Combination of targeting gene-viro therapy with recombinant Fowl-pox viruses with HN and VP3 genes on mouse osteosarcoma

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Abstract. – BACKGROUND: Osteosarcoma is an aggressive cancerous neoplasm arising from primitive transformed cells of mesenchymal origin that exhibit osteoblastic differentiation and produce malignant osteoid. With the rapid development of tumor molecular biology, gene and viral therapy, a highly promising strategy for the treatment, has shown some therapeutic effects.

OBJECTIVES: To study the strategy of cooperative cancer gene therapy, previously, we explored the antitumor effects of recombinant Fowl-pox viruses (FPVs) with both HN (hemagglutinin-neuramidinase) and VP3 genes on mouse osteosarcoma.

MATERIALS AND METHODS: We constructed vFV-HN, vFV-VP3 and vFV-HN-VP3 inserting CAV VP3 gene, NDV HN gene into fowlpox virus. S180 osteosarcoma were transfected with Recombinant Fowl-pox viruses (FPVs). These cell lines stably expressing tagged proteins were selected by culturing in medium containing puromycin (2 µg/ml) and confirmed by immunoblotting and immunostaining. S180 osteosarcoma model with BALB/c mice and nude mice were established and the vFPV viruses as control, vFV-HN, vFV-VP3, vFV-HN-VP3 were injected into the tumor directly. The rate of tumor growth, tumor suppression and the sialic acid levels in serum were examined and the tumor tissues were analyzed by the method of immunohistochemistry. Flow cytometric analysis was performed using a FACSCalibur flow cytometer. A total of 100,000 events were analyzed for each sample and the experiment was repeated at least twice.

RESULTS: Our data indicated that vFV-HN, vFV-VP3 and vFV-HN-VP3 all had growth inhibition effects, the inhibition rate of vFV-HN-VP3 group was 51.7%, which was higher than that of vFV-HN, vFV-VP3 group and control group ($p < 0.01$). The sialic acid level of vFV-HN-VP3 group in mouse serum was 4.22 ± 0.27 mmol/l, which was lower than that of other groups ($p < 0.01$).

CONCLUSIONS: These results suggest that genes into mouse osteosarcoma cancer cells can cause cell a specificity anti-tumor immune activity, suppress tumor growth, and increase the survival rate of the tumor within host.

Introduction

Osteosarcoma is an aggressive cancerous neoplasm arising from primitive transformed cells of mesenchymal origin that exhibit osteoblastic differentiation and produce malignant osteoid[1,2]. It is the most common histological form of primary bone cancer and is the eighth most common form of childhood cancer, comprising 2.4% of all malignancies in pediatric patients, and approximately 20% of all primary bone cancers[3,4]. There is a preference for origination in the metaphyseal region of tubular long bones, with 42% occurring in the femur, 19% in the tibia, and 10% in the humerus[5,6]. About 8% of all cases occur in the skull and jaw, and another 8% in the pelvis[7]. Neoplastic cells arise osteoblastic differentiation and

form tumoral bone, and have the high incidence, the high malignant degree, the high rate of disability and the high-speed migration. Lung metastasis of osteosarcoma will occur rapidly without therapy and the patients will die within 18 to 24 months after contracting this disease[8]. The osteosarcomatous patient must face to resect the trouble bone, even amputation for saving his life. And sometimes amputation is the inevitable way of the large focal lesion, but the five-year survival rate of the osteosarcomatous amputation is only 17.4% to 21.8% to the early and mid-term patients[9].

Gene and viral therapy, a highly promising strategy for the treatment, has shown some therapeutic effects, but the therapeutic effect *in vivo* is less obviously than *in vitro*, still having been a lack of real breakthrough[10]. Osteosarcomatous is due to involve the high heterogeneity caused by the interaction of multifactor[11].

Apoptin is a chicken anemia virus-derived, p53-independent, bcl-2-insensitive apoptotic protein with the ability to specifically induce apoptosis in tumor cells[12]. The avian paramyxovirus Newcastle disease virus (NDV) selectively replicates in tumor cells and is known to stimulate T-cell, macrophage, and NK cell-mediated responses[13]. Apoptin and Newcastle disease virus have reported their specific and effective destruction of osteosarcomatous cells[14,15]. To improve further the therapeutic effect of oncolytic viruses, therapeutic genes have been incorporated into the Recombinant fowlpox viruses (FPVs). Exploring the strategy of cooperative cancer gene therapy on mouse osteosarcoma, previously we have constructed the recombinant virus, vFV-HN, vFV-VP3 and vFV-HN-VP3 with fowlpox virus and in order to gain some considerable significance in developing safe and/or effective anti-tumor medicine.

Materials and Methods

Materials

Recombinant Fowl-pox virus (vFV-HN, vFV-VP3 and vFV-HN-VP3), Newcastle disease virus (NDV) and S180 osteosarcoma mouse cell line were from Genetic Engineering Laboratory of PLA, Academy of Military Medical Sciences. Alkaline Phosphates Color Development Kit was from Sangon Biotech Co. Ltd. (Shanghai, China) Acrylamide, N, N'-Methylenebisacrylamide, β-Mercaptoethanol, N, N, N, N-tetramethylethylene diamine (TEMED) and ammonium persulfate were purchased from GIBCO. Anti-apoptin

and anti-neuraminidase antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture

S180 osteosarcoma mouse cell line was cultured *in vitro* under the condition: Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium supplemented with 10% fetal bovine serum (Gibco) plus 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C/5% CO_2, saturated humidity.

Western Blot Analysis

S180 osteosarcoma cells were infected by Recombinant Fowl-pox virus for 72 hours. After that, vFV-Apoptin-infected cells were washed with cold phosphate-buffered saline (PBS), scraped into 400 μl lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 5 mM NaF, 1 mM phenylmethylsulphonylfluoride, 1 μg/ml leupeptin, 200 μg/ml trypsin inhibitor, 1 μg/ml pepstatin), transferred to a microfuge tube and kept on ice for 30 min. Samples were boiled in sample buffer (50 mM Tris, pH 7.4, 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 10% glycerol) for 3 min and cell debris was removed by centrifugation at 12,000 g at 4°C for 10 min. Samples were loaded onto 12% SDS polyacrylamide gels, separated by electrophoresis and electroblotted onto Hybond-C membranes (Amersham Bioscences, Buckinghamshire, UK). The membrane was then blocked with 5% milk in Tris-buffered saline. Blots were incubated with mouse polyclonal anti-Apoptin antibody (mouse anti human HN monoclonal antibody and mouse anti NDV positive serum) for 2 hr followed by incubation with goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) labeled with alkaline phosphatase for 2 hr. Signals were visualized by chemiluminescence using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) solutions (0.3% NBT, 0.3% BCIP, 100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5). Extracts of pV-Apoptin-transfected cells was used as a positive control and fowlpox virus-infected and noninfected cells were used as negative controls.

Co-immunoprecipitation

For co-immunoprecipitation assays, constructs encoding SFB tagged (streptavidin-binding pep-

tide) and Myc-tagged proteins were transiently co-transfected into S180 cells. Cells were lysed with NETN buffer containing 20 mM NaF, 1 g/ml of pepstatin A, and 1 g/ml aprotonin on ice for 20 min. After removal of cell debris by centrifugation, the soluble fractions were collected and incubated with S-protein beads for 2 h at 4°C. Beads were washed three times with NTEN buffer, boiled in 2× SDS loading buffer, and resolved on SDS-PAGE. Membranes were blocked in 5% milk in TBS containing 0.05% Tween-20 (TBST) and then probed with antibodies as indicated.

MTT Colorimetric Assay

The MTT colorimetric assay was performed to detect tumor cell viability after infection. Digested the S180 osteosarcoma cell in the logarithmic growth phase were seeded in 96-well plates (1 × 104 cells/well) 1 day before cells were infected with various concentrations (1 MOI, 10 MOI and 100 MOI) (multiplicity of infection, MOI) of vFV-Apoptin or fowlpox virus in serum-deprived RPIM-1640 culture medium. After infected S180 osteosarcoma cells with different concentration for 2h, the medium was replaced by 10% RPIM-1640 and incubated for 24h, 48h, 72h, and 96h. The culture media were removed and the crystals formed were dissolved by adding 100 μl/well dimethylsulfoxide (DMSO). Then methyl thiazolyl tetrazolium (MTT) (5 mg/ml, dissolved in PBS in, 0.22 μm filter filtration except bacteria) 20 μl was added to the medium and incubated under the condition 37°C, 5% CO_2 for 4 hours. Then added DMSO 100 ml/well, and carefully removed the supernatant, then cells were divided into control group and test groups with 3 replications each group at different concentrations and different time points. The absorbance at 490 nm was measured with a enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices Spectramax 190, Sunnyvale, CA, USA). Untreated cells were used as controls and all measurements were performed in triplicate. The percent cell survival was expressed with respect to control values (untreated cells) using the following formula:

$$\frac{(1\text{-test group A490 value})}{\text{Control group A490 value}} \times 100\%$$

DNA Ladder Experiment

Cells were harvested by scraping and centrifuging and then lysed with lysis buffer (5 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% Triton X-100) on ice for 15min. Fragmented DNA in the supernatant after centrifugation at 12,000 rpm was extracted twice with phenol/chloroform/iso-propanol (25/24/1, v/v) and once with chloroform and then precipitated with ethanol and 5 M NaCl. The DNA pellet was washed once with 70% ethanoland resuspended in Tris-EDTA buffer (pH 8.0) with 100 g/ml RNase at 37°C for 2 h. The DNA fragments were separated by 1% agarose gel electrophoresis

The establishment of S-180 Osteosarcoma Model in BALB/C Mice

Acquire S-180 cells in logarithmic growth phase from BALB/C mice, wash them twice with serum-free Hanks, and make cell concentration to 5×10^7 per milliliter using Trypan Blue staining method. Hypodermic inoculate right-rear limb with S-180 cells after one hour of their collection with the amount of 100 μL for each mouse, namely 5×10^6 tumor cells for each one.

Fluorescence Activated Cell Sorting (FACS)

For cell cycle analysis, cells were washed with PBS, resuspended in 300 μl of PBS and then fixed with the addition of 700 μl of 100% ethanol. After stored at −20°C overnight, fixed cells were washed and incubated in RNAse A in sodium citrate buffer for 30 minutes, and then stained with propidium iodide (50 μg/ml) for 30 minutes. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). A total of 100,000 events were analyzed for each sample and the experiment was repeated at least twice.

Animal Grouping and Treatment

10 days after hypodermic inoculation tubercles will grow out, which means inoculation success rate is 100%. Randomly divide these mice with their tumors grow to about 5 mm in diameter into 5 groups, and there are 7 mice in each group. The results are: No. 1 group is for control with PBS; No. 2 is for vFV treatment; No. 3 is for vFV-HN treatment; No. 4 is for vFV-VP3 treatment; No. 5 is for vFV-HN-VP3 treatment. Intratumoral inoculation will be carried out fortnightly, and there are 3 treatments altogether. The methods are as follows: Dilute viruses with PBS to 1×10^{10} PFU/ml (plaque forming units/ml). For treatment groups, inoculate inside the tumors with 100 ul viruses (1×10^9PFU) per mice for each treatment. For control group, inoculate inside the tumor

with 100 ul PBS per mice for each treatment. 7 days after the last treatment, kill the mice, and check all the indexes.

Measurements of Tumor Growth Rate and Tumor Inhibition Rate

Observe the mental and food taking state of the mice every day. Measurement of the tumor volume will be started after the cancer cells were successfully bore into the mice, and the measurement of the major (L) and minor (S) diameters including the thickness of the skin with Vernier calipers should be taken every two days. Calculate the volume (V) of the tumor with the following formula:

$$V = \frac{(S2 \times L)}{2}$$

Calculate the tumor inhibition rate according to the final measurement of the tumor volume of the mice. Tumor inhibition rate % = (Tumor volume of control group – Tumor volume of treatment group)/Tumor volume of control group × 100%.

Measurement of the Serum Sialic Acid Amount of the Mice

Acquire blood from sinus of the tumor bearing mice, separate out the serum, and make measurement of the sialic acid amount according to the methods in papers. With 721 ultraviolet spectrophotometer, read the OD (optical density) value at 560 nm, and then calculate the mean value. The amount of the sialic acid will be calculated with the following formula. The amount of sialic acid = A_{560} value of measurement tube/A_{560} value of standard tube ×1.94.

Pathological Analysis of the Tumor Tissue

Kill all the mice using cervical vertebra dislocation method, quickly dissect the mice to acquire the tumor tissue, and quickly put them into cold neutral fixation liquid with 10% formaldehyde. Fixation should last for 24-72 hours at 4°C. use conventional methods to dehydrate, embed and cut the tissues into slices, and then dye them with hematoxylin and eosin. Finally mount them with neutral gum. Thus, they are ready for microscope observation.

Statistical Analysis

All the data were shown as mean ± SD, and analyzed by one-way independent *t*-test or ANOVA. *p* < 0.05 was considered as statistically significant.

Results

Successful Expression of vFV-HN, vFV-VP3 and vFPV-HN-VP3 in S180 cells

In order to investigate the anti-tumor effect of the recombinant FPV, we first detected the expression of recombinant virus genes in S180 cells. Protein mixtures were extracted from vFV-HN and vFPV-HN-VP3 infected S180 cells, analyzed by means of SDS-PAGE electrophoresis and followed by Western blotting carried out on the NC membranes with HN-specific antibody. The result in vFV-HN group showed that there were specific bands about 63 kD, and were equal for NDV (Newcastle disease virus) positively infected bands (Figure 1 A), implying the same immunogenicity between proteins expressed by vFV-HN and NDV infected cells. In vFV-HN-VP3 group, a band with molecular weight much higher than HN was clearly detected by HN antibody, showing that HN-VP3 protein was abundantly expressed (Figure 1 A).

Total RNA were extracted from vFV-HN, vFV-VP3 and vFPV-HN-VP3 infected S180 cells, and amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) with HN and VP3 gene-specific primes. The results of PAGE electrophoresis showed that HN gene expressions could be detected in both vFV-HN and vFPV-HN-VP3 groups (Figure 1 B). And VP3 gene expressions was detected in both vFV-VP3 and vFPV-HN-VP3 groups (Figure 1C). All these proved that vFV-HN, vFV-VP3 and vFPV-HN-VP3 were successfully expressed in S180 cells.

Inhibition Rates of S180 cells by Combination Fowl-pox Viruses

By successfully expressing recombinant FPV genes into S180 cells, we detected the cells inhibition rates *in vitro*. In a certain period of time, the inhibition rates of S180 cells increased while the number of FPV and vFPV within S180 cells increasing. However, the inhibition rates reached the peak at 72 hours, and then decreased (Figure 2A). vFV-HN-VP3 had the highest killing rate. Given a certain period of time, for example 72 hours, the inhibition rates increased, not following a strict dose-effect relationship (*p* > 0.05) (Figure 2B).

To determine whether recombinant FPV genes killed S180 by inducing apoptosis, after 72 hours' infection, we isolated the genomic DNA of about 6×10⁵ S180 cells and the genomic DNA were submitted to agarose gel electrophoresis. For normal S180 cells, a small quantity of ge-
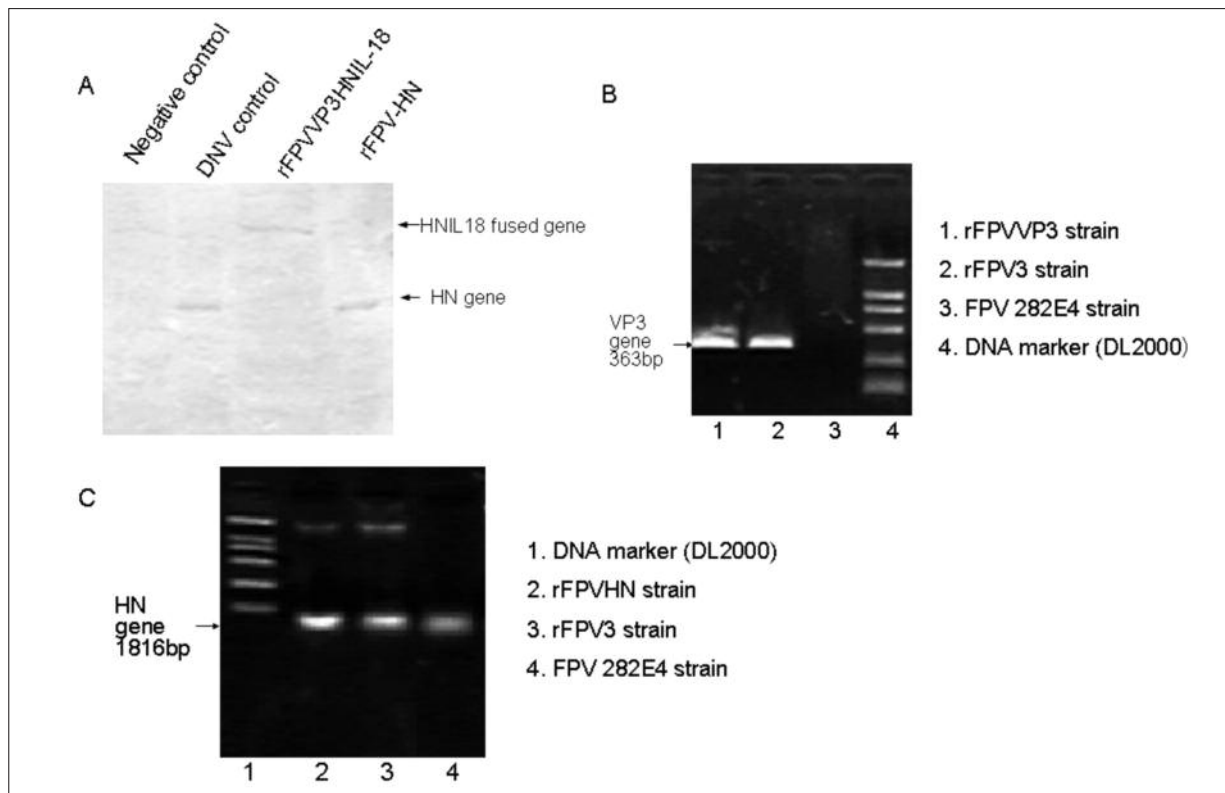
Figure 1. Identification of recombinant Fowl-pox viruses expression in S180 cells by western and RT-PCR. S180 cells were infected by FPV or recombinant Fowl-pox viruses (vFV-HN, vFV-VP3 and vFV-HN-VP3, respectively) for 72 hours, cell extracts were subjected to western blot using anti-HN antibody to detect vFV-HN and vFV-HN-VP3 expression *(A)*. Genome DNA was used as RT-PCR template. HN primers were used to detect vFV-HN and vFV-HN-VP3 expression *(B)* while VP3 primers were used to detect vFV-VP3 and vFV-HN-VP3 expression *(C)*.

nomic DNAs might be damaged during the procedure of DNA extraction. However, for S180 cells after 72 hours' infection of vFV, the result of agarose gel electrophoresis showed a nonspecific smear (Figure 2C), revealing that infection of vFV3 ruptured the S180 genome and thus induced the apoptosis of S180 cells.

Osteosarcoma Model Established in BALB/C Mice and Nude Mice

Tumors could be seen at the inoculation position in the BALB/C mice and nude mice 4 days after injection of S180 cells. And the long and short diameter of the tumors were 5 mm and 4 mm, respectively. So the osteosarcoma model was established.

The growth tendency of each experimental group after treatment of recombinant virus tumor volume of each group showed no significant difference before treatment of recombinant virus. As the tumor grew during the treatment, the rate of tumor growth was not the same between the experimental group and control group (Figure 3A). The growth speed of tumors was highest in the PBS treated group, while, lowest in the vFPV-HN-VP3 virus (Figure 3A). Using the very last measurement of tumor volume to calculate the rate of tumor suppression of each group, the suppression rate of the vFPV-HN-VP3 treated group was 51.7% compared to the PBS treated control group (Figures 3B). In groups treated with virus which carried HN or VP3 only, the tumor suppression rate were 26.7% and 34.2%, respectively. The wild type virus also had suppressing effect to some extent; however, the ratio is very low, 4.32% only (Figures 3B). For cell cycle analysis, these findings suggest that genes into mouse osteosarcoma cancer cells can cause cell a specificity anti-tumor immune activity, suppress tumor growth, and increase the survival rate of the tumor within host (Figures 3C).

Sialic acid (SA) is a generic term for the N- or O-substituted derivatives of neuraminic acid and it plays a special role in the malignant tumors. SA
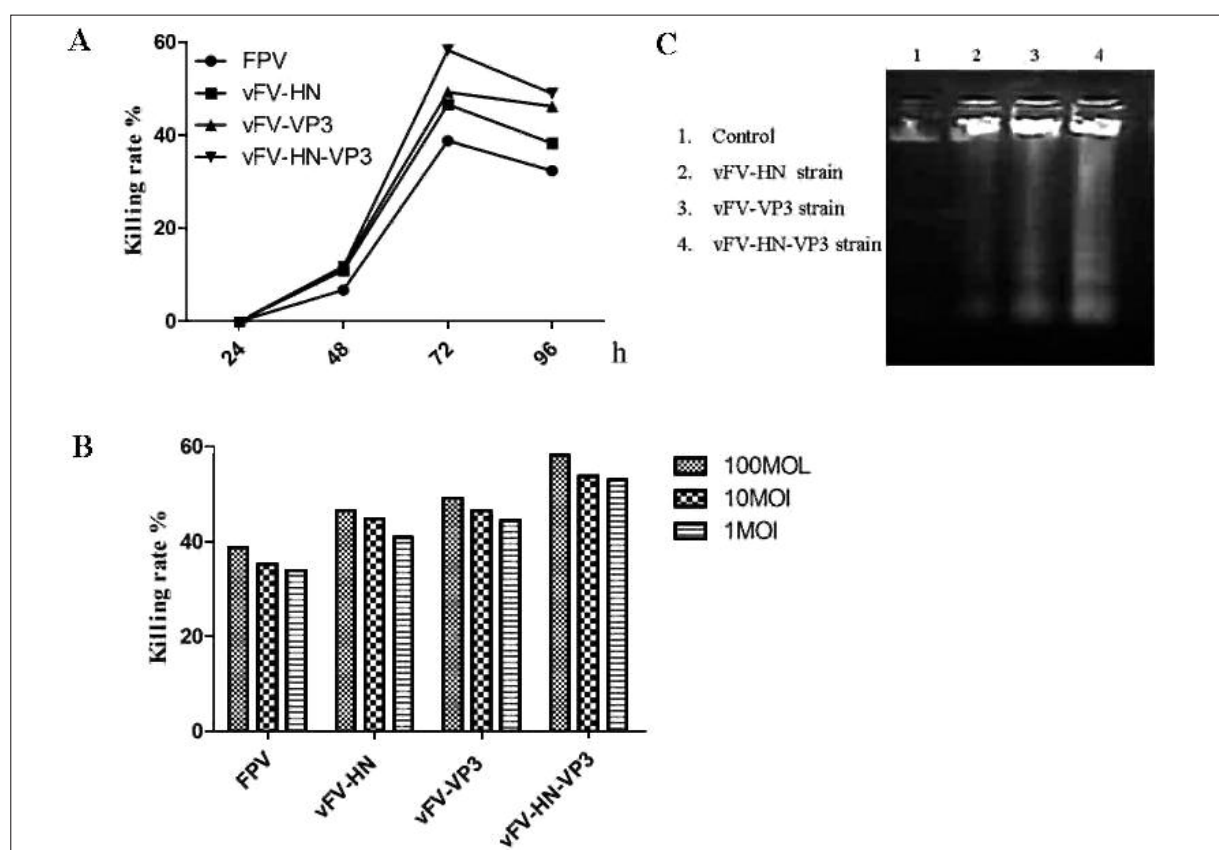
Figure 2. The inhibition rates of S180 cells by combination Fowl-pox viruses. S180 cells were infected by FPV or recombinant Fowl-pox viruses for the indicated periods, the inhibition rates of each groups were measured *(A)*. S180 cells were infected by FPV or recombinant Fowl-pox viruses for the indicated dosages of 72 hours, the inhibition rates of each groups were measured *(B)*. S180 cells were infected by 100MOI FPV (control) or recombinant Fowl-pox viruses for 72 hours, Genome DNA was isolated and subjected to 1% agarose gel, the DNA ladder represent the digested DNA fragment of apoptosis cells *(C)*.

widely used as a tumor marker, is closely correlated with tumor cells proliferation, metastasis, invasion, reduced adherence, tumor antigenicity and escape of the immune surveillance of host cells. The serum sialic acid content of the vFV-HN group was much lower than the control group ($p < 0.01$), which manifested the neuraminidase (NA) activity of HN gene *in vivo*. The serum sialic acid content of the vFV-HN group had no statistical significance compared to the control group ($p > 0.05$), and it indicated VP3 only cannot degrade SA. The fFV-HN-VP3 group held the lowest serum sialic acid content among all the groups and this meat combination of HN and VP3 had the greatest effects on SA degradation (Figures 4).

Pathological Analysis of Osteosarcoma Tissues

These studies was to develop a metastatic osteosarcoma nude mouse model to evaluate the *in vivo* efficacy of new targeting gene-viro therapy with recombinant fowl-pox viruses with HN and VP3 genes on mouse (Figure 5). S180 osteosarcoma model in nude mice were established and did the pathological analysis of the osteosarcoma tissues by safranin O staining (Figure 5B), alcian blue staining (Figure 5C), alkaline phosphatase staining (Figure 5E), oil red-O staining (Figure 5F). After that, the vFPV viruses as control, vFV-HN, vFV-VP3, vFV-HN-VP3 were injected into the tumor directly. The tumor growth (Figure 5), tumor suppression were examined and the tumor tissues were analyzed by the method of morphological dying analysis.

The immunohistochemical results of the osteosarcoma tissues showed different cellular morphology. In the PBS treated control group, the cancer cells were closely arranged with distinctive cellular outline, and at the same time no cell degeneration and necrosis were observed (Figures 6A). Importantly, there was a great extent of vacuolization in cancer cells and some of them
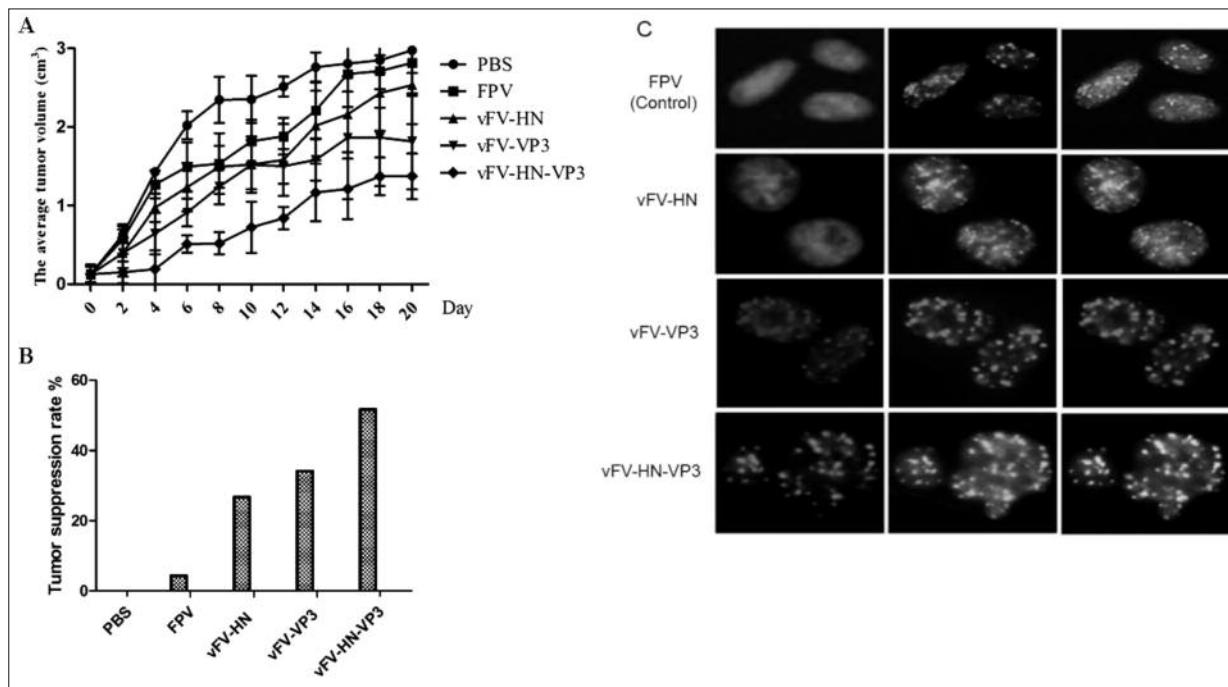
Figure 3. Regression of established osteosarcoma in C57BL/6 mice. Tumor-bearing mice (10 days after tumor cell implantation) were injected into the tumor directly with 100 ul PBS (●), 100 ul 1×1010 PFU/ml FBV (■), 100 ul 1×1010PFU/ml vFV-HN (▲), 100 ul 1×1010 PFU/ml vFV-VP3 (▼) or 100 ul 1×1010 PFU/ml vFV-HN-VP3 (◆), respectively, once every 2 days for 10 times *(A)*. It was evident that, compared with the PBS control groups, the vFV-HN-VP3 treated mice showed significant tumor volume decrease and the suppression rate of the vFV-HN-VP3 treated group was 51.7%. *B,* ($p < 0.01$). In groups treated with virus which carried HN or VP3 only, the tumor suppression rate were 26.7% and 34.2%, respectively. *C,* S180 cells forms the gene-induced foci. S180 cells transfected with SFB-tagged (streptavidin-binding peptide) were treated with IR, fixed, and immunostained with anti-Flag and anti-HN antibodies, anti-VP3 antibodies.
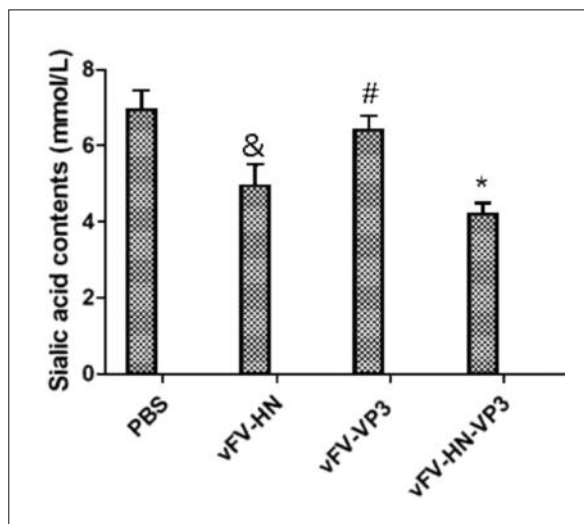

Figure 4. Test of Sialic acid level in mouse serum. After PBS control or recombinant Fowl-pox viruses treatment, the serum sialic-acid content of each group were measured. Histograms show the measured data from there experiments expressed as the mean ± SEM of control, #$p > 0.05$ vs. vFV-VP3 group, & $p < 0.01$ vs. vFV-HN group, *$p < 0.01$ vs. vFV-HN-VP3 group.

showed necrosis in the vFV-HN treatment groups (Figures 6B) and similar cellular morphology was seen in the vFV-HN-VP3 treatment groups (Figures 6D). While in the vFV-VP3 treatment groups, large scale of necrosis were observed and some cancer cells exhibited karyorrhexis (Figures 6C).

Discussion

To investigate the strategy of cooperative cancer gene therapy on mouse Osteosarcoma, previously, we constructed the recombinant virus, vFV-HN, vFV-VP3 and vFV-HN-VP3 within fowlpox virus, and reported their specific and effective destruction of osteosarcomatous cells. Some data clearly demonstrated that the combination anti-cancer gene was more effectively rather than single antitumor gene. In this study, in order to further explore the antitumor effects of Recombinant Fowl-pox viruses with both HN and VP3 genes on mouse osteosarcoma. Os-
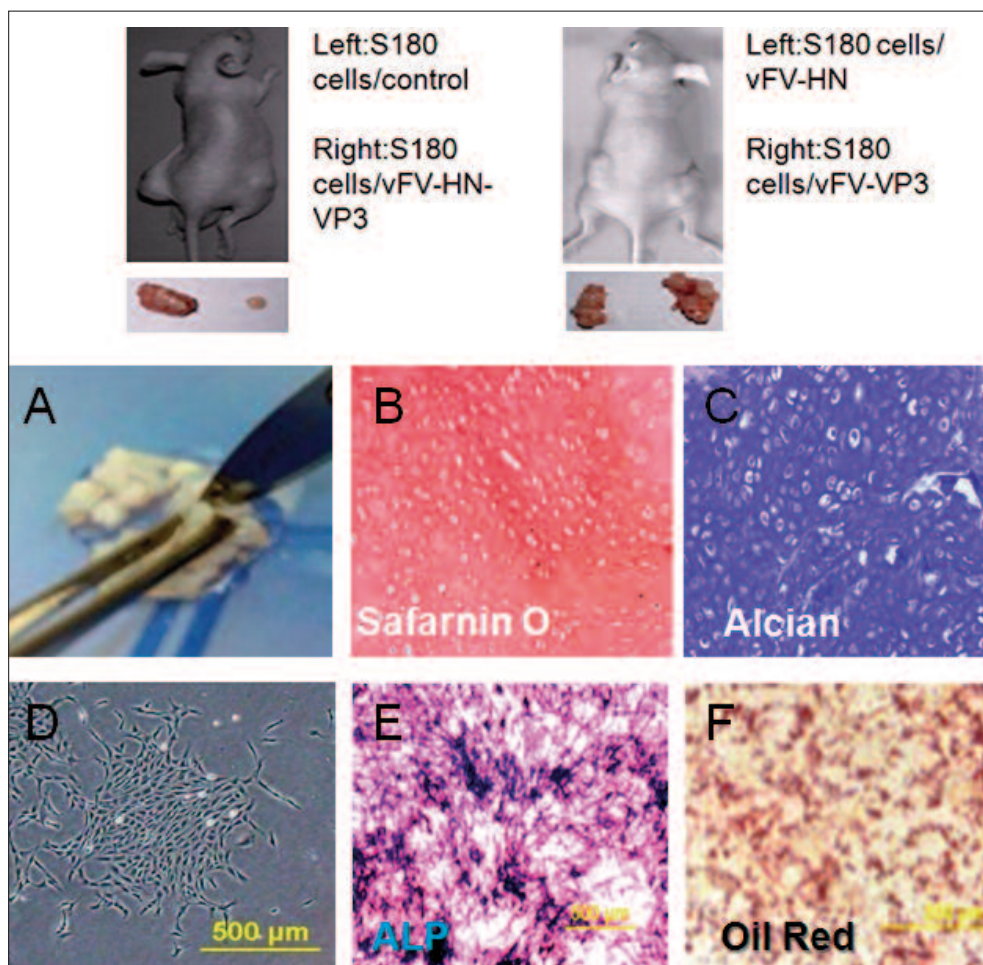
Figure 5. S180 osteosarcoma model in nude mice were established and did the pathological analysis of the osteosarcoma tissues by safranin O Staining (Figure 5B), alcian blue staining (Figure 5C), alkaline phosphatase staining (Figure 5E), oil red-O staining (Figure 5F). After that, the vFPV viruses as control, vFV-HN, vFV-VP3, vFV-HN-VP3 were injected into the tumor directly. The tumor growth (Figure 5), tumor suppression were examined and the tumor tissues were analyzed by the method of morphological dyeing analysis.

teosarcoma model S180 with BALB/c mice was established and the vFPV viruses were injected into the tumor directly. The rate of tumor growth, tumor suppression and the sialic acid levels in serum were examined and the tumor tissues were analyzed by the method of immunohistochemistry. The vFV-HN, vFV-VP3 and vFV-HN-VP3 all had growth inhibition effects, the inhibition rate of vFV-HN-VP3 group was 51.7%, which was higher than that of vFV-HN, vFV-VP3 group and control group ($p < 0.01$). The sialic acid level of vFV-HN-VP3 group in mouse serum was 4.22±0.27 mmol/l, which was lower than that of other groups ($p < 0.01$). The study results demonstrated that VP3 gene and HN gene had collaborative anti-tumor effects on mouse osteosarcoma.

In cancer gene therapy, restricted expression of the therapeutic gene in the tumor is important. If the therapeutic gene is expressed in all cells, it will affect tumor and normal cell. Use of the tumor specific promoter system will solve this problem. However, true tumor specific promoters are rare, and often these promoters are useful only in the particular types of cancers from which they are derived. The tumor specific cell killing nature of apoptin makes it a new alternative for cancer gene therapy[16].

The genome of Fowl-pox virus is so large that it can carry lots of exogenous genes, without weakening its infection ability[9]. Therefore, it gradually becomes the new vector for tumor gene therapy. In this study, the Authors recombine the genes which have different contribution to tumor
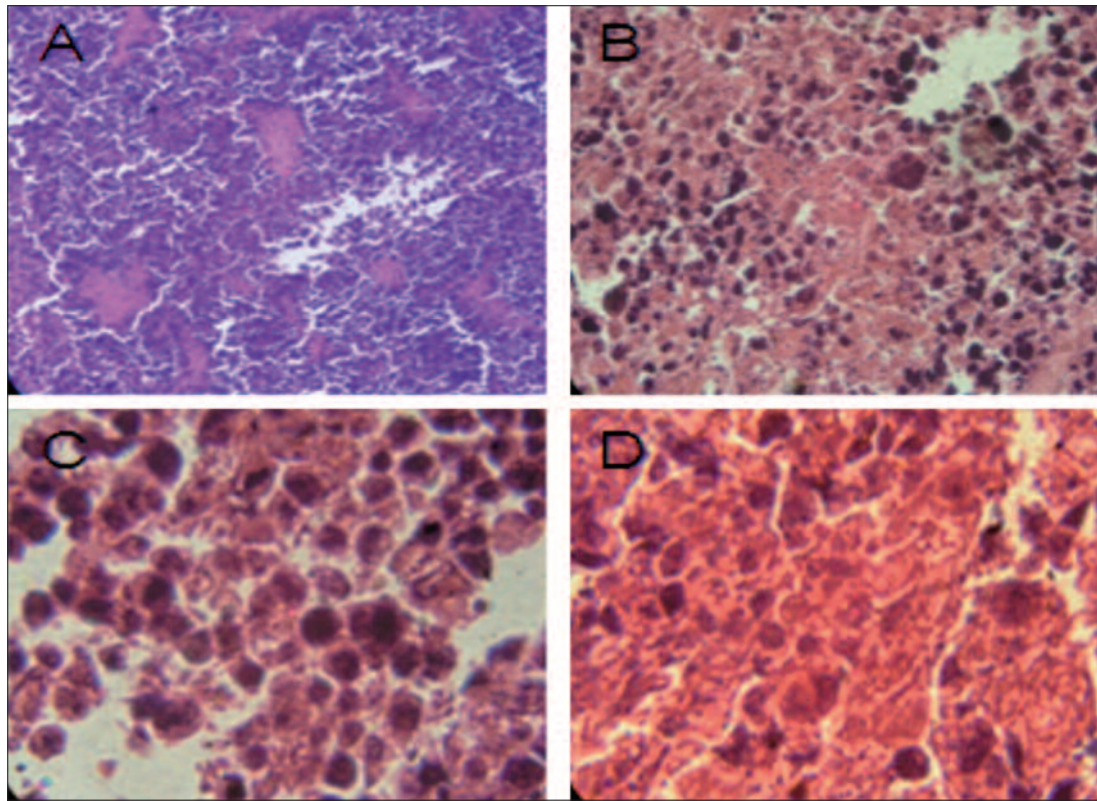
Figure 6. Pathological analysis of the osteosarcoma tissues by H&E staining. In the PBS treated control group, the cells showed normal morphology and good proliferation *(A)*, while the osteosarcoma of the vFV-HN treated group showed apoptosis appearance *(B)*. In the vFV-VP3 treated group, the osteosarcoma cells appeared the nuclear fragmentation and most of cells dead *(C)*, while the osteosarcoma cells of the vFV-HN-VP3 group were undergone severe apoptosis, and some tumor tissue even had necrosis appearance *(D)*. Original magnification, × 400.

suppression so as to detect whether this fusion gene has better tumor suppression effect. The inhibition effect on the growth of mouse osteosarcoma *in vivo* is tested by recombinant Fowl-pox viruses involving NDV-derived hemagglutinin-neuraminidase (HN) gene and VP3 gene with the FPV vector. To achieve the goal of complete elimination of tumor xenograft in animal models, we have developed a new strategy called Targeting Gene-Virotherapy of Cancer, which aims to combine the advantages of both gene therapy and virotherapy.

The previous reports demonstrated that the level of sialic acid on the surface of tumor cells is high than that on normal cells[11]. It's the high level of sialic acid on the surface which can anti-immune system that can promote tumor cells proliferation[7]. HN protein had NA activity and can hydrolyses sialic acid on the surface of tumor cells. Because of this effect, the antigen of tumor cells fully exposed to the body immune systems and then the tumor cell will be recognized and killed

by immune system[12,13]. The chicken anemia virus protein apoptin, which is a 14-kDa basic and proline-rich protein derived from the chicken anemia virus, can induce a p53-independent type of apoptosis in human tumor cells or transformed cells and cannot be blocked by Bcl-2[14]. Apoptin fails to induce apoptosis in normal cells. Therefore, apoptin is a potential antitumor agent. And VP3 induced apoptosis, which is p53-independent, may be the better way to cure human tumor.

Here we combine HN gene and VP3 gene with the Fowl-pox viruses vector to test the anti-tumor effect. We found that the fusion protein can induce the death of tumor cells formed in nude mice by injecting the recombinant virus 3 times continuously. Our results demonstrated the recombinant Fowl-pox viruses can efficiently infect mouse S180 cells and the vFV-HN-VP3 can kill tumor cells by inducing apoptosis of s180 cells. To further test the anti-tumor effects of vFV-HN-VP3 *in vivo*, here we succeeded to establish s180 osteosarcoma model of BALB/C

mouse and inject the Fowl-pox viruses into the tumors of the mouse. In the experiments we measured the tumor volume regularly to test the growth state of tumors. The date demonstrated that there is no significant difference at the beginning ($p > 0.05$). But as time passed, the speed of tumor growth in vFV-HN-VP3 group is lowest, comparing with that in control group (PBS), which is the fasted. Compared with control group, recombinant Fowl-pox viruses with vFV-HN or vFV-VP3 can both inhibit the growth of s180 tumor; but the inhibition effects are significantly worse than that with combination of vFV-HN and vFV-VP3. The growth curve of tumor volume show that the anti-tumor effects of vFV-HN-VP3 are significantly more efficient than that of control, vFV-HN or vFV-VP3. These results demonstrated vFV-HN-VP3 injection can efficiently inhibit tumor growth. At the same time, the level of sialic acid in mouse serum in vFV-HN-VP3 group is lower than that in other groups; this demonstrated that VP3 can enhance the effects of HN playing the role of NA *in vivo*; and can cut down the level of sialic acid in mouse serum significantly, which can further improve the ability of mouse immune system to recognize and kill tumor cells.

Conclusions

Our study demonstrated that VP3 gene and HN gene had collaborative effects on tumor growth inhibition and gene combination therapy can indeed improve the inhibition effect. Therefore, it may have good application prospects in clinical research; although the anti-tumor mechanism of the two genes in vivo need to be further studied.

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Conflict of Interest

The Authors declare no conflict of interest for this article.

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