

# A novel recombinant immunocasp-6 fusion gene specifically and efficiently suppresses HER2-overexpressing osteosarcoma

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**Abstract.** Osteosarcoma is the most common primary malignant tumor of bone for adolescent or children. The poor prognosis of patients, due to its remote metastasis, has led to the exploration of more effective and less toxic treatments. Immunotherapy is a promising strategy for the treatment of human epidermal growth factor receptor 2 (HER2)-overexpressing tumors. Herein, we describe experiments conducted with a fusion gene, immunocasp-6, which was generated by fusing a HER2-specific single-chain Ab, a single-chain *Pseudomonas* exotoxin A and an active caspase-6 which can directly cleave lamin A leading to nucleus damage inducing programmed cell death. We demonstrated that immunocasp-6 can specifically and efficiently recognize and induce apoptosis in HER2-overexpressing osteosarcoma cells *in vitro*. The immunocasp-6 was transferred into BALB/c athymic mice bearing human osteosarcoma by i.m. injection of liposome-encapsulated pCMV-immunocasp-6. Expression of immunocasp-6 not only strongly inhibited tumor growth and significantly prolonged animal survival, but also greatly prevented tumor metastasis. Our data showed that the immunocasp-6 can specifically recognize HER2-overexpressing osteosarcoma cells, can also promptly attack their nucleus and induce apoptotic death, suggesting the potential of this strategy for the treatment of human HER2-overexpressing tumors.

## Introduction

The human epidermal growth factor receptor-2 (HER2), a member of the epithelial growth factor receptor family, transduces cell signaling and plays key roles in cell differentiation, adhesion, and motility (1). Sufficient evidence has suggested that patients with HER2-overexpressing tumors exhibit a reduced response to conventional treatments (2). The HER2 protein is reportedly overexpressed in several human malignant tumor, including human breast and ovarian cancer (3), salivary gland adenocarcinoma (4), gastric cancer (5) and osteosarcoma (6-9). Since it is overexpressed in tumor cells but is not detected in normal cells, HER2 is an ideal target molecular for cancer gene therapy to exploit differences at the molecular level between normal and malignant cells (10).

Caspases are vital elements in transferring apoptotic signals and executing apoptosis in mammalian cells (11). Caspase-6 is one of effective caspases during the cell apoptotic program (12). Activation of caspase-6 induces apoptosis by cleaving lamin A and other substrates (13). Unlike its wild-type zymogen counterpart, active caspase-6 constructed with subunits in reverse order, is capable of autocatalytic processing *in vitro* independent of apoptotic signals, and can induce apoptosis of tumor cells, which thereby makes it an attractive candidate for gene therapy (14).

As a well-recognized Ab, e23sFv, derived from a mouse mAb against human HER2, has been confirmed to bind the extracellular domain of HER2 protein with high affinity and to be internalized by endocytosis (15,16). The highly specific antibody to antigen suggests that we can construct a fusion gene, immunocasp-6, consisting of NH<sub>2</sub>-terminal leader sequence to promote secretion of the recombinant immunocasp-6 fused with an anti-HER2 single-chain Ab, the translocation domain (domain II) of *Pseudomonas* exotoxin A (PEA) and an active caspase-6, to specifically and efficiently suppress the HER2 overexpressing tumors. PEA is a single-chain toxin consisting of three major domains (I, II and III) responsible for binding of the molecule to target cells, translocation of the molecule to the cytosol, and the induction of cell death, respectively (17).

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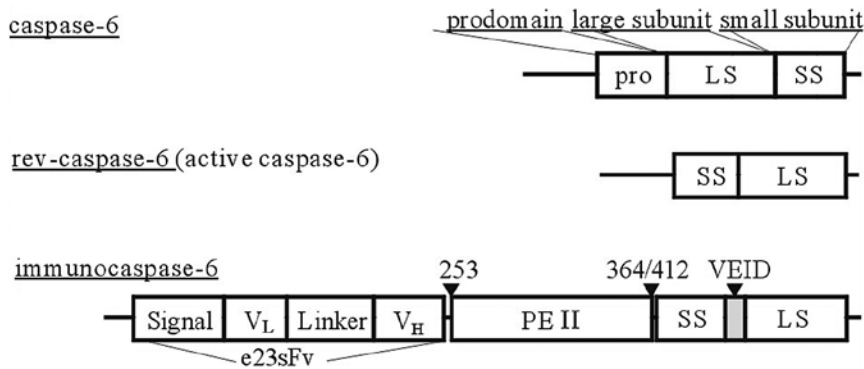


Figure 1. Schematic diagram of immunocaspase-6 constructs. Immunocaspase-6 construct was generated by fusing the e23sFv gene in frame with the 5' end of the fusion genes encoding the PEA domain II (amino acids 253-412) and active caspase-6.

Domain II of PEA has been reported to efficiently transfer the cellular toxicity domain to the cytoplasm (18-20). By replacing the cellular toxicity domain of PEA with active caspase-6, we sought to translocate the caspase into tumor cells in which it would induce tumor cell apoptosis. Even though this novel immunocaspase-6 has been proven to be effective in inducing apoptosis in the HER2-overexpressing human breast tumor cell line, SKBR-3, their effects on human osteosarcoma is still unclear. Thus the purpose of the present study is to extend our immunocaspase-6 strategy to the treatment of osteosarcoma *in vitro* as well as *in vivo* and to verify that the immunocaspase-6 can specifically and efficiently suppress the HER2-overexpressing tumors.

## Materials and methods

**Plasmid and DNA construct.** Recombinant immunocaspase-6 was generated by sequential fusion of the genes of a signal peptide (Met-Lys-His-Leu-Trp-Phe-Phe-Leu-Leu-Leu-Val-Ala-Ala-Pro-Arg-Trp-Val-Leu-Ser-) consisting of a single chain HER2 antibody (e23sFv), a *Pseudomonas* exotoxin A (PEA) translocation domain (from aa 253 to 412) and an active caspase-6 (Fig. 1). The immunocaspase-6 was cloned into a pCMV plasmid, namely pCMV-immunocaspase-6.

**Cell culture and transfection.** Human osteosarcoma cell line SOSP-9607-E10, with relatively high metastatic potential, was derived from a 17-year-old male patient who had been diagnosed of tibial osteosarcoma and underwent osteotomy and established from these cells by continuous *in vitro* cultivation for over 120 transfer generations in one year. SOSP-9607-E10 cells were maintained in DMEM or RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 4 mmol/l L-glutamine. At 24 h before transfection, cells were seeded in 12 or 96-well plates at  $1 \times 10^5$  or  $5 \times 10^3$  cells per well. pCMV-immunocaspase-6 or pCMV vector alone, 1  $\mu$ g, encapsulated by 2  $\mu$ l Lipofectamine 2000 (Invitrogen) were mixed, incubated for 20 min at room temperature to form DNA-liposome mixture. Then the mixture was administered to cells and incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub> for 6 h, then the medium was removed and cells were resuspended in complete medium.

**Cell viability assay.** Viability of the transiently transfected cells was tested by using the 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, after the cells adhered to the low lamber of 96-well plates, the cells were divided randomly into three groups, namely the mock group, the control group and the immunocaspase-6 group, and transfected with pCMV-immunocaspase-6 or pCMV vector. Thereafter, the cells were cultured in 96-well plates for 12 to 96 h, then incubated with 20  $\mu$ l of 1.5 mg/ml MTT for 4 h. After that, the cells were treated with 150  $\mu$ l DMSO. OD at A490 nm were determined using the Sunrise microplate reader (Tecan). Each assay was performed in triplicate on at least three independent occasions.

**Flow cytometry assay for detection of apoptosis.** SOSP-9607-E10 cells were seeded at a density of  $1 \times 10^5$  cells per well on slides in 12-well Costar transwell plates, and transfected with pCMV-immunocaspase-6 or pCMV vector when cell number was at a density of  $3 \times 10^5$  cells per well. After 48 h of transfection, cells in the lower chamber were collected, stained with Annexin V-FITC/PI following standard procedures and finally analyzed by FCM.

**Immunofluorescence.** After transfection SOSP-9607-E10 cells were incubated in complete medium for an additional 48 h. Then, the cells in the lower chamber were fixed in paraformaldehyde solution (4% in phosphate buffered saline (PBS), pH 7.4), permeabilized with PBS containing 0.1% Triton X-100, and blocked with 2% normal rabbit serum. Then the cells were stained with antibodies recognizing caspase-6 (C20, 1:200; Santa Cruz Biotechnologies) as the primary antibodies, with biotin-linked anti-goat IgG (1:100; Santa Cruz Biotechnologies) and FITC-linked anti-rabbit IgG (1:100; Sigma) as the secondary antibodies. The staining was examined using a fluorescence microscope (Japanese Olympus Co.).

**Electronic microscopy assay.** SOSP-9607-E10 cells were harvested 48 h after transfection, and then fixed in 2.5% glutaraldehyde, dehydrated and embedded to observe morphologic change with a transmission electron microscope.

**Immunohistochemistry assay.** The transfected cells were cultured on coverslips as mention above, and then fixed with a freshly prepared paraformaldehyde solution for 30 min at room temperature, and permeabilized with 0.1% Triton X-100 for 15 min on ice.

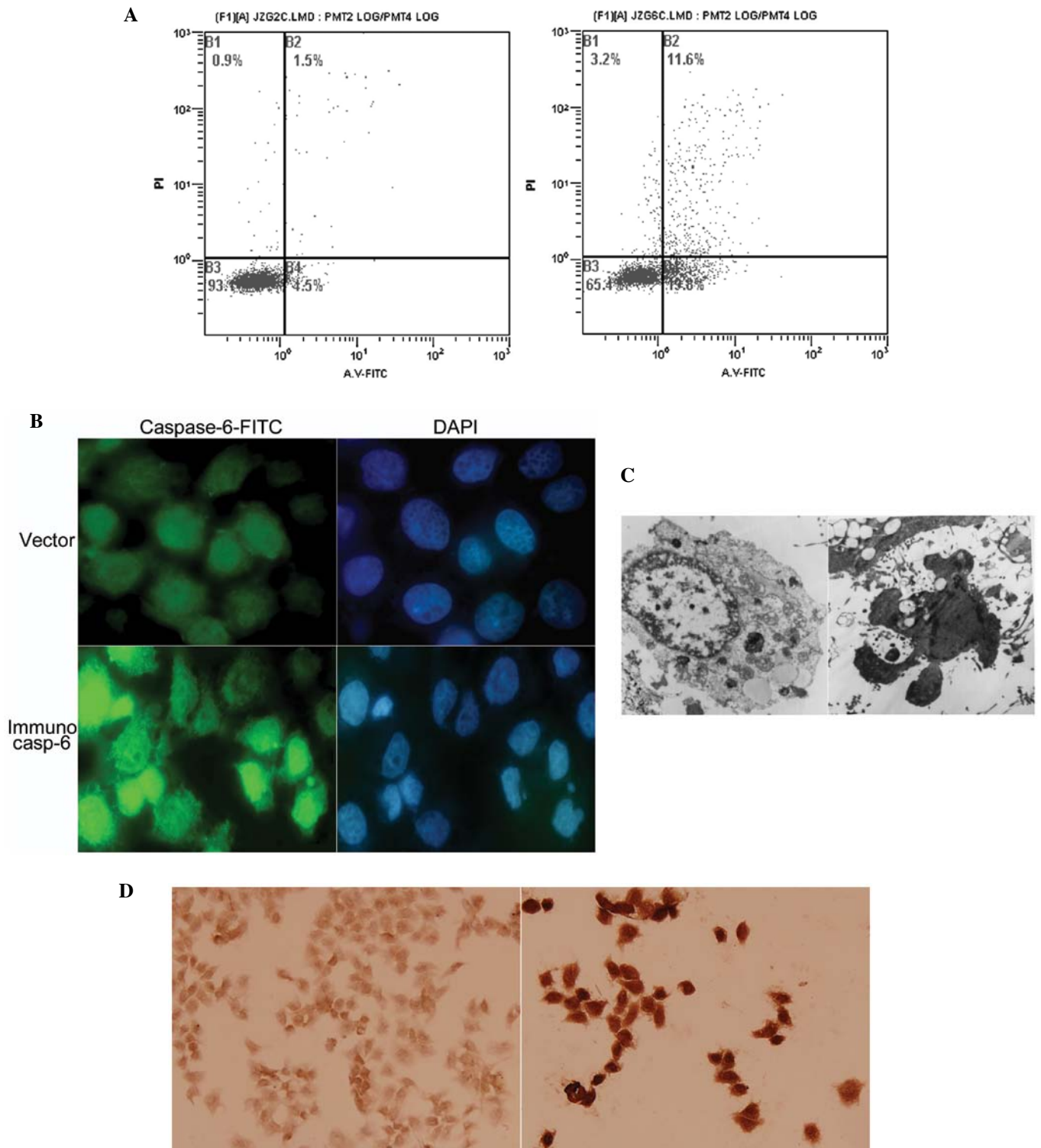


Figure 2. Immunocasp-6 induced apoptosis in HER2-overexpressing osteosarcoma cells. SOSP-9607-E10 cells were transiently transfected with immunocasp-6, and cell viability were tested using Annexin V-FITC staining (A). Morphological change of transfected cells were tested by immunofluorescence assay (B), and electronic microscopy (C). Immunohistochemistry staining was performed on the transfected SOSP-9607-E10 cells with anti-caspase-6 antibody (D). The left part of (A-D) is the result of cells in the vector group while the right part is the result of cells in the immunocasp-6 group.

Xenograft tumors and muscle tissues were fixed in paraformaldehyde solution and embedded in paraffin after treatment, paraffin-embedded tissue sections were dewaxed, hydrated, and incubated in 0.3% methanol-H<sub>2</sub>O<sub>2</sub> for 20 min to remove endogenous peroxidase. Next, they were dried and blocked for 1 h with the appropriate serum in a humidified chamber. Primary antibody was added overnight at 4°C.

The samples were probed with primary antibody-recognizing caspase-6 (C20, 1:200; Santa Cruz Biotechnologies), followed by biotin-linked antirabbit IgG (1:100; Santa Cruz Biotechnology) as the secondary antibody and then processed with the Vectastain Elite ABC kit per the manufacturer's instructions prior to digital photography under an Olympus Eclipse E600 microscope with a Spot RT slider camera and imaging software.

Table 1. SOSP-9607-E10 cells transiently transfected with immunocasp-6, and cell OD value (490 nm) generated using the MTT assay (mean ± SD, h).

Time after transfection (h)	n	12	24	36	48	60	72	84	96
Mock <sup>a</sup>	3	0.580±0.107	0.663±0.915	0.801±0.099	0.992±0.117	1.039±0.063	1.048±0.177	1.205±0.113	1.322±0.098
Vector <sup>b</sup>	3	0.603±0.132	0.663±0.117	0.907±0.069	1.071±0.115	1.065±0.188	1.076±0.260	1.215±0.116	1.341±0.180
Immunocasp-6 <sup>c</sup>	3	0.541±0.053	0.564±0.060	0.644±0.524	0.775±0.081	0.601±0.165	0.595±0.240	0.562±0.123	0.458±0.214

<sup>a,c</sup>P=0.013, <sup>b,c</sup>P=0.007, <sup>ab</sup>P=0.989 (comparison between every two groups).

*TUNEL staining.* TUNEL staining was performed on paraffin sections, using the TdT-FragEL™ DNA Fragmentation Detection kit (Calbiochem) in accordance with the manufacture's instructions. Hematoxylin was used to counterstain the sections.

*Antitumor activity of immunocasp-6 in vivo.* Six- to eight-week-old BALB/c athymic mice were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch (Shanghai, China), and were cared and used in compliance with institutional guidelines. The mice were inoculated s.c. with 2x10<sup>6</sup> SOSP-9607-E10 cells. Tumors were allowed to grow until they reached a diameter of 5-7 mm (day 0). The mice were then randomly divided into different two groups, namely the immunocasp-6 group and vector group.

The mice bearing SOSP-9607-E10 tumors were subjected to liposome-mediated immunocasp-6 or vector treatments. pCMV-immunocasp-6 or pCMV vector alone, 10 µg, encapsulated by 20 µl Lipofectamine 2000 was administered i.m. to mice. Nine mice were utilized for each treatment, each mouse was administered every 3 days for a total of five times. The volume of the tumor, the body weight of the mice and the net weight of the tumor were observed and analyzed by statistics.

*Assessment of immunocasp-6 effects on human osteosarcoma lung metastasis.* Eighteen athymic, six- to eight-week-old Balb/c mice were inoculated with 2x10<sup>5</sup> SOSP-9607-E10 cells into thighbone marrow. The mice were then divided randomly into two groups, nine mice in each group, for i.m. liposome-mediated immunocasp-6 or vector treatments as indicated above. The treatment was performed once every 3 days for two weeks, then once a week thereafter for five weeks. The number of the neonatal tumors in lungs were counted, mouse survival times were recorded and the neonatal mass was tested by H&E staining.

*Statistical analyses.* The data are expressed as the mean ± SD. Statistical analyses were performed with the SPSS13.0 software package for Windows (SPSS, Chicago, IL). Cell viability assay were analyzed by the analysis of covariance (ANCOVA, dunnett T3) method. The volume of the tumor, the body weight of the mice and the net weight of the tumor were analyzed using independent-samples t-test (the data of the volume of the tumor and the body weight of the mice were obtained from the data before treatment subtracted by the data after treatment). The survival rates were analyzed using the Kaplan-Meier method, and comparisons among treatment groups were obtained using the log-rank test. Statistical significance was based on a value of P<0.05.

**Results**

*Immunocasp-6 effectively and specifically suppresses the growth of SOSP- 9607-E10 cells in vitro.* Active caspase-6 is expressed and secreted from the transfected cells, binds to HER2-overexpressing breast tumor cells, internalizes, undergoes autoprocessing between PEA Arg279 and Gly280, and induces cell apoptosis (21). To investigate whether the same cytotoxic effect could be achieved in osteosarcoma cells, we first tested the cell viability of the transiently transfected

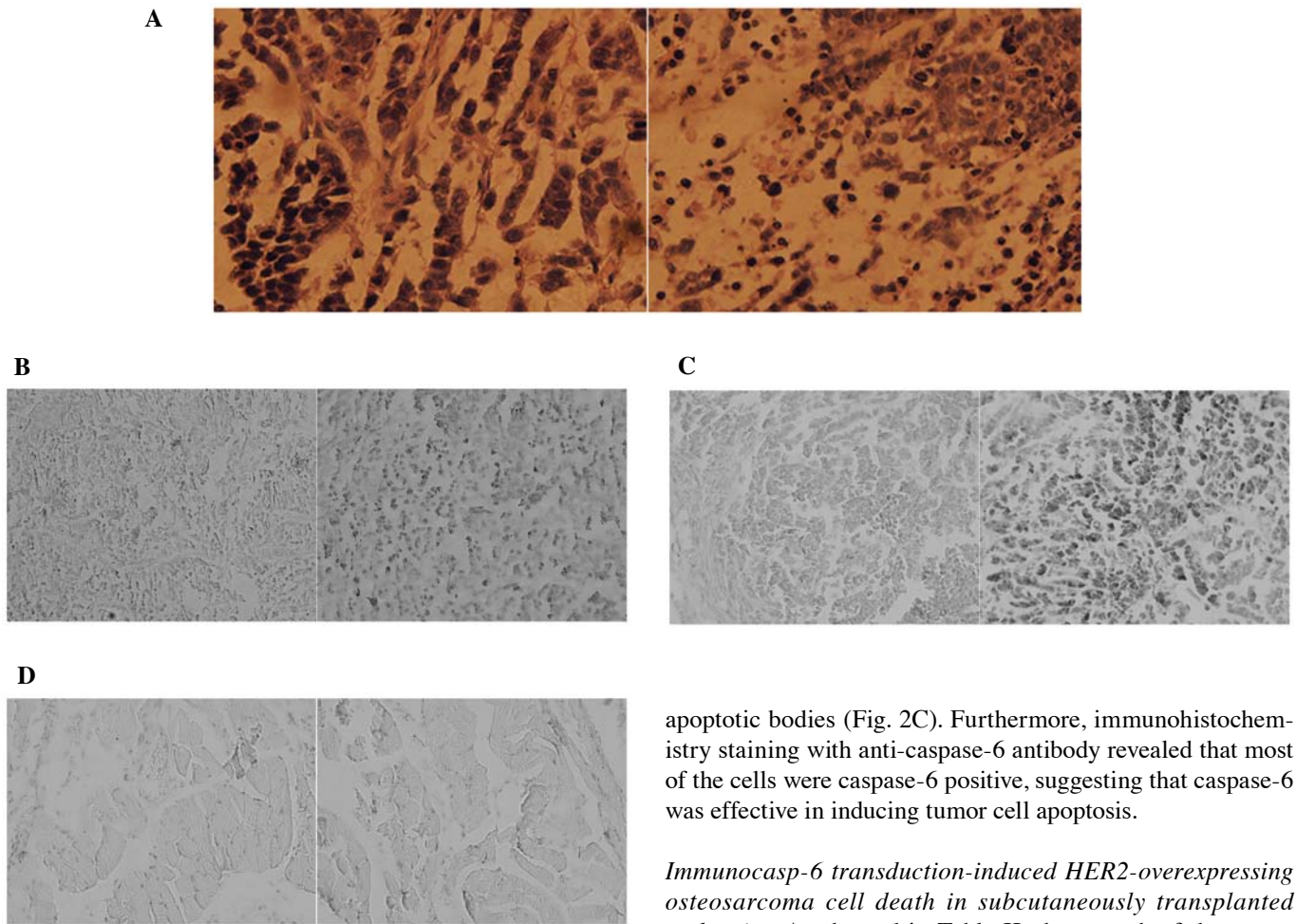


Figure 3. Immunocasp-6 suppressed the growth of osteosarcoma *in vivo*. The SOS-9607-E10 nude mice were randomly divided into two groups. They received i.m. injections of 10  $\mu$ g of lipofectamine-encapsulated pCMV-immunocasp-6 or pCMV vector plasmid in the right posterior limb every 3 days for a total of five times. The body volume of the tumor, body weight of the mice and net weight of the tumor were observed and analyzed by statistics (Table II). The tumor tissues were collected and subjected to H&E staining (A), TUNEL staining (B). The tumor and muscle tissues were tested by immunohistochemistry staining with caspase-6 antibody (C and D). The left part of (A-D) is the result of tissue in the vector group, while the right part is the result of tissue in the immunocasp-6 group.

cells by MTT assay, Table I shows that transient expression of the immunocasp-6 led to an apparent delay in cell viability. In other words, tumors cells in the immunocasp-6 treatment group have weaker viability than those in the mock or control group ( $P=0.013$ ,  $P=0.007$ , respectively), while there is no significantly difference between the mock group and control group ( $P=0.989$ ).

The Annexin V-FITC staining 48 h after the transfection revealed that the percentages of apoptotic cells in the immunocasp-6 group were 31.4%, while only 6% in the vector group (Fig. 2A). When tumor cell growth was stunted, the morphological change of transfected cells were tested. Immunofluorescence test discovered that transiently transfected cells had enriched or chipped nuclear (Fig. 2B). Transmission electron microscopy presented typical apoptotic changes in cells, including chromatin condensation and its margination at the nuclear periphery, cellular shrinkage and blebbing, and formation of so-called

apoptotic bodies (Fig. 2C). Furthermore, immunohistochemistry staining with anti-caspase-6 antibody revealed that most of the cells were caspase-6 positive, suggesting that caspase-6 was effective in inducing tumor cell apoptosis.

*Immunocasp-6 transduction-induced HER2-overexpressing osteosarcoma cell death in subcutaneously transplanted nude mice.* As showed in Table II, the growth of the tumor in the liposome-mediated pCMV-immunocasp-6 treatment group was significantly slower than that of the pCMV vector group ( $P=0.001$ ), the body weight of the mice in the immunocasp-6 group was significantly heavier than that of the vector group ( $P=0.0002$ ), and the net weight of the tumor in the immunocasp-6 group was significantly less than that of the vector group ( $P=0.0006$ ). Then the osteosarcoma tissues were collected and H&E (hematoxylin and eosin) stain was performed. The staining discovered tumor tissues were in poor condition in which most of tumor tissue was dead (Fig. 3A). Furthermore, terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) staining, demonstrated that most of the tumor tissue in the immunocasp-6 group were in the state of apoptosis (Fig. 3B). Thereafter, immunohistochemistry analysis confirmed the presence of caspase-6 in tumor tissues in the immunocasp-6 group, but not in those treated with vector (Fig. 3C) and muscle tissues in either immunocasp-6 group or vector group (Fig. 3D). These findings suggest that immunocasp-6 can specifically and efficiently induce tumor tissue apoptosis.

*Inhibitory effect of immunocasp-6 on lung metastasis of HER2-overexpressing osteosarcoma in vivo.* The neonatal mass were diagnosed as osteosarcoma by H&E staining (data not shown). As shown in Fig. 4A, the number of neonatal tumors in lungs in the immunocasp-6 group was significantly less than that in the vector group. Furthermore, the mice in the immunocasp-6 group survived longer than that in the vector group (Fig. 4B). SOS-9607-E10 tumors were more likely to metastasize to the

Table II. Comparison between control and treatment group of the tumor volume, tumor net weight and mouse body weight (mean  $\pm$  SD).

Group	n	Tumor volume (mm <sup>3</sup> )	Tumor net weight (g)	Mouse body weight (g)
Control	9	975.09 $\pm$ 49.76	1.08 $\pm$ 0.16	6.20 $\pm$ 1.14
Treatment	9	376.01 $\pm$ 265.18 <sup>a</sup>	0.64 $\pm$ 0.18 <sup>b</sup>	4.07 $\pm$ 0.49 <sup>c</sup>

<sup>a</sup>P=0.001, <sup>b</sup>P=0.0006, <sup>c</sup>P=0.0002 (compared with the control group).

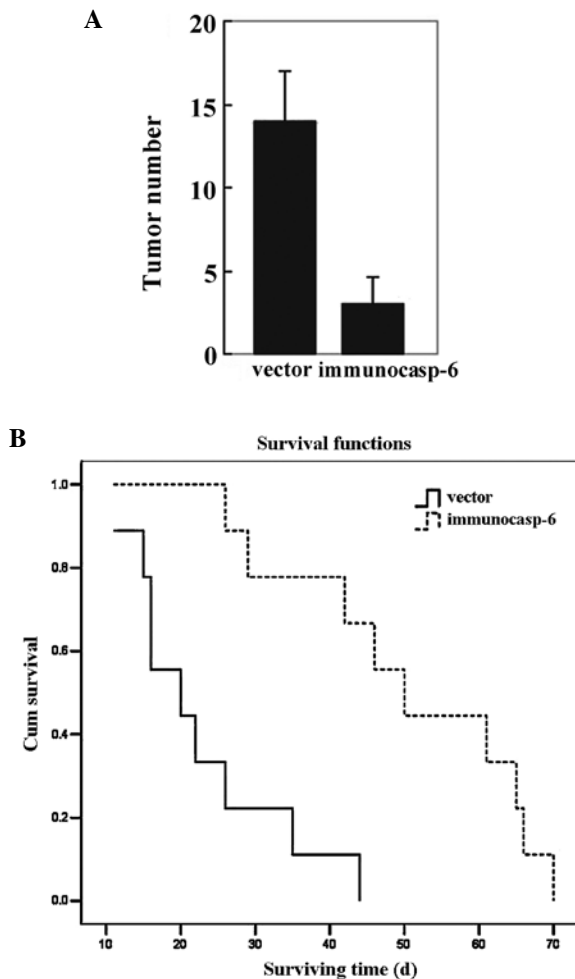


Figure 4. Immunocasp-6 treatment suppresses the metastasis of HER2-overexpressing osteosarcoma *in vivo*. Tumor numbers in lung tissue were counted (A). Survival rates of immunocasp-6 treated and control mice were evaluated (B).

lung in the absence of immunocasp-6 treatment, suggesting that immunocasp-6 can prevent or slow osteosarcoma metastasis.

## Discussion

Many strategies of gene therapy have been designed to kill cancer cells, including their transduction with suicide genes or tumor suppressor genes or activation of the immune system against the tumor cells (22-25). Although many problems have impeded the practical use of gene-based therapy for human

cancers, the search for safe, effective and tissue-specific gene therapies continues. In the past decade, we carried out a series of studies on antibody-directed and cell-mediated cancer immunotherapy by combining the specificity of antibodies and the potent cytotoxicity of pro-apoptotic proteins. Up to now, a number of pro-apoptotic effectors, including caspase-3 (26), caspase-6 (21), granzyme B (27), tBid (10) and apoptosis inducing factor (AIF) (28), have been used to construct immunoproapoptotic proteins and have been confirmed to be efficient in inducing targeted apoptosis both *in vitro* and *in vivo*. In the present study, we generated a novel immunocasp-6 gene construct by fusing a leader sequence, single-chain HER2 (e23sFv) Ab and the translocation domain of PEA to the active caspase-6 and extended the strategy to HER2 overexpression osteosarcoma.

In the study, we verified that the HER2-targeted suppressing effect of the novel immunocasp-6 on osteosarcoma cells *in vitro*. Firstly, we transfected the fusion gene into SOSP-9607-E10 cells and compared the vitality of these tumor cells. The effective destruction of SOSP-9607-E10 cells by the novel immunocasp-6 was confirmed by both MTT assay and flow cytometry assay. These findings suggested that the immunocasp-6 can strongly inhibit the growth of SOSP-9607-E10 cells. Morphological examination including immunofluorescence assay and electron microscopy verified that SOSP-9607-E10 cells in the immunocasp-6 group presented the typical characteristics of apoptosis, which suggested that immunocasp-6 might inhibit the growth of the tumor cells by inducing tumor cell apoptosis. In order to further explore the factors responsible for tumor cell apoptosis, the immunohistochemistry analysis were applied. The findings verified that caspase-6 can lead to tumor cell apoptosis. Thus, it can be concluded that the novel immunocasp-6 can specifically recognize the HER2-overexpressing osteosarcoma cells, induce tumor cell apoptosis and strongly inhibit the growth of tumor cells.

In order further to verify HER2-targeted suppressing effect of the novel immunocasp-6 on the osteosarcoma cells *in vivo*, the mouse SOSP-9607-E10 tumor xenograft model was constructed. The mice in the immunocasp-6 group showed better condition than that in the vector group, the growth of the tumor became slower (P=0.001), the weight of the nude mice was heavier (P=0.0006), the net weight of the tumor was lighter (P=0.0002), which suggested the immunocasp-6 can suppress the growth of the tumors. Thereafter, H&E staining and TUNEL staining revealed the tumor tissue treated by immunocasp-6 was also in poor condition and presented the character of apoptosis, suggesting that immunocasp-6 suppressed the growth of the tumor by inducing tumor tissue apoptosis. Furthermore, immunohistochemistry analysis

confirmed the presence of caspase-6 in tumor tissues treated with immunocasp-6, while caspase-6 were not found in those treated with pCMV vector and muscle tissues in either treatment group or control group. Thus, it can be concluded that the novel immunocasp-6 can specifically recognize and efficiently suppress the growth of HER2-overexpressing osteosarcoma, without damaging the normal tissues.

Moreover, in order to verify if the immunocasp-6 can prevent the metastasis of SOSP-9607-E10 tumors, we counted the number of neonatal tumors in lungs after mice died and recorded the survival time. The findings showed that the neonatal tumors in lungs in the immunocasp-6 group were significantly less than that in the vector group. Furthermore, the mice in the immunocasp-6 group survived longer than that in the vector group. SOSP-9607-E10 tumors were more likely to metastasize to the lung in the absence of immunocasp-6 treatment suggesting that immunocasp-6 prevents or slows osteosarcoma metastasis.

In summary, we described a novel immunocasp-6 therapeutic gene construct, which can kill HER2-overexpressing osteosarcoma specifically and efficiently; this novel immunocasp-6 holds promise for the generation of a novel therapy for HER2-overexpressing tumors.

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