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Original Article

Targeted treatment of liver metastasis from gastric cancer using specific binding peptide

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Abstract: Gastric cancer ranks the first in China among all gastrointestinal cancers in terms of incidence, and liver metastasis is the leading cause of death for patients with advanced gastric cancer. Tumor necrosis factor (TNF) is a cytokine commonly chosen as the target for gene therapy against cancers. The specific binding peptide pd20 of gastric cancer cells with a high potential for liver metastasis was fused with human TNF to obtain the pd20-TNF gene using DNA recombinant technique. The expression of the fusion protein was induced and the protein was purified. In vitro activity test showed that the fusion protein greatly improved the membrane permeability of liver cells in nude mice with liver metastasis from gastric cancer. The tumor implantation experiment in nude mice showed that the fusion protein effectively mitigated the cancer lesions. The results provide important clues for developing the drugs for targeted treatment of liver metastasis from gastric cancer.

Keywords: Liver metastasis from gastric cancer, specific binding peptide, targeted treatment

Introduction

The prevalence of gastric cancer (GC) ranks the fourth among all malignancies worldwide. Furthermore, GC is also the most prevalent gastrointestinal cancer in China. The GC patients who were diagnosed at the early stage account for less than 5% [1, 2] and most patients were diagnosed at the advanced stage. The incidence of liver metastasis from GC can reach as high as 44.5%, accounting for 15% among all metastases from GC. Liver metastasis from GC is the leading cause of death in advanced gastric cancer patients [3-5]. Unfortunately, the therapy for the GC patients with liver metastasis is limited. In 1975, Carswell et al. discovered a substance in the serum named tumor necrosis factor (TNF), which was secreted by activated macrophages, NK cells and T lymphocytes [6]. TNF is the first cytokine used in biological therapies for tumors. However, TNF was only confined to local treatment due to lack of targetability [7].

With advances in the understanding of the molecular mechanism of GC, targeted treatment now provides a new solution for cancer therapy. The specific binding peptide pd20 of GC cells have a high potential of liver metastasis. Therefore, in the present study, a pd20-TNF gene which was fused with TNF using recombinant DNA technique was considered as a selective substance in the GC therapy. We performed a tumor implantation experiment in nude mice to determine whether the fusion protein pd20-rmhTNF was an effective therapy for the GC patients with liver metastasis.

Materials and method

Materials

Target genes

In the present study, we utilized specific binding peptide pd20 and TNF-α gene (human-derived) as the target genes.

Experimental animals and vectors

Balb/c nude mice, host bacteria *E. coli* DH5α and *E. coli* BL21 (DE3), pET28a(+) vector containing the kanamycin-resistance gene were

provided by Western Biotechnology (Chongqing, China).

Methods

Construction of pET28a(+)-pd20-TNFα vector

We designed the sequence of specific binding peptide pd20 in the study. The nucleic acid fragment encoding the specific binding peptide pd20 was designed and synthesized using codon in *E. coli*. At the 5'- and 3'-terminal of the synthesized fragment, the cutting sites for the restriction enzyme NdeI and XhoI were added, respectively. The DNA products obtained from restriction enzyme digestion were separated by 1.5% agarose gel electrophoresis. The fragment was recovered, ligated to pMD18 vector and identified by NdeI+XhoI digestion. The 20 μl of reaction system was shaken well and placed in a 37°C water bath for 3 h. The fragment was recovered and ligated to the vector. The nucleic acid fragment encoding the specific binding peptide pd20 was subjected to denaturation at 65°C for 5 min and annealing, and then ligated to pBV220 plasmid treated by mutant TNF and NdeI+XhoI. The ligation product was used to transform the competent DH5α cells. After restriction enzyme digestion and DNA sequencing, the prokaryotic expression vector containing pd20-TNF fusion gene was obtained. The NdeI/XhoI double digestion system was as follows: 2.0 μL of NdeI, 2.0 μL of XhoI, 2.0 μL of 10 × M Buffer, 4.0 μL of Plasmid/pd20-TNFα, and 10.0 μL of ddH_2O. The ligation reaction system included 4.5 ul of pd20-TNFα gene, 0.5 ul of pET28a(+) vector, and 5 ul Ligation solution.

Transformation of DH5α host bacteria and BL21 (DE3) E. coli cells using pET28a(+)-pd20-TNFα

E. coli DH5α/BL21 was picked under aseptic condition and streaked onto the LB agar plate. The LB plate containing Kana was used as control. The bacteria were grown on the plate at 37°C overnight, and the fresh, rejuvenated single colonies were inoculated to 10 mL LB liquid medium for bacterial culture at 37°C overnight. Then 2 ml of the above bacterial liquid was drawn and added into the 250 mL culture flask containing 50 mL LB liquid medium. The bacteria were cultured on a shaker at 37°C for 2 h (until OD600=0.6). Then the bacteria were transferred to a 50 mL centrifuge tube using aseptic technique, placed in an ice bath for 20 min, and centrifuged at 3500 r/min at 4°C for 10 min. The supernatant was discarded and the bacterial precipitate was collected. The cells were resuspended in 25 ml precooled 0.1% $CaCl_2$ and placed in an ice bath for 30 min. Centrifugation was performed at 3500 r/min at 4°C for 10 min, with supernatant discarded and the bacterial precipitate collected. Then the cells were resuspended in 1 ml precooled 0.1% $CaCl_2$, subpackaged at the volume of 100 μL /EP tube and left to stand at 4°C for 12-24 h. The ligation product pET28a(+)-pd20-TNFα was used to transform the DH5α/BL21 competent cells. The mixture was gently shaken and placed in an ice bath for 30 min. Heat shock was performed by placing the cells in 42°C bath for 1.5 min, followed by ice bath for 2 min. Then the cells were added with 800 ml LB liquid medium and cultured on a shaker at 37°C for 1 h. The thalli were separated and collected by centrifugation. The bacterial cells were resuspended in 200 μL LB liquid medium and coated onto the LB plate (containing Kana). Then the bacteria were cultured with the dish placed upside down in a 37°C incubator.

Identification of pET28a(+)-pd20-TNFα recombinant plasmid

Single colonies were picked on the plate, and the plasmid was extracted with Triton (Biotech, Beijing, China). The products were sequenced after PCR. The PCR system is as follows: 1 uL of recombinant plasmid DNA; 1 uL of P1 (20 pmol/μL), 1 uL of P2 (20 pmol/μL), 9.5 uL of Sterilized deionized water, and 12.5 uL of 2 × Taq PCR Master Mix. The primers were shown as follows: P1: 5'CCAAATCATATGGGTCGTCGTACCCG TTCTCGT3'; P2: 5'ATTTGGCTCGAGTCACAGGG-CAATGATCCCAAA3'.

Condition of PCR reaction was as follows: predenaturation at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, 35 cycles; final extension at 72°C for 10 min. Then 3 μL PCR products were subjected to 1% agarose gel electrophoresis. The length of the amplified fragment was determined.

The plasmid was double digested with NdeI and Xholat 37°C water bath for 3 h. The system included 2.0 μL of NdeI, 2.0 μL of XhoI, 2.0 μL

of 10 × M Buffer, 2.0 μL of pET28a(+)-pd20-TNFα plasmid, and 12.0 μL of ddH_2O.

Sequencing of pET28a(+)-pd20-TNFα recombinant plasmid

The positive recombinant plasmid was screened and sequenced. The sequenced products were analyzed against NM_000594.2 using BLAST on GenBank.

Induction of expression in positive clones, SDS-PAGE and western blotting

The positive clones were inoculated to 5 mL LB containing Kana (50 ug/ml) and cultured overnight at 37°C. Then 0.5 mL culture solution was inoculated to 50 mL LB containing Kana (100 ug/mL) and incubated on a shaker at 37°C until OD600 reached 0.6. The clones were added with 1 M IPTG to the final concentration of 1.0 mM, and they were further incubated for 4 h. After that, 1.5 ml bacterial liquid was added into the Eppendorf tube, and the bacteria were precipitated by centrifugation. The precipitate was resuspended in 20 uL double distilled water and added with 20 uL 2 × SDS loading buffer containing DTT. The mixture was placed in boiling water for 3 min, centrifuged and analyzed by SDS-PAGE. Using the method described in Molecular Cloning (Version 3), SDS-PAGE was performed using a vertical electrophoresis tank. First 15 mL of 12% polyacrylamide gel was added into the tank for the separation and the uppermost layer was sealed with 2 mL of ddH_2O. It was ensured that the liquid was leveled off and the polymerization was performed at room temperature for 3 h. Then the water was removed from the separating gel, and a comb was inserted between the glass plates. Then 4 mL of 5% polyacrylamide stacking gel was added and the polymerization was initiated at room temperature for 30 min. After that the comb was removed. SDS-PAGE was carried out for 10 μL supernatant obtained. With Tris-glycine buffer added into the electrophoresis tank, electrophoresis was carried out at 4°C. The stacking gel was run at voltage 8 v/m and the separating gel at 15 v/cm. When bromphenol blue was at the lower part of the tank, the electrophoresis was terminated. The gel was recovered and stained with Coomassie brilliant blue at room temperature for 4 h. Next the gel was immersed in the destaining solution until the target band became distinct. The gels were scanned using the imaging system.

Detection of the solubility of fusion protein

The positive recombinant bacteria were cultured with IPTG for 4 h to induce the expression of the fusion protein. Centrifugation was performed at 13000 r/min for 1 min to collect the thalli which were ultrasonically disrupted at the following conditions: intermittent ultrasound treatment at 4°C in an ice bath, ultrasound disruption for 2 s, followed by an interval of 5 s, for a total of 10 min, amplitude 30%. When the bacterial liquid became clear, it was centrifuged at 13000 r/min at 4°C for 10 min, with supernatant and precipitate collected separately. To determine whether the expressed products were solved in the supernatant or existed as inclusion bodies in the precipitate, SDS-PAGE was performed [8].

Purification of the fusion protein using Ni-affinity chromatography

Induced culture was carried out. The thalli were disrupted and the supernatant was collected (by the same procedures as above). Purification was done using Ni-affinity chromatography, the procedure was as follows:

(1) The Ni column was washed with 5 volumes equilibrium buffer at the flow rate of 2 mL/min. (2) The bacterial culture supernatant was diluted and filtered, and loaded onto the column at the flow rate of 1 mL/min. (3) The Ni column was washed again with 5 volumes equilibrium buffer at the flow rate of 2 mL/min so that the target protein would completely bind to the column. (4) The heteroproteins were washed off using elution buffer 1 at the flow rate of 2 mL/min and the impurity peak was detected. (5) Elution was performed using elution buffer 2 at the flow rate of 1 mL/min. The elution peak was detected and the elution liquid was collected. (6) The elution liquid was subjected to ultrafiltration concentration for several times using 15 mL ultrafiltration tube. The concentrates collected each time were combined and subjected to ultrafiltration concentration again. The product was subpackaged to the tubes (1.5 ml per tube), and 1 mL was preserved at 4°C with the remaining preserved at -80°C. (7) The column was washed with impurity elution buffer, elution buffer and 2 × loading buffer (1 mL each), followed by boiling water bath for 10 min. The purified proteins were analyzed by SDS-PAGE.

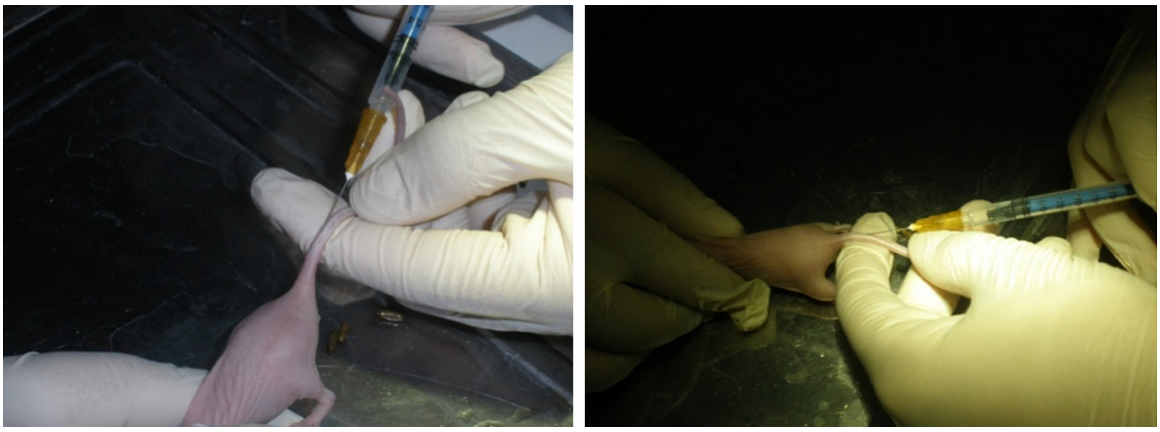

Figure 1. Injection of pd20 via the tail vein.

Western blot detection of the fusion protein

Wet transfer: The purified fusion protein was subjected to SDS-PAGE. The gel containing the target protein was cut to appropriate size and the gel was balanced with transfer buffer three times for 10 min each time. The Whatman 3 mm filter paper and NC membrane of the same size as the gel was also immersed into the transfer buffer for 10 min. The transfer cassette was opened and placed in a shallow dish. The fiber pad soaked in the transfer buffer was spread over the inner side near the cathode. Then several Whatman 3 mm filter papers soaked in the transfer buffer were placed over it. The gel containing the target protein was transferred to the filter papers. The gel was wetted with the transfer buffer and the NC membrane was spread over it. Then several Whatman 3 mm filter papers soaked in the transfer buffer and the fiber pad were also placed over it successively. It was ensured that no air bubbles were present. A straw was used as the roller to remove all the air bubbles if necessary. Then the transfer cassette was closed and placed into the tank. The transfer buffer was poured into the tank, and the power was turned on to initiate the electrophoresis at the constant current of 100 mA for 3 h. After electrophoresis, the transfer cassette was taken out and the NC membrane was moved into the TBST buffer using tweezers. The membrane was washed with TBST buffer three times for 5 min each time.

Immunoreaction: The product was added with 3% bovine serum albumin (3% BSA in TBS buffer) to completely cover the membrane. The membrane was sealed for 4 h, and the membrane was washed with the sealing liquid discarded. Then TBST buffer was added to wash the membrane three times for 5 min each time. The primary antibody was added, and the TBST buffer was discarded. The mouse anti-TNF-α antibody (diluted 1000 times in 0.01 mol/L pH 7.2 PBS) was added until the membrane was completely covered. The reaction proceeded at 4°C with gentle shaking for 4 h. The diluted primary antibody was discarded and the membrane was washed with TBST buffer three times for 5 min each time. Next HRP-labeled goat anti-mouse IgG (1:5000) was added to completely cover the membrane. The reaction proceeded at room temperature with gentle shaking for 4 h. The diluted IgG was discarded, and the membrane was washed with TBST buffer three times for 5 min each time.

Color development: The TBST buffer was discarded, and DAB reagent was added with gentle shaking for 1-3 min. The color development was monitored until the band turned brownish red. The color development reaction was terminated by washing the membrane in the stop solution three times for 10 min each time.

Imaging and storage of NC membrane: The NC membrane was scanned using the gel imaging system and properly stored. The water was removed from the NC membrane using the absorbent paper and left to air dry. The NC membrane was placed in the plastic bag and sealed.

Establishment of animal model of liver metastasis from gastric cancer

The Balb/c nude mice were fasted for 12 h and received intraperitoneal injection of 40 mg/kg

A catatgGGTCGTCGTACCCGTTCTCGTCGTCTGCGTCGTTCTATG
AGCACTGAAAGCATGATCCGGGACGTGGAGCTGGCCGAGG
AGGCGCTCCCCAAGAAGACAGGGGGGCCCCAGGGCTCCAG
GCGGTGCTTGTTCCTCAGCCTCTTCTCCTTCCTGATCGTGGCA
GGCGCCACCACGCTCTTCTGCCTGCTGCACTTTGGAGTGATC
GGCCCCCAGAGGGAAGAGTTCCCCAGGGACCTCTCTCTAAT
CAGCCCTCTGGCCCAGGCAGTCAGATCATCTTCTCGAACCCC
GAGTGACAAGCCTGTAGCCCATGTTGTAGCAAACCCTCAAG
CTGAGGGGCAGCTCCAGTGGCTGAACCGCCGGGCCAATGCC
CTCCTGGCCAATGGCGTGGAGCTGAGAGATAACCAGCTGG
TGGTGCCATCAGAGGGCCTGTACCTCATCTACTCCCAGGTCC
TCTTCAAGGGCCAAGGCTGCCCCTCCACCCATGTGCTCCTC
ACCCACACCATCAGCCGCATCGCCGTCTCCTACCAGACCAA
GGTCAACCTCCTCTCTGCCATCAAGAGCCCCTGCCAGAGGG
AGACCCCAGAGGGGGCTGAGGCCAAGCCCTGGTATGAGCC
CATCTATCTGGGAGGGGTCTTCCAGCTGGAGAAGGGTGACC
GACTCAGCGCTGAGATCAATCGGCCCGACTATCTCGACTTTG
CCGAGTCTGGGCAGGTCTACTTTGGGATCATTGCCCTGTGActcgag

B HMGRRTRSRRLRRSMSTESMIRDVELAEEALPKKTGGPQGSRRC
LFLSLFSFLIVAGATTLFCLLHFGVIGPQREEFPRDLSLISPLAQAV
RSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELR
DNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTK
VNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAE
INRPDYLDFAESGQVYFGIIAL-LE

Figure 2. The sequences of nucleic acid (A) and protein (B).

pentobarbital. The skin at the surgical site was disinfected, and a paramedian incision of about 1cm was made on the left to expose the greater curvature of stomach. The serous layer of the stomach was detached, and the subserosal injection of the suspension of gastric cancer cell line AGS or SGC7901 (2×10^6) was performed. The gastric serosa near the injection site was disinfected using cotton ball soaked in 75% alcohol for 2 min so as to kill the tumor cells. Then the stomach was returned to the peritoneal cavity and the peritoneum was sutured (containing the muscular layer). After modeling, the nude mice were sent back to the cages [9, 10].

Immunofluorescence detection of binding specificity of peptide pd20 in tumor-bearing nude mice

For the experimental group, Bio-pd20 100 μl was injected via the tail vein of each nude mouse (1 mg/ml, **Figure 1**). The time of injection was recorded. Intraperitoneal anesthesia with 150 mg/kg pentobarbital sodium was performed at 0.5 h, 2 h, 18 h, 24 h and 48 h after injection, respectively. The skin was cut open at the level of diaphragm to expose the entire thoracic wall. The sternum was cut off, and attention was paid not to damage the heart and the great vessels. After full exposure of the heart, an intravenous needle was inserted into the aorta via the left ventricle. After the blood was drawn into the needle, 50 ml normal saline preheated to 37°C was gently injected in the aorta. A small cut was made in the right auricle to make it bleed until the blood flowing out turned pale red. The tumors, liver metastatic lesions, heart, kidney, lung, spleen and muscle tissues were harvested and placed in 25% sucrose. The tissues were embedded in OTC, made into frozen sections and fixed in cold acetone for 20 min. The protein was detected by immunofluorescence conventionally. The cells were incubated at 4°C overnight and washed with PBS under oscillation three times for 5 min each time. Then the cells were incubated with FITC-avidin for 1 h, washed with PBS under oscillation three times for 5 min each time and observed under the fluorescence microscope.

Purification of pd20-TNF fusion protein

Every 1 g thalli were resuspended in 7 ml lysis buffer and disrupted ultrasonically. Then fractional salting out using ammonium sulfate was performed at 30%-65% saturation. The precipitate obtained with 65% saturated ammonium sulfate was used for the dialysis of solution A (0.02 mol/L PB pH 6.5), followed by purification through the Sp-Sepharose FF column. Continuous gradient elution was performed using 0%-100% solution B (0.02 mol/L PB, 1 mol/L NaCl, pH 6.5). The elution peaks were detected and the activity was determined. On this basis, dialysis was performed for solution C (0.02 mol/L Tris•Cl, 1 mmol/L EDTA pH 8.5), and purification was done through the Q-Sepharose FF column. Continuous gradient elution was performed using 0%-50% solution D (0.02 mol/L Tris•Cl, 1 mmol/L EDTA, 1.0 mol/L NaCl pH 8.5). The elution peaks were detected and the activity was determined. SDS-

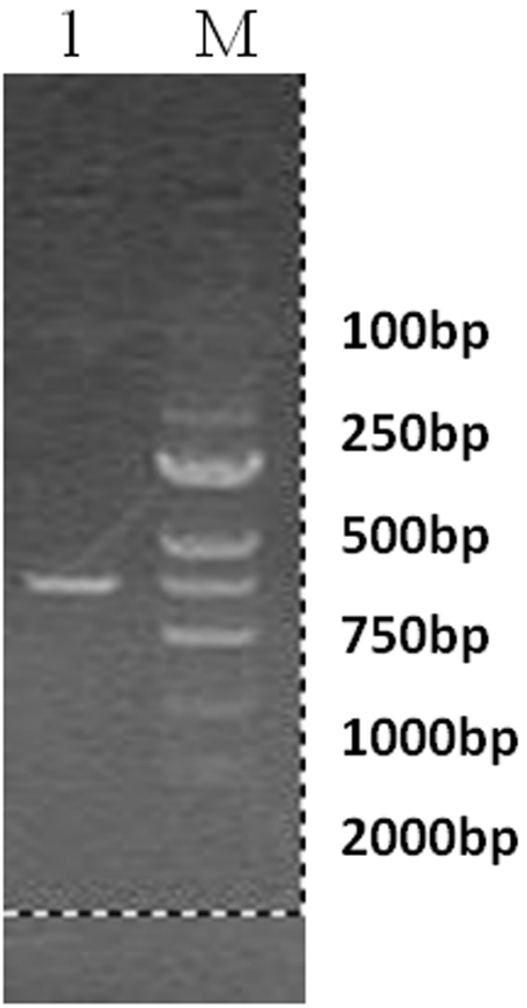

Figure 3. PCR identification of pd20-TNFα recombinant plasmid. Note: 1 is the amplified product for pd20-TNFα recombinant plasmid; M is DL2000.

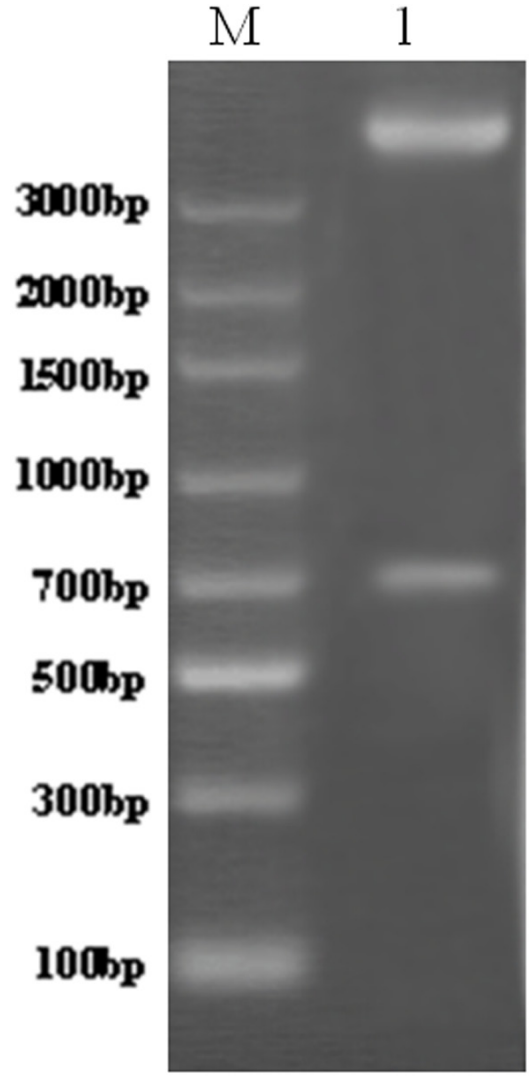

Figure 4. Profile of double digestion of the recombinant plasmid. Note: 1 is the product of double digestion of the pd20-TNFα recombinant plasmid; M is DNA marker.

PAGE was performed and the purity was detected.

In vitro activity detection

The activity of the fusion gene was detected using the L929 cytotoxity assay. The L929 cells were inoculated to the 96-well plate at 100 μl per well and cultured at 37°C in a 5% CO_2 incubator overnight until the cells reached over 80% confluence. Two wells were detected using each dose of the TNF standard, with 0.1 ml added into each well. After that, 4-fold gradient dilution was performed using DMEM medium containing 3% fetal bovine serum for each well. The samples were first prediluted to the initial concentration of about 100 1 U/ml, and then subjected to 4-fold gradient dilution to the final volume of about 100 μl/well. The cells were further cultured for 16 h. With crystal violet staining, the OD570 nm was measured and the activity of the sample was calculated by the following formula: potency of the standard × (predilution factor of the sample/predilution factor of the standard) × (half effective dilution of the standard/half effective dilution of the sample equivalent to the standard).

In vivo experiment in nude mice

The model of liver metastasis from gastric cancer in nude mice was established using in-situ tumor implantation [11]. The nude mice were

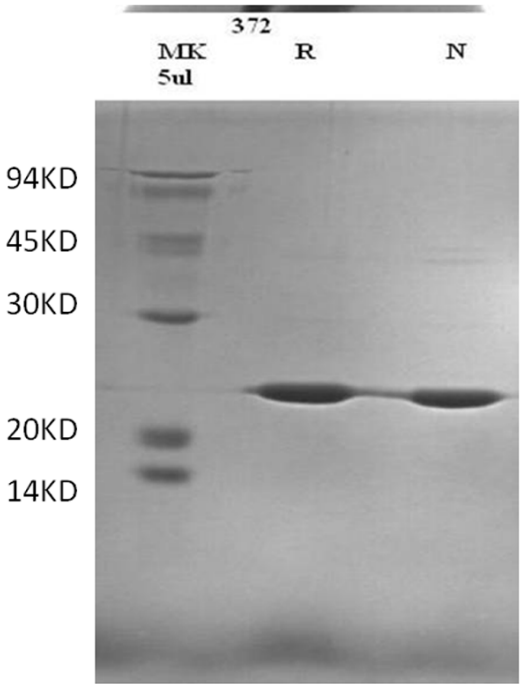

Figure 5. Induced expression of pd20-TNFα fusion protein. Note: R and N are the two replicates after 4 h of induced culture with pET28a(+)-pd20-TNFα vector; MK is the protein marker.

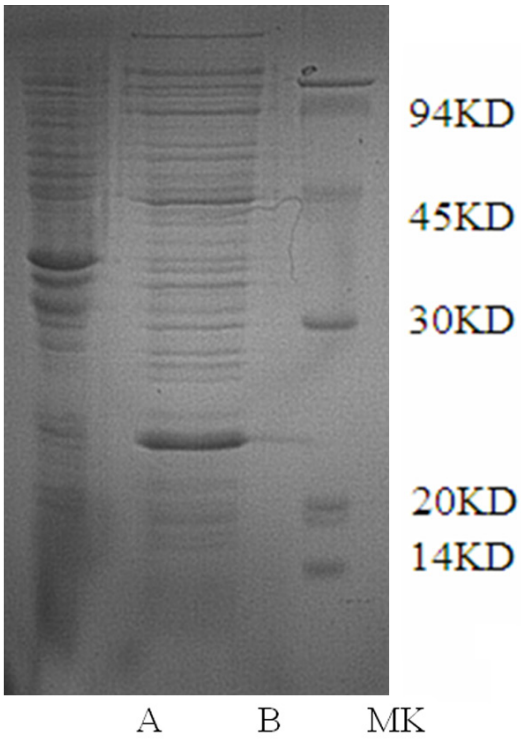

Figure 6. Solubility of the pd20-TNFα fusion protein. Note: A is the precipitate after 4 h of induced culture with pET28a(+)-pd20-TNFα vector; B is the supernatant after 4 h of induced culture with pET28a(+)-pd20-TNFα vector; MK is protein marker.

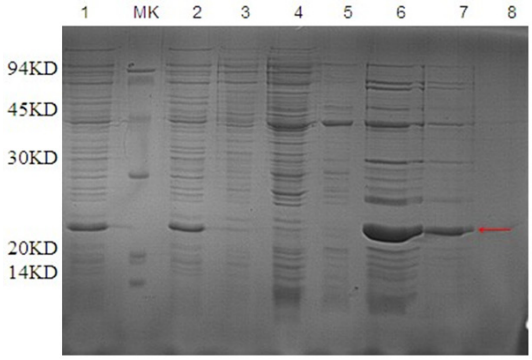

Figure 7. Purification of the pd20-TNFα fusion protein. Note: MK: Molecular weight standards, Lane 1: loading sample, Lane 2: Flow-through, Lane 3: Elution 1-1, Lane 4: Elution 1-2, Lane 5: Elution 1-3, Lane 6: Elution 2-1, Lane 7: Elution 2-2, Lane 8: Elution 2-3.

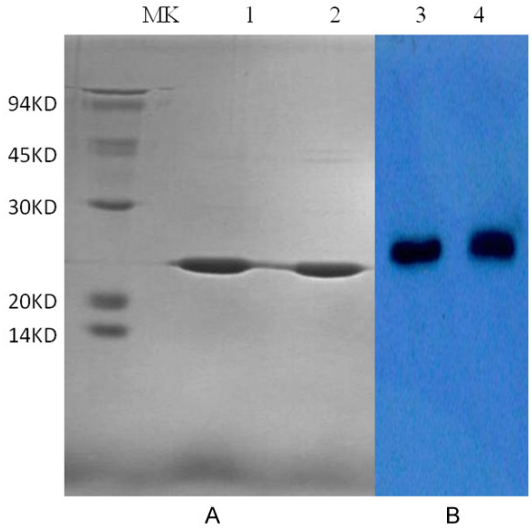

Figure 8. Western Blot detection of the pd20-TNFα fusion protein. Note: A is the PAGE image of the sample; B is WB image; 1-2 are purified protein; 3-4 are protein blots; MK is protein marker.

randomly divided into 3 groups (pd20-TNF fusion protein group 1.2 × 10^6 u/kg·d, TNF group 1.2 × 10^6 u/kg·d, normal saline group), with 10 mice in each group. For the prevention group, the drug was administered via the tail vein at the given dose the next day after inoculation every day for 7 days; for the treatment group, the drug was administered via the tail vein at the given dose 3 weeks after inoculation every day for 7 days. HE staining was performed for the tumors 4 weeks after inoculation. The tissues were observed under the light microscope, and the effect of pd20-rmhTNF fusion protein in preventing liver metastasis

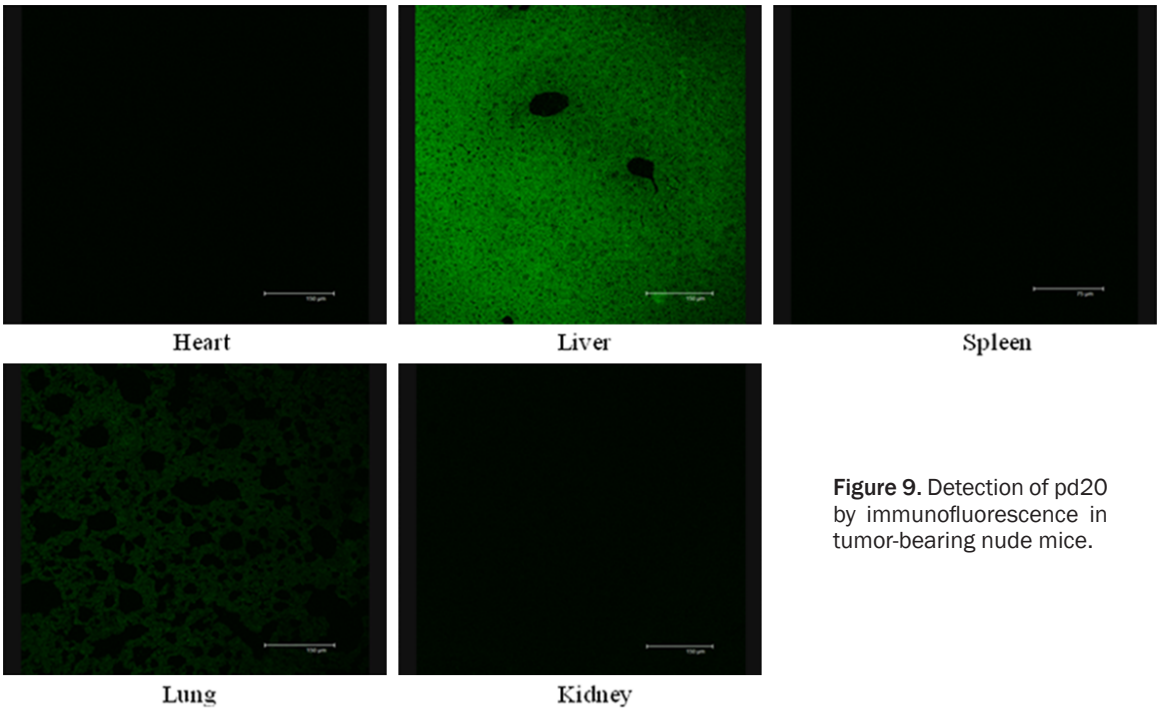

Figure 9. Detection of pd20 by immunofluorescence in tumor-bearing nude mice.

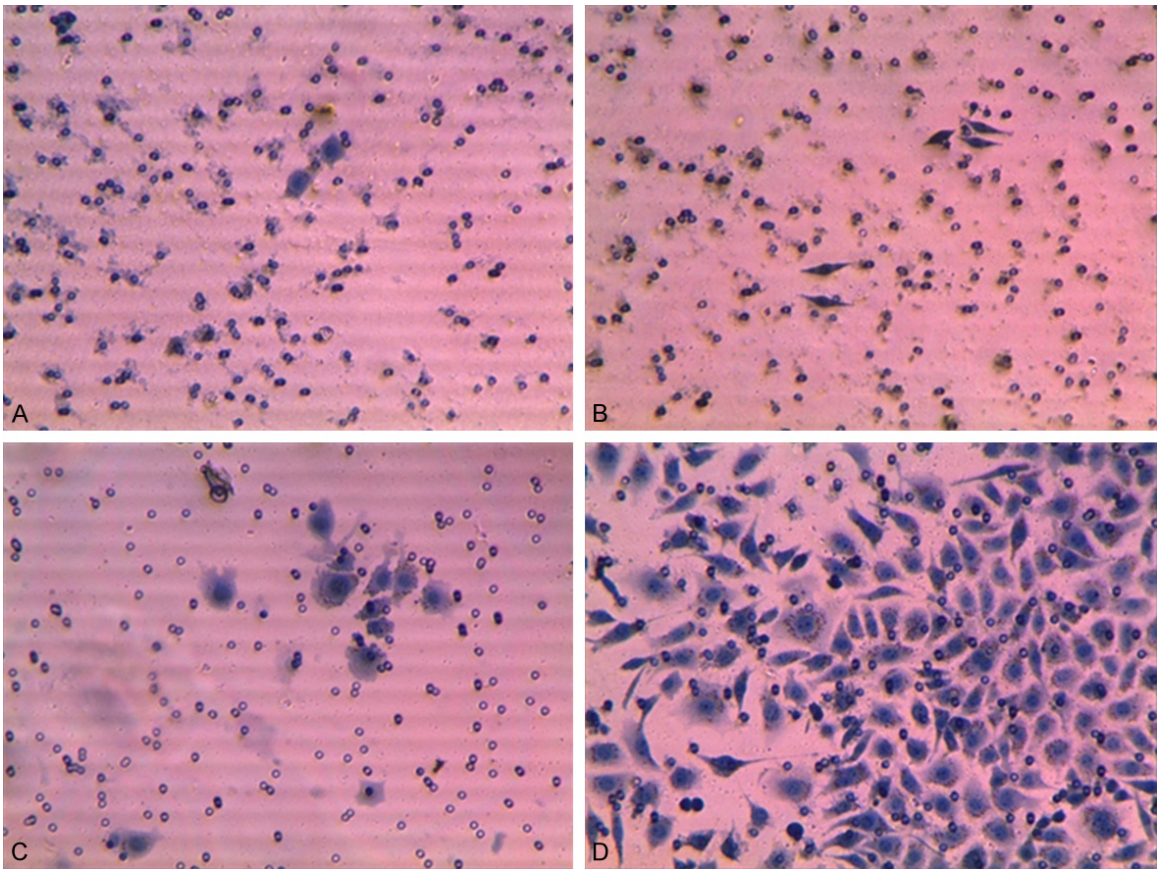

Figure 10. In vitro activity detection of the pd20-TNF fusion protein. Note: A was treatment group 1 with drug concentration of 0.002 mg/ml; B was treatment group 2 with drug concentration of 1/3*0.002 mg/ml; C was treatment group 3 with drug concentration of 1/3*1/3*0.002 mg/ml; D was the blank control group with no drug administered. The 3-fold dilution was performed in group 1, 2 and 3.

Table 1. The cell count and the cell membrane permeability in each group under the inverted microscope

Group	Cell count					Average cell membrane permeability
No. 1	5	0	2	1	2	2.0
No. 2	8	7	3	12	7	7.4
No. 3	18	15	9	30	28	20.0
No. 4	180	120	140	90	120	130.0

from gastric cancer in nude mice was evaluated.

Results

Cloning of pd20-TNF fusion gene

The full-length nucleic acid sequence of the pd20-TNFα was synthesized as follows: the nucleic acid sequence of the specific binding peptide pd20 was marked in blue; the nucleic acid sequence of TNF-α was in black and written in uppercase letters; the cutting sites for NdeI and XhoI were underlined and written in lowercase letters (**Figure 2A**).

The corresponding protein sequence was shown in **Figure 2B**.

PCR identification of the recombinant plasmid

The recombinant plasmid was identified by PCR, and the desired sequence was obtained (**Figure 3**).

Double digestion of the recombinant plasmid

The pET28a(+)-pd20-TNFα plasmid was identified by double digestion with EcoRI and BamH1. The length of the bands obtained was the same as expected (**Figure 4**), indicating that the target gene was successfully cloned to the pET28a(+) vector.

Prokaryotic expression of the pET28a(+)-pd20-TNFα fusion gene

The positive recombinant bacteria were cultured with IPTG for 4 h, and the thalli were collected by centrifugation and subjected to SDS-PAGE (**Figure 5**). It can be seen from the figure that the band of the fusion protein was distinct at about 21 kDa after the induced culture.

Detection of solubility of the fusion protein

The positive recombinant bacteria were cultured with IPTG for 4 h, and the thalli were collected by centrifugation. The thalli were disrupted ultrasonically and centrifuged, and the supernatant and the precipitate were collected separately for SDS-PAGE. The results are shown in **Figure 6**. From the supernatant collected from lysis, a band of about 21 kD was detected, but this band was indistinct for the precipitate. The length of this band was the same as the length predicted of the fusion protein. It was thus proved that the fusion protein was expressed and soluble in the supernatant after the induced culture with pET28a(+)-pd20-TNFα vector.

Purification of the fusion protein by Ni-affinity chromatography

After purification through the Ni-NTA column, the target fusion protein was obtained. Electrophoresis indicated that the protein purity reached over 85% (**Figure 7**).

Western blot detection of the fusion protein

The purified fusion protein was transferred to the NC membrane after SDS-PAGE (**Figure 8A**). The protein reacted with the mouse anti-TNF-α antibody and a distinct band appeared at 21.2 kD (**Figure 8B**). This indicated that the fusion protein was recognized by the mouse anti-TNF-α antibody after induced expression in *E. coli*.

Detection of binding specificity of peptide pd20 in tumor-bearing nude mice

The binding specificity of peptide pd20 in tumor-bearing nude mice was detected by immunofluorescence, and the result is shown in **Figure 9**. Immunofluorescence indicated that the expression of pd20-TNFα fusion gene was specific and strong in the liver tissue; pd20-TNFα fusion gene was weakly expressed in lung and not expressed in heart, spleen and kidney.

In vitro activity detection of the pd20-TNF fusion protein

The in vitro activity of the pd20-TNF fusion protein was detected according to the specified

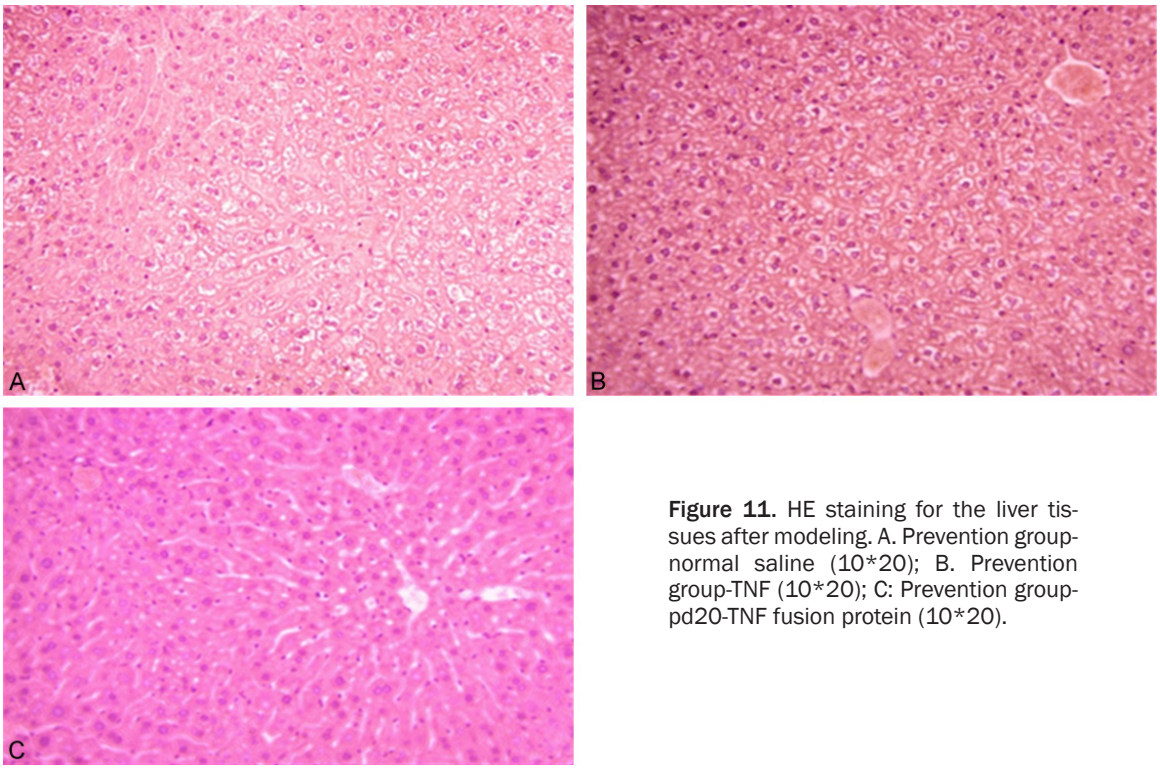

Figure 11. HE staining for the liver tissues after modeling. A. Prevention group-normal saline (10*20); B. Prevention group-TNF (10*20); C: Prevention group-pd20-TNF fusion protein (10*20).

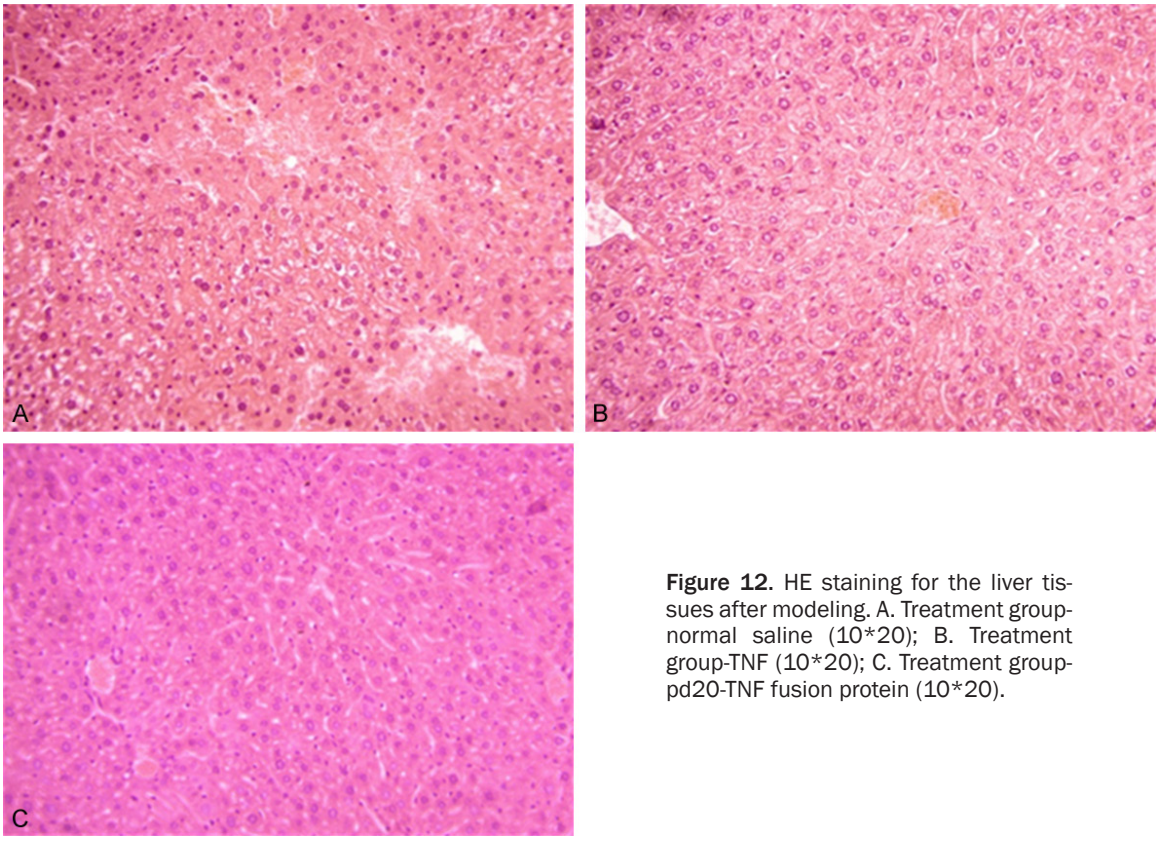

Figure 12. HE staining for the liver tissues after modeling. A. Treatment group-normal saline (10*20); B. Treatment group-TNF (10*20); C. Treatment group-pd20-TNF fusion protein (10*20).

procedures, and the result is shown in **Figure 10**.

The cell count and the cell membrane permeability in each group under the inverted micro-

scope are shown in **Table 1**. The in vitro protein activity detection indicated that the pd20-TNF fusion protein had high activity in vitro.

Effect of pd20-TNF fusion protein in preventing liver metastasis from gastric cancer in nude mice

Liver metastasis from gastric cancer was mimicked in nude mice by in-situ tumor implantation. The nude mice were randomly divided into 3 groups (pd20-TNF fusion protein group 1.2 × 10^6 u/kg·d, TNF group 1.2 × 10^6 u/kg·d, normal saline group), with 10 mice in each group. For the prevention group, the drug was administered via the tail vein at the given dose the next day after inoculation every day for 7 days. HE staining was performed 4 weeks after inoculation, and the tissue was observed under the light microscope (**Figure 11**).

HE staining showed that the best preventive effect was achieved in the group administered with pd20-TNF fusion protein with fewer cancer lesions; the second best effect was achieved in the group administered with TNF; the largest number of cancer lesions was seen in the normal saline group. Thus the pd20-TNF fusion protein had certain preventive effect on liver metastasis from gastric cancer in nude mice.

Effect of pd20-TNF fusion protein against liver metastasis from gastric cancer in nude mice

Liver metastasis from gastric cancer was mimicked in nude mice by in-situ tumor implantation. The nude mice were randomly divided into 3 groups (pd20-TNF fusion protein group 1.2 × 10^6 u/kg·d, TNF group 1.2 × 10^6 u/kg·d, normal saline group), with 10 mice in each group. For the prevention group, the drug was administered via the tail vein at the given dose the next day after inoculation every day for 7 days. HE staining was performed 4 weeks after inoculation, and the tissue was observed under the light microscope (**Figure 12**).

HE staining showed that the best preventive effect was achieved in the group administered with pd20-TNF fusion protein with fewer cancer lesions; the second best effect was achieved in the group administered with TNF; the largest number of cancer lesions was seen in the normal saline group. Thus the pd20-TNF fusion protein had certain treatment effect on liver metastasis from gastric cancer in nude mice.

Discussion

Targeted cancer therapy involves the design of the drug targeting at specific cancer-related proteins or gene fragments, which is delivered to the targeted site using vectors. In the targeted therapy, only the cancer cells are killed and the normal cells are left unaffected [12, 13]. Though new targeted drugs are being developed constantly, trastuzumab is by far the only targeted drug having significant efficacy against advanced gastric cancer according to phase III clinical trials. Trastuzumab is a recombinant DNA-derived humanized monoclonal antibody which works by selectively acting on the extracellular region of HER-2, blocking the downstream signal transduction. However, only about 20% of the gastric cancer patients can benefit from trastuzumab [14-16]. Gastric cancer is associated with complex biological mechanism, and identifying more biomarkers with higher sensitivity and specificity and developing the targeted drugs on this basis are the primary concerns [17, 18]. In this study, the specific binding peptide pd20 of gastric cancer cells with a high potential of liver metastasis was fused with human TNF to obtain the pd20-TNF gene using DNA recombinant technique. The expression of the fusion protein was induced, and the fusion protein was purified. In vitro activity detection indicated that the fusion protein greatly improved the membrane permeability of the liver cells in nude mice with liver metastasis from gastric cancer. As indicated by in-situ tumor implantation experiment, the fusion protein effectively reduced the cancer lesions. This result lays the theoretical and experimental foundation for developing targeted drugs against liver metastasis from gastric cancer.

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