TRAIL mutant membrane penetrating peptide alike-*MuR6*-TR enhances the antitumor effects of TRAIL in pancreatic carcinoma both *in vitro* and *in vivo*

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Received November 6, 2016; Accepted April 10, 2017

DOI: 10.3892/ijmm.2017.2968

Abstract. To remedy the drug resistance of natural tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and enhance its antitumor effects, we prepared a type of TRAIL mutant membrane penetrating peptide alike (TMPPA)-TRAIL mutant R6 (*MuR6*-TR) by mutating the N-terminal of the soluble TRAIL gene sequence. The expressed *MuR6*-TR protein was purified to treat pancreatic carcinoma cell lines BxPC-3 and PANC-1. The inhibitory effects on the proliferation of BxPC-3 and PANC-1 cells was assessed with CCK-8 assay and compared with natural TRAIL. The antitumor effect of *MuR6*-TR was assessed on implant tumors derived from PANC-1 cells in nude mice and compared with gemcitabine. Finally, the soluble *MuR6*-TR gene was successfully mutated with 4 amino acids in the N-terminal of TRAIL and had a molecular size of 513 bp. The mutant *MuR6*-TR was connected to pET32a and verified by enzymatic digestion and sequencing. The recombinant *MuR6*-TR was transformed and expressed in *Escherichia coli*. The CCK-8 assay results indicated that *MuR6*-TR inhibited the growth of BxPC-3 and PANC-1 cells in a dose-dependent manner, with IC_{50} values of 4.63 and 7.84 ng/ml, respectively, which were much lower than that of natural TRAIL. MuR6-TR demonstrated a higher inhibitory effect on tumor growth (24.2%) than natural TRAIL (14.4%) and an effect similar to that of gemcitabine at an early period. Thus, the mutant *MuR6*-TR exhibited a stronger antitumor effect than that of natural TRAIL both *in vivo* and *in vitro* and may have potential therapeutic value for pancreatic carcinoma, which requires further validation.

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Key words: tumor necrosis factor-related apoptosis-inducing ligand, cell-penetrating peptides, antitumor effect, pancreatic carcinoma, apoptosis

Introduction

Pancreatic carcinoma is a neoplasm formed by abnormal proliferation of pancreatic cells due to the dysregulation of cellular growth under the effect of multiple tumorigenic factors. Due to its biological complexity and serious threat to patients, it is necessary to develop new therapeutic strategies for pancreatic carcinoma since current approaches have limited efficacy. For example, gemcitabine is associated with serious side effects and resistance is observed in various cases (1). Among tumorigenic factors, a deficiency in cellular apoptosis, the programmed death of cells, plays a critical role in the onset and development of tumors (2). Caspase activation is a key step in apoptosis and can be activated by both intrinsic and extrinsic pathways to induce a catalytic reaction and mediate cellular apoptosis (3). It has been proposed that an increase in the apoptotic threshold through alteration of molecules contributes to the therapeutic resistance of pancreatic carcinoma including apoptosis inducers or antitumor medications (4). Therefore, enhancing the sensitivity of tumor cells to apoptosis inducers is a potential strategy for the development of novel therapeutic strategies.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also termed as Apo2 ligand (Apo2L), is a member of the tumor necrosis factor (TNF) superfamily and the immune regulator of congenital and acquired immunity. TRAIL can initiate apoptotic signaling by binding to the death receptor (DR) to induce apoptosis targeting multiple tumor cells but without obvious killing of normal cells (5). However, treating different types of tumors simply dependent on TRAIL/Apo2L has limited efficacy. Studies have indicated that normal cells and more than half of passaged tumor cells (even >60%) demonstrate tolerance to TRAIL (4,6). The reason for the tolerance is due to the existence of a deficiency and mutations in the apoptotic signaling pathways of tumor cells, which include pro-apoptotic factors or anti-apoptotic factors to increase the apoptotic threshold of drug-resistant tumor cells to escape apoptotic scavenging. Increasing the sensitivity of

DR to TRAIL in tumor cells and the activity of pro-apoptotic factors, or removing the inhibition of anti-apoptotic factors can promote the efficacy of TRAIL to induce the apoptosis of tumor cells and reverse the tolerance of tumor cells to TRAIL.

Cell-penetrating peptides (CPPs) exhibit high transportation efficacy, low toxicity and no permanent damage to the cellular membrane, and therefore show potential value in reprogramming and gene editing (7). As one member of the CPPs, the basic amino acid polyarginine (pAr) carries cations under physiological pH and can bind to the negative glycosaminoglycan or lipids on the cellular membrane. Because of the small structure and higher penetrating efficacy, pAr can transport molecules into the cytoplasm to bind to downstream signals and to display its biological effects. The features of CPPs demonstrate that the targeting protein can be transported to the cytoplasm through CPPs to decrease the clearance rate of drugs in the blood and to ensure that the drugs directly react with the targeting signal in cells, which simultaneously activate extracellular and intracellular signaling to play a role in the effects of drugs. Therefore, pAr has been used to design antitumor peptides (8,9).

In the present study, we modified the N-terminal of TRAIL to R6 by mutating 4 loci at domain 114-281aa which is enriched in arginine to form a CPP-like amino acid sequence [TRAIL mutant R6 (*MuR6*-TR)] and investigated the *in vivo* and *in vitro* antitumor effects of *MuR6*-TR in pancreatic carcinoma. This study provides evidence for the modification of TRAIL in order to enhance the sensitivity of pancreatic carcinoma cells to apoptosis inducers.

Materials and methods

Design and synthesis of the primers. According to the literature (10) and GeneBank (http://www.ncbi.nlm.nih.gov/nuccore/), the soluble TRAIL sequence 114-281 at the N-terminal was selected. In reference to the prefered adjusting coding sequence of *Escherichia coli* (*E. coli)* synonym codon, the TRAIL sequence was inserted with the initiation codon ATG (M) and termination codon TAA to harvest *E. coli* preference codon with 513 bp. The amino acid sequence VRERGP located at 114-119 was mutated into RRRRRR, i.e., the N-terminal of natural TRAIL was mutated into R6 with 4 mutation loci to obtain the TRAIL mutant R6 (*MuR6*-TR). The amino acid sequence of *MuR*6-TR at 114-281 was: MRRRRRRQRVAAHITGTRGRSNTLSSPNSKNEKALGR KINSWESSRSGHSFLSNLHLRNGELVIHEKGFYYIYSQ TYFRFQEEIKENTKNDKQMVQYIYKYTSYPDPILLMK SARNSCWSKDAEYGLYSIYQGGIFELKENDRIFVSVTN EHLIDMDHEASFFGAFLVG.

The existing TRAIL sequence was used as a template for PCR to achieve local mutation with upstream *Nde*I and downstream *Eco*RI as restriction enzyme cleavage sites. The primers were upstream MuR6-TR-*Nde*I (48 bp), GGTCATATGCGT CGTCGTCGTCGTCGTCAGCGTGTGGCTGCTCACATC and downstream TR-*Eco*-R (41 bp) GTTGAATTCTTATTA ACCAACAAGGAAAGCACCGAAGAAAG.

Amplification of the MuR6-TR segment with PCR. The *MuR6*-TR was amplified with PCR using 50 μl of the total reaction system containing plasmid DNA (Pmd19/TRAIL) 1 μl, 10X PCR buffer for KOD-Plus-Neo 5 μl, dNTPs (2 mM) 5 μl, 25 mM $MgSO_4$ 3 μl, KOD-Plus-Neo 1 μl, MuR6-TR-*Nde*I/TR-*Eco*-R (10 pmol/μl) 1 μl, MuR6-TR-*Nde*I/TR-*Eco*-R (10 pmol/μl) 0.5 μl of each and RNase-free water 33 μl. The reaction condition included an initial denaturation at 94˚C for 2 min followed by 25 cycles of denaturation at 94˚C for 15 sec and annealing/extension at 68˚C for 30 sec, with a final extension at 68˚C for 5 min.

Transformation and identification of MuR6-TR. The vector and target gene were digested with *Nde*I and *Eco*RI, harvested with OMEGA recovery kit, eluted with 30 μl deionized water, electrophoresed and images were captured, respectively. The target segment and vector were linked together and 10 μl of the linked product was added to 100 μl Top10 competent cells for transformation. Then the transformed competent cells were smeared on LB solid medium containing ampicillin (Amp) at 37˚C overnight. The bacterial colonies were selected and digested with enzyme for identification. These positive colonies were stored for sequencing.

Expression of MuR6-TR in bacterial colony. The *E. coli* BL21(DE3) (in 1,000 μl) treated with pET3a-MuR6-TR at 37˚C overnight was added to 50 ml LB-Amp^+ medium and incubated on a shaking plate (250 rpm) at 37˚C for 3 h and then the temperature was decreased to 24˚C. Then, IPTG (0.1 M) was added at a 1% ratio for culture induction overnight. Samples of 0.5 and 0.15 ml collected before and after induction were centrifuged and the sediments after removal of the supernatant were re-suspended with 50 μl H_2O, followed by addition of 50 μl 2X loading buffer for electrophoresis. The remaining bacterial solution was centrifuged at 12,000 rpm for 5 min to obtain the bacterial collection which was re-suspended with 8 ml Na_2HPO_4 (50 mM) and lysed with ultrasound. The ultrasound lysis condition was: Φ6 probe, sonication with 200 W pulses for 2 sec with an interval of 2 sec, and repetition for 10 min. The 1 ml bacterial lysis was centrifuged at 12,000 rpm for 10 min to harvest the supernatant and sediment. Then, the supernatant and sediment re-suspended with 1 ml H_2O (20 μl for each) were added to 30 μl H_2O and 50 μl 2X loading buffer for electrophoresis. The samples for electrophoresis were heated in boiled water for 10 min and centrifuged at 12,000 rpm for 10 min. Finally, 10 μl of the supernatants was used for SDS-PAGE electrophoresis.

Purification of the targeting protein. The protein was firstly purified with cation SP Sepharose FF XK16 column (GE Healthcare, Piscataway, NJ, USA), according to the manual, followed by elution with an anion XK26/20 column filled with Sephadex G-25 medium and anion exchanging buffer (GE Healthcare). Then the protein was purified using a Q Sepharose FF XK16 column (GE healthcare), according to the manufacturer's manual. The penetrating and eluting components were collected, respectively, and 50 μl 2X loading buffer was added in a 1:1 ratio for electrophoresis. The same volume (50 μl) of original solution served as the control. The purified MuR6-TR and TRAIL proteins were subjected to western blotting for identification.

Activity of MuR6-TR protein in inhibiting proliferation of tumor cells. TRAIL-insensitive pancreatic carcinoma cell

lines (BxPC-3 and PANC-1) were cultured in a 5% CO_2 incubator at 37˚C with different media and density in a 96-well plate as shown in Table I. The medium was changed every 2-3 days, and the cells were passaged with medium containing 0.25% trypsin 0.02% EDTA at a 1:1 ratio. Cells in a logarithmic growth stage were used for experiments.

The *MuR6*-TR and TRAIL proteins were diluted with sterile PBS buffer to a final concentration of 5 mg/ml and sterilized with a filter. The cells were then treated with the different proteins with initial concentrations of 1 μg/ml and 200 μg/ml (the concentration of natural TRAIL protein was adjusted according to preliminary data) followed by a 3-fold dilution (total 10 dilution concentration). The experiment was repeated triple times.

The inhibitory effects on the proliferation of cells were measured with CCK-8 (cat. no. CK04-13; Dojindo), according to the manufacturer's instructions, analyzed with the equation $y = A2 + (A1 - A2)/[1 + (x/x0)^p]$ using OriginPro 9.0 software and fitted with growth/digmoidal-logistics for the inhibition rate curve to calculate IC_{50}. The *in vitro* therapeutic effect of the drugs was defined as sensitive killing with IC_{50} <10 μg/ml, dose-dependent cytotoxicity and maximal inhibition ratio >80%.

Inhibition of the growth of the implanted tumors in PANC-1-loaded nude mice by MuR6-TR. All animal procedures were approved by the Animal Care and Scientific Committee of Sichuan University. Balb/c nude female mice (SPF, 6-8 weeks, 18-22 g) were provided by Shanghai Sippr-BK Laboratory Animal Ltd. and bred at 23±2˚C, with a humidity of 40-70% and a 12/12 light/dark cycle with free access to food and water.

The PANC-1 cells were cultured in DMEM containing 10% FBS in a 5% CO_2 incubator at 37˚C. The cells at logarithm growth stage were digested with 0.25% trypsin, rinsed with PBS and re-suspended with serum-free medium to adjusted the cell density to $2.5x10^7$ cells/ml (1:1 Matrigel).

Under sterile conditions, each nude mouse was subcutaneously implanted with a 0.2 ml cell suspension ($5x10^6$ cells/mouse) in the right axillary. When tumors grew to a size of 150-250 mm^3, 32 mice with a healthy appearance and with a similar tumor size (single, global tumor without irregular shape or cluster tumors) were divided into 4 groups: i) saline; ii) gemcitabine; iii) natural TRAIL; and iv) MuR6-TR (n=8 for each group). The mice in the different groups were injected via tail vein with the different agents according to Table II consecutively for 5 days at fixed times.

The growth of tumors was measured twice weekly with the long (Y) and short (X) diameters to calculate the tumor growth inhibition (TGI, %), and simultaneously the body weight of mice was determined consecutively for 4 weeks. The TGI (%) was calculated according to the formula:

$$TGI\ (\%) = (1 - \text{tumor volume}_{treatment}/\text{tumor volume}_{vehicle}) \times 100\%,$$

where $\text{volume}_{treatment}$ is the tumor volume in the treatment groups and tumor $\text{volume}_{vehicle}$ is the tumor volume in the vehicle group.

After the last injection of the agents, the animals were sacrificed with an over-dosage of CO_2 and the tumors were removed to measure the weight. If the animals appeared moribund or the tumor size was >3,000 mm^3 during the experiment, the animal was sacrificed with an over-dosage of CO_2 to examine the pathological change in organs.

Table I. Conditions for cell culture.

Cell line	Medium	Density
BxPC-3	RPMI-1640 + 10% FBS + 1.0 mM sodium pyruvate	$4x10^3$/well
PANC-1	Low-glucose DMEM + 10% FBS	$5x10^3$/well

Table II. Treatments of mice in the different groups.

Group	Concentration (mg/ml)	Volume (ml/kg)	Strategy
Vehicle (saline)	-	10	i.v., q.d. x 5 days
Gemcitabine	5	10	i.v., q.o.d. x 3 times
TRAIL	6	10	i.v., q.d. x 5 days
MuR6-TR	6	10	q.d. x 5 days

TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MuR6-TR, TRAIL mutant R6.

Statistical analysis. All data are expressed as mean ± SEM only when denoted differently. Comparisons were performed with the Student's t-test and P<0.05 was considered to indicate a statistically significant difference. The mortality rate was expressed as a percentage and compared with the Chi-square test.

Results

Amplification and transformation of the MuR6-TR targeting gene. As shown in Fig. 1A, the resulting *MuR6*-TR segment by one-round PCR with primers MuR6-TR-*Nde*I/TR-*Eco*-R was ~510 bp which was in agreement with the size of the designed gene product (513 bp).

The segments from *MuR6*-TR and pET32a digested by *Nde*I and *Eco*RI were 550 bp and 5.4 kb, respectively, as shown in Fig. 1B. The sequence of the target gene *MuR6*-TR was confirmed by Beijing Genomics Institute (Shenzhen, China) and was in agreement with the designed sequence as shown in Fig. 1C.

After transformation by connection of MuR6-TR/pET32a, the bacteria grew well with normal density. The transformed plasmid carrying pET32a/MuR6-TR in the bacteria was digested with *Xba*I and *Eco*RI, resulting in an ~5.4 kb vector segment and ~550 bp target segment. As shown in Fig. 1D, the target segment of MuR6-TR was positive in 8 of 10 samples.

Expression and purification of pET32a/MuR6-TR in bacteria. The expression of pET32a/MuR6-TR in bacteria was high. The supernatant of lysate contained 80% target protein and the sediment contained 20% target protein (Fig. 2A).

The concentration of *MuR6*-TR protein in the cation-exchange eluting solution, desalination eluting solution and anion-exchange penetrating solution was measured (Table III). According to Table III, the recovery rate from

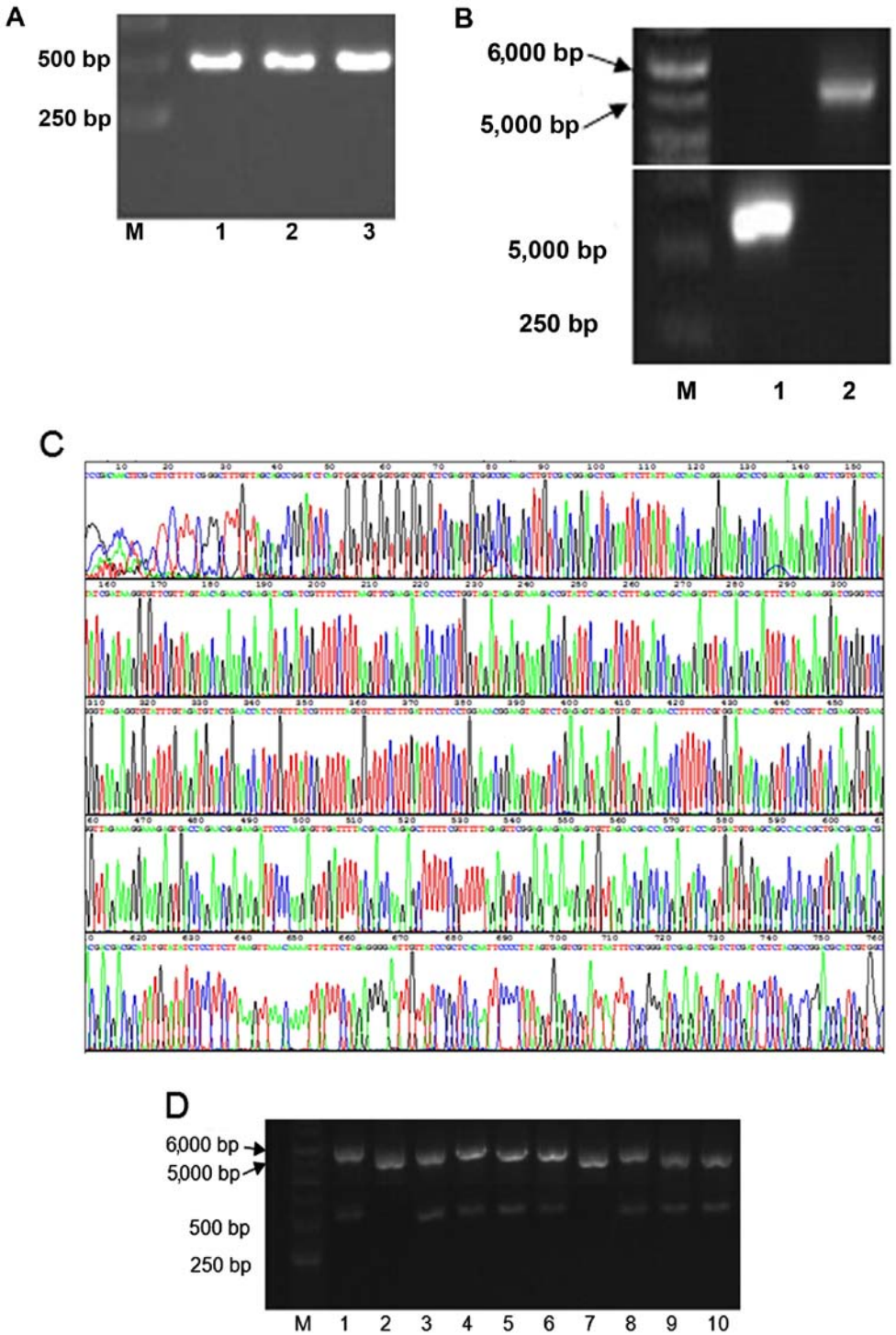

Figure 1. PCR production of (A) TRAIL mutant R6 (*MuR6*-TR) digested segment by *Nde*I/*Eco*RI, (B) MuR6-TR/pET32a, (C) sequencing and (D) transformation of bacteria. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

cation-exchange to desalination, from desalination to anion-exchange and from cation-exchange to anion-exchange was 91.33, 87.68 and 80.08%, respectively, which was within the acceptable range. The solution during exchange was clear and the purified protein was subjected to electrophoresis as shown in Fig. 2B. There was a visible protein band in the cation-exchange eluting solution; in the final anion-exchange penetrating solution, there was a clear protein band and few artificial bands.

The expression of TRAIL and *MuR6*-TR proteins was confirmed by western blotting (Fig. 2C).

Inhibition of cell growth in culture by MuR6-TR protein. The antitumor activity of *MuR6*-TR protein was measured in pancreatic carcinoma cells BxPc-3 and PANC-1. The results indicated that the inhibition rate of both cell lines by *MuR6*-TR was significantly higher when compared to that by natural TRAIL when *MuR6*-TR was at a similar range of

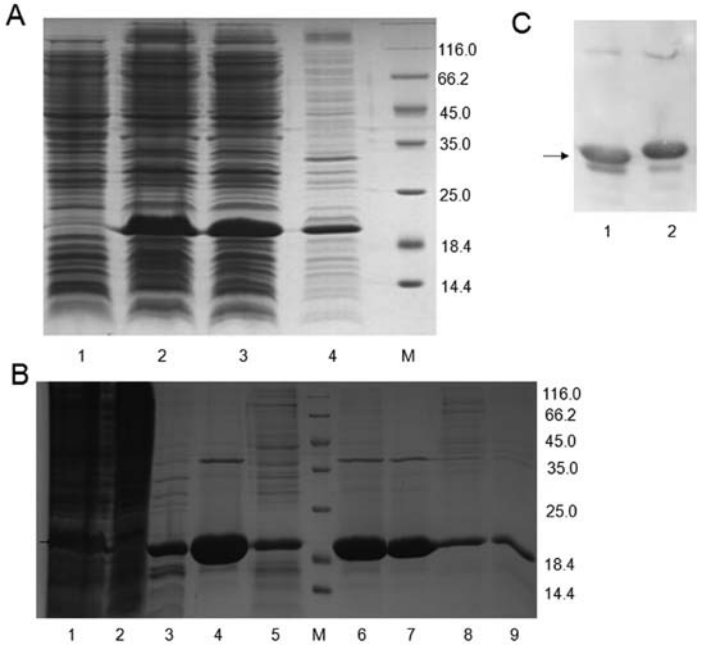

Figure 2. Identification of *MuR6*-TR targeting protein. (A) Electrophoresis of pET32a/MuR6-TR protein from transformed bacteria: lane 1, before induction; lane 2, after induction; lane 3, supernatant; lane 4, sediment. (B) Proteins in different eluting solution: lane 1, original MuR6 solution in SP column; lane 2, penetrating solution in SP column; lane 3, 0.8 M NaCl eluting solution in SP column; lane 4, 1.5 M NaCl eluting solution in SP column; lane 5, NaOH eluting solution in SP column; lane 6, original anion-exchange solution; lane 7, anion-exchange penetrating solution; lane 8, anion-exchange eluting solution; lane 9, 0.5 M NaOH eluting solution; M, marker. (C) Expression of TRAIL (lane 1) and *MuR6*-TR (lane 2) determined by western blotting. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; *MuR6*-TR, TRAIL mutant R6.

Table III. Measurement of protein in the different chromatography solutions.

Samples	Volume (ml)	Concentration (mg/ml)
Cation-exchange eluting solution	26	7.7267
Desalination eluting solution	44	4.1701
Anion-exchange penetrating solution	69	2.3315

concentrations (P<0.05; Table IV). The fitting curve showed that the IC_{50} of *MuR6*-TR for both BxPC-3 and PANC-1 cell lines was significantly lower than that of natural TRAIL (P<0.05; Table V, Fig. 3).

Inhibition of tumor growth in nude mice by MuR6-TR. The effects of *MuR6*-TR on the body weight, tumor growth, tumor inhibition rate and mortality rate of mice are shown in Fig. 4 and Table VI. As shown in Fig. 4A, the body weight of the mice in the 4 groups was not significantly increased during the observation time and there was no significant different among the different groups (P>0.05); but with exception in the gemcitabine group at day 7 when there was a significant decrease noted in body weight (P<0.05).

The results of tumor growth indicated that the tumor volumes at the different time-points and in the different groups were variable. At day 7 after treatment, the tumor volume in the gemcitabine group was significant smaller than that in the other groups (P<0.05). Furthermore, the tumor volume in the *MuR6*-TR group was significant smaller than that in the vehicle group and TRAIL group (P<0.05; Fig. 4B). Although the inhibition rate of tumor growth by *MuR6*-TR was significantly lower than gemcitabine, the inhibition rate of tumor growth by *MuR6*-TR was significantly higher than that by TRAIL at all observation time-points (P<0.05; Fig. 4C).

For the mortality rate, the results indicated that the mortality rate in the *MuR6*-TR group was significantly lower than that in the TRAIL group at days 7-28 and similar to that in the gemcitabine group at days 11-28.

Discussion

In the present study, we mutated the natural TRAIL protein at 114-119 residues with CPP-like change to form TRAIL mutant *MuR6*-TR which was verified by western blotting and demonstrated the antigen determinant of TRAIL. The *MuR6*-TR protein also displayed antitumor effects in pancreatic carcinoma cell lines and in implanted pancreatic carcinoma tumors in a nude mouse model. These results indicated that natural TRAIL mutant *MuR6*-TR enhanced the sensitivity of tumor cells to apoptosis inducers and could be a potential targeting therapy for pancreatic cancer.

Human natural TRAIL is a type II transmembrane glycoprotein composed of 281 amino acids. The structural domain of TRAIL C-terminal at residues of 114-281 has several β-motifs to form hollow tubular structure. Three TRAIL monomers can form homogenous trimer through the β-motif to display biological activity (11). The C-terminal of natural TRAIL can be hydrolyzed by metalloprotease into soluble functional segments (sTRAIL) while the trimer of either entire

Table IV. Proliferation inhibition of BxPC-3 and PANC-1 cells by natural TRAIL and MuR6-TR at different concentrations.

Natural TRAIL			MuR6-TR		
	Inhibition rate (%)			Inhibition rate (%)	
Concentration (μg/ml)	BxPc-3	PANC-1	Concentration (μg/ml)	BxPc-3	PANC-1
200	79.890	94.191	1	96.659	92.408
66.667	80.561	91.061	0.333333333	95.747	92.832
22.222	79.657	86.793	0.111111111	92.254	92.872
7.407	81.259	74.223	0.037037037	80.832	88.602
2.469	66.873	40.823	0.012345679	59.624	65.288
0.823	59.125	5.747	0.004115226	41.020	22.361
0.274	37.56	3.834	0.001371742	19.372	7.291
0.091	29.993	1.147	0.000457247	-1.694	-1.418
0.030	18.294	-6.277	0.000152416	-5.981	3.178
0.010	4.838	-8.491	0.0000508052	-	1.037

TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MuR6-TR, TRAIL mutant R6.

Table V. IC_{50} of natural TRAIL and MuR6-TR in the BxPC-3 and PANC-1 cell lines.

	Natural TRAIL		MuR6-TRAIL	
	BxPC-3	PANC-1	BxPC-3	PANC-1
IC_{50} (μg/ml)	0.284	2.817	$4.63x10^{-3a}$	$7.84x10^{-3b}$

[a]P<0.05 vs. BxPC-3 in natural TRAIL; [b]P<0.05 vs. PANC-1 in natural TRAIL. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MuR6-TR, TRAIL mutant R6.

TRAIL or sTRAIL can induce the apoptosis of tumor cells. The defect of N-terminal amino acid of TRAIL has no effect on the protein function, which provides the possibility for the directed reformation of TRAIL.

In a primary study, we modified sTRAIL by forming CPPs in the N-terminal and found that different CPPs in classification and molecular weight demonstrated large variation in the stability, soluble expression and biological activities of TRAIL (data not shown). After studying the structure of TRAIL, we found that the arginine (R) dominant VRERGPQR domain in 114-121 residues of C-terminal has a similar structure with the non-amphipathic pAr of CPPs and is not involved in the specific spatial conformation of TRAIL. Therefore, we proposed to directly reform the N-terminal of sTRAIL with CPPs in 114-121 residues of the C-terminal.

In the study of pAr cell-penetration, R9 is commonly used because both the linear and branched structures have cell-penetrating function (12). A previous study indicated that the cell-penetrating efficacy of the peptide having <5 or >15 R is obviously decreased (13). With the success in mutating VRERGPQR with 5 amino acid into RRRRRRRRR8 (R8) in 114-121 residues of TRAIL (data not shown), we further reduced the mutation by mutating 4 residues of sTRAIL N-terminal (R6) in the present study and this mutant was confirmed by PCR. This soluble R6 mutation increased the stability while minimizing the change of the primary structure of TRAIL to exhibit biological activities.

The expression of exogenous protein by *E. coli* is affected by many factors, such as IPTG concentration, induction time and stability of exogenous protein in the host. The commonly used concentration of IPTG is 0.1-1.0 mmol/l while a higher concentration of IPTG can increase the cost and inhibit bacterial growth (14). Therefore, minimal IPTG should be used under the prerequisite that there is no effect on the expression of protein. Our primary study indicated that 0.1 mM IPTG could achieve ideal expression of protein while a higher concentration did not increase the expression but decreased the bacterial sum. Therefore, we set the IPTG concentration to 0.1 mM. In addition, the induction time is also critical because earlier induction can inhibit the growth of bacteria while later induction results in aging bacteria and is bad for expression. We found that induction after 3 h is the best time for ideal bacterial production and protein expression. The fermentation of *E. coli* at a high density requires reduction of the production of acetic acid which can inhibit the growth and expression of bacteria, and the glucose can be replaced by glycerol which produces less acetic acid (15). Under our current condition, the final total expression of MuR6-TR was high (80%) which is helpful for the next purification.

In bacteria, there are nucleic acid, polysaccharose and other proteins which are existent in complex forms with the expressed targeting proteins. Therefore, extraction and purification of the targeting protein is critical. The MuR6-TR protein has an isoelectric point at 9.96 but no disulfide bond, is lowly adhesive to regular purifying gels under low-salt condition and resistant to high-salt (11). While high ion intensity under high-salt condition can deviate the practical pI from theoretical pI, which is helpful to explore ideal condition for ion exchange and eluting to obtain targeting proteins with higher concentration and purity. For the safety of animals in

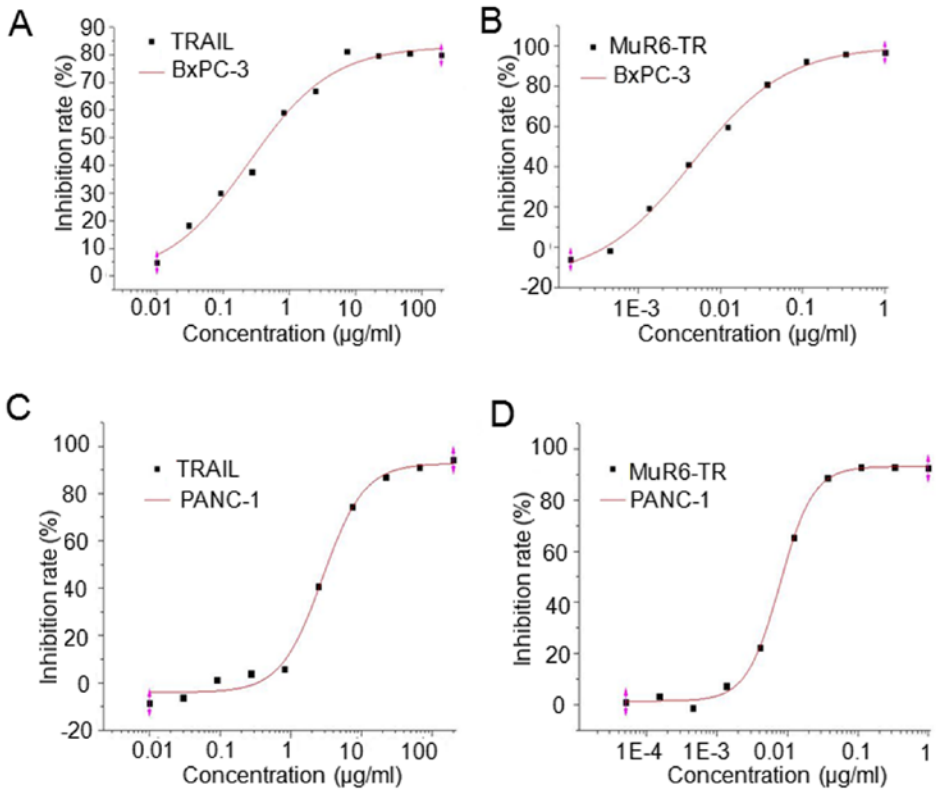

Figure 3. The fitting curves of (A and C) natural TRAIL and (B and D) MuR6-TR on the proliferation inhibition of (A and B) BxPC-3 cells and (C and D) PANC-1 cells. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MuR6-TR, TRAIL mutant R6.

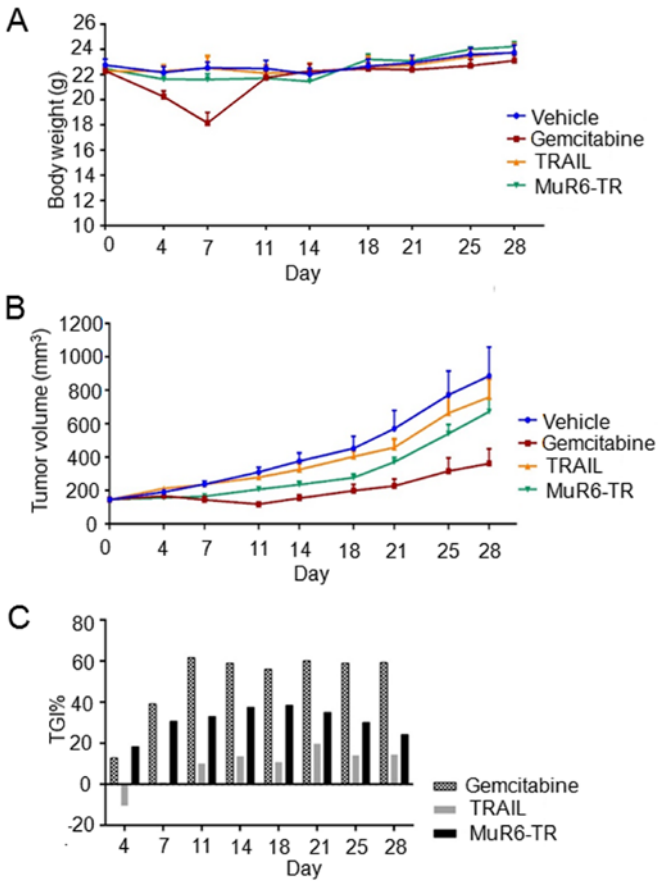

Figure 4. Effects of different treatments on (A) body weight, (B) tumor volume and (C) tumor growth inhibition (TGI). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MuR6-TR, TRAIL mutant R6.

Table VI. Mortality rate of mice in different groups.

	Mortality rate (%)								
	Day								
Group	0	4	7	11	14	18	21	25	28
Vehicle	0	0	0	0	0	0	0	0	0
Gemcitabine	0	0	12.5	25	25	25	25	25	25
Natural TRAIL	0	12.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5
MuR6-TR	0	25	25	25	25	25	25	25	25[a]

[a]P<0.05 vs. TRAIL. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand. MuR6-TR, TRAIL mutant R6.

subsequent experiments, it is necessary to remove the pyrogen which is a metabolic endotoxin produced by microbes. The first step of cation-exchange is mainly to elute the targeting protein from the added samples containing many unspecific proteins which are mostly negative-charged. Therefore, it is necessary to perform the second step of anion-exchange for purification to obtain targeting protein with high purity. The western blot result indicated that the purified protein after two-step elution in the present study had the antigenic determinant and was suitable for the next experiment in animals.

TRAIL mainly exhibits its effect by binding to superficial DR4 and DR5 on the cell membrane. In pancreatic tumor tissues, there is universal expression of TRAIL receptor which

plays an important role in modulating the apoptosis of pancreatic carcinoma and there is differential expression of the TRAIL receptor. For example, DR4 and DR5 are expressed much higher in pancreatic tumor tissues than pancreatic normal tissues. The expression of DR5 in pancreatic carcinoma tissue is related to the differentiation degree and malignant degree of tumor tissues while the expression of DR4 and decoy receptor 1 and 2 (DcR1, DcR2) in pancreatic carcinoma tissue is not related with the differentiation degree and clinical stage (16,17). According to a review by Di Pietro and Zauli (6), Apo2L/TRAIL was sensitive to 61 of 92 studied primary or passaged tumor cell lines, having a sensitivity rate of 66.3% and a resistance rate of 33.7%. Other studies indicated that approximately 50% tumor cell lines are resistant to TRAIL (18,19). In the present study, both TRAIL-insensitive pancreatic carcinoma cell lines BxPC-3 and PANC-1 showed a higher growth inhibition rate and lower IC_{50} with *MuR6*-TR than natural TRAIL, suggesting that the mutant protein with N-terminal CCPs had an *in vitro* advantage in inhibiting the growth of pancreatic carcinoma.

As a platform to mimic the intrinsic environment of the body, animal experiments can primarily explore the effect and safety of novel drugs in the body. According to previous studies, the TRAIL receptor agonists (including recombinant soluble TRAIL) and the monoclonal antibodies against TRAIL-R1 and TRAIL-R2 which are specific for TRAIL-induced apoptosis have been evaluated in the early stage of clinical experiments in hematologic and solid tumors including pancreatic carcinoma (20,21). However, most pancreatic carcinoma cell lines showed low sensitivity to apoptosis induced by TRAIL although they express basic signaling molecules of the TRAIL system (22). According to the *in vitro* experiment, we selected the pancreatic carcinoma cell line PANC-1 which is resistant to natural TRAIL but sensitive to MuR6-TR. The present study indicated that MuR6-TR showed an antitumor effect in inhibiting the growth of implanted pancreatic tumors in nude mice. Although lower than gemcitabine, MuR6-TR demonstrated a more effective effect than natural TRAIL protein in inhibiting the growth of tumors. There are several explanations for the lower effect of *MuR6*-TR than gemcitabine. Firstly, the mutant MuR6-TR had a higher therapeutic effect but a shorter time of bioactivity in the body, which is supported by the finding that *MuR6*-TR had a similar effect with gemcitabine during the first 4-7 days. We will conduct continuous administration of the drug and to change the frequency in future experiments to observe the antitumor effect. Secondly, the optimal dosage for the mutant protein requires further investigation. Notably, the IC_{50} of *MuR6*-TR was much lower than TRAIL in the *in vitro* experiment and the lower dosage *MuR6*-TR demonstrated a similar effect with gemcitabine *in vivo* at 4-7 days. Therefore, we can increase the dosage to enhance the antitumor activity after determining the safe dosage of the drug. Thirdly, the *in vitro* sensitivity of cell lines to mutant protein may not represent the *in vivo* sensitivity. It is possible to combine current first-line clinical medicines to investigate the potential antitumor effects.

During the selection of the drug dosage and administration frequency, we considered the tolerance of the mice, the convenience for patients in future clinical experiments and the quantity of *MuR6*-TR entering cells in order to prolong its half-life period in the body. These considerations approached the scheme of q.d. x 5 days which will be further improved in future experiments. Moreover, 2 of 8 mice (25%) died during the early stage of the experiment (day 1-4 after administration of MuR6-TR); however, the mice did not die immediately after the administration of MuR6-TR. Thus, it was not due to an acute allergic reaction. We tried to ascertain the reasons of the death by dissection of the two dead mice. We identified a large area of white necrotic foci in the livers of the dead mice. Thus, we diagnosed liver toxicity. We will add a toxicity test in future reseach. In addition, a higher requirement for the purification protocol in the future is needed. On the other hand, the mortality rate in the gemcitabine group was the same at 25%, suggesting that the dosage of gemcitabine should be adjusted in future experiments.

In summary, the present study mutated TRAIL with CPPs at the N-terminal and demonstrated that the mutant *MuR6*-TR had improved antitumor effects both *in vitro* and *in vivo* compared to the natural TRAIL. The mechanism was not explored in this study. However, since the structure of mutant *MuR6*-TR was similar to natural TRAIL, it is likely to also display its antitumor effects by binding to the TRAIL receptors on the tumor cell membrane. There was another TRAIL mutant membrane penetrating peptide alike (TMPPA) which showed significantly stronger affinity to the cancer cell membrane compared with natural TRAIL (data not shown). Thus, we believe that TMPPA enhances the affinity to the cancer cell membrane. Then, it massively aggregates on the cancer cell membrane and increases the signal transduction finally increasing the antitumor effects. Nevertheless, the therapeutic effect of *MuR6*-TR and the detailed mechanism warrant further research.

Acknowledgements

This study was supported by grants from the National Natural Scientific Foundation of China (nos. 81301962 and 81372444).

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