病毒滴度。利用 Western blotting 检测 TRAIL 蛋白在 HepG2 中的表达。结果:酶切以及测序证实,成功构建 TRAIL 基因重组慢病毒载体,纯化的慢病毒滴度为 1.02×10^4 ifμ/μL。利用嘌呤霉素筛选获得稳定表达 TRAIL 的细胞系,经 Western blot 方法检测到 TRAIL 蛋白的稳定高表达。结论:成功构建了带有 TRAIL 基因的慢病毒载体,并实现其在 HepG2 的稳定高表达。

关键词:TRAIL;慢病毒载体;HepG2

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作者简介:栾坤业(1984-),男,硕士研究生,研究方向:肝胆外科专业,E-mail:luankunye-qd@163.com

△通讯作者:孙传东,E-mail:sunchuandong@hotmail.com.

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