RESEARCH ARTICLE

Enhancement of Anti-tumor Activity of Newcastle Disease Virus by the Synergistic Effect of Cytosine Deaminase

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Abstract

This study was conducted to investigate enhancement of anti-tumor effects of the lentogenic Newcastle disease virus Clone30 strain (NDV rClone30) expressing cytosine deaminase (CD) gene against tumor cells and in murine groin tumor-bearing models. Cytotoxic effects of the rClone30-CD/5-FC on the HepG2 cell line were examined by an MTT method. Anti-tumor activity of rClone30-CD/5-FC was examined in H22 tumor-bearing mice. Compared to the rClone30-CD virus treatment alone, NDV rClone30-CD/5-FC at 0.1 and 1 MOIs exerted significant cytotoxic effects (P<0.05) on HepG2 cells. For treatment of H22 tumor-bearing mice, recombinant NDV was injected together with 5-FC given by either intra-tumor injection or tail vein injection. When 5-FC was administered by intra-tumor injection, survival for the rClone30-CD/5-FC-treated mice was 4/6 for 80 days period vs 1/6 , 0/6 and 0/6 for the mice treated with rClone30-CD, 5-FC and saline alone, respectively. When 5-FC was given by tail vein injection, survival for the rClone30-CD/5-FC-treated mice was 3/6 vs 2/6 , 0/6 and 0/6 for the mice treated with rClone30-CD, 5-FC or saline alone, respectively. In this study, NDV was used for the first time to deliver the suicide gene for cancer therapy. Incorporation of the CD gene in the lentogenic NDV genome together with 5-FC significantly enhances cell death of HepG2 tumor cells in vitro, decreases tumor volume and increases survival of H22 tumor-bearing mice in vivo.

Keywords: Cytosine deaminase - NDV - HepG2 - H22 - suicide gene therapy

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Introduction

Traditional therapies for cancers are nothing less than the radiotherapy and chemotherapy, however both of them have many shortcomings and patients are prone to relapse (Zhao et al., 2003). Thence, better therapies for treating cancers are still unmet medical needs.

Newcastle disease virus (NDV) is a non-segment, negative single strand virus and belongs to the Avulavirus genus in the Paramyxoviridae family (Knipe et al., 2006). Accumulating evidence indicates that NDV is a powerful anti-tumor agent, and could be alternative way for cancer therapy (Zamarin et al., 2009). NDV has been successfully used for treatment of head and neck squamous cell carcinomas (Karcher et al., 2004), tumors of digestive tract (Liang et al., 2003), glioblastoma multiforme (Schneider Tetal., 2001; Steiner et al., 2004), malignant melanoma (Batliwalla et al., 1998; Cassel et al., 1988; Wallack et al., 1998), colorectal carcinoma (Schlag et al., 1992; Ockert D et al., 1996) and other advanced cancers. Furthermore, several of the naturally occurring NDV strains have been used in multiple clinical trials against advanced human cancers. Based on their virulence, NDV viruses can be divided into three categories: velogenic (high), mesogenic (medium) and lentogenic (low) groups. Most oncolytic studies are done using either velogenic or mesogenic NDVs (Peeters et al., 1999; Zamarin et al., 2009). Large scale production and clinic application of these viruses would be a threat to poultry industries. However, the oncolytic efficiency of the lentogenic virus is hard to reach the expected standard. The aim of the study intends to enhance the anti-tumor activity of the lentogenic virus by integrating a suicide gene cytosine deaminase into the viral genome (Romer-Oberdorfer et al., 1999; Nakaya et al., 2001).

Suicide gene therapy (SGT), also known as enzyme-activating prodrug therapy. The proteins expressed by the suicide gene are non-toxic substance and play roles as the catalysts in chemical reactions. During the catalytic reaction process, the suicide gene products convert a non-toxic or low-toxic prodrug to a drug that has anti-tumor functions (Bentires-Alj et al., 2000; Fuchita et al., 2009).

The cytosine deaminase (CD) gene product convert the prodrug 5-flucytosine (5-FC) into cytotoxic substance 5-fluorouracil (5-FU), which has anti-tumor activity (Topf et al., 1998; Pierrefite-Carle et al., 1999; Negroni

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et al., 2007). The CD/5-FC system demonstrates stronger bystander effect and the gap-junctional intercellular communications than other suicide gene system, which results in strong inhibition of tumor growth (Khatri et al., 2006; Stolworty et al., 2008; Dachs et al., 2009). However, there is no efficient method to deliver the CD gene into the tumor target cells, which hampers the application of the suicide gene therapy.

In this study, the lentogenic NDV was utilized as a vector to deliver the CD gene into the cancer cells. The NDV/CD can specifically infect the cancer cells (Ravindra et al., 2009), meanwhile the CD is expressed in the cancer cells, then CD converts the prodrug 5-FC into 5-FU to achieve the anti-tumor purpose. Our results clearly demonstrate that the incorporation of CD gene into lentogenic viral genome in combination with 5-FC significantly enhance the anti-tumor activity of the virus.

Materials and Methods

Cell lines and biochemical reagents

Human hepatic cancer cells (HepG2) and mice hepatic cancer cells (H22) were purchased from ATCC. Baby hamster kidney cells (BHK-21) were kindly given by Dr. B. Moss. Dulbecco's modified Eagle's minimal essential medium (DMEM) was purchased from GIBCO (Karlsruhe, Germany). 5-fluorocytosine (5-FC, batch number: 2011072761) was purchased from Huiyinbi Biological Pharmaceutical Co., Ltd. (Jiangxi, China), 5-fluorouracil (5-FU, batch number: 1112301) purchased from Jinyao Amino Acid Co., Ltd. (Tianjin, China), Saline (batch number: 111006A4) was purchased from Medisan Pharmaceutical Co., Ltd. (Harbin, China). The NDV genome of the lentogenic strain LaSota Clone30 (pBrClone30) was cloned by this laboratory. All RE enzymes, Ampicillin, pMD18-T simple vector and DNA ligase etc. were purchased from Takara Biotechnology Co., Ltd (Dalian, China).

Experimental animals and embryos

Level II Kunming mice were purchased from Slaccas Experimental Animal Co., Ltd (Shanghai, China), animal license number SCXK (Shanghai, China) 2012-0002, 9 days SPF chick embryos were purchased from Harbin Veterinary Research Institute (Harbin, China).

Construction of pBrClone30-CD and rescue of the virus by reverse genetics system

The total genome DNA of E.coli JM109 was extracted (Frederick et al., 2002) and the CD gene was obtained by PCR method. The PCR primers, Sence: 5' CAGTTAACGCCACCATGT-CGAATAACGCTTTACA 3', Anti-sence: 5' GACGCGTTCAACGTTTGTAATCGA-TGG 3' were synthesized in Sangon Company (Shanghai, China). The CD gene was ligated into the pMD18-T simple vector and sequenced, the correct CD fragment was ligated into the pBrClone30 vector as pBrClone30-CD plasmid. The CD gene was inserted between the F gene and HN gene in the viral genome.

The reverse genetics system has been established to rescue the NDV in our laboratory. pBrClone30-CD, pTM1-NP, pTM1-P and pTM1-L were co-transfected into 70-80% BHK-21 monolayer in 6-well plate by Lipofectmine2000 at 37 °C 5% CO_2. BHK-21 cells were freeze and thawed between -80 °C and 37 °C 3 times at 72 h post-transfection. Supernatant was harvested after centrifugation (1500 r/min) at 4 °C for 3 min and filtered through a 0.22 μm filter. 200 μl of the supernatant was inoculated into the allantoic cavities of 9-day-old embryonated SPF chicken eggs. 3 days later, the allantoic fluid was harvested and titrated by a rapid plate hemagglutination test and hemagglutinate inhibition test. A positive hemagglutination test indicated that virus was present in the allantoic fluid of inoculated eggs, the positive samples were conserved at -80 °C.

Extraction of the viral RNA and RT-PCR

The viral RNA was extracted by TRIzol (Promega, Madison, WI). The equal amount (4 μg) of the viral RNA was used to reverse-transcribe in different volume of reaction system (50 μl and 100 μl) at 42 °C for 2 h referencing the manual of M-mlV (Promega, Madison, WI). Amplifications of the cDNA were performed by PCR method (25 μl PCR reaction system) with the sence and anti-sence primers.

The examination of cytotoxic effect of the NDV rClone30-CD in HepG2 cells

The survival of HepG2 cells was measured by the 3- (4, 5-dimethylthiazol-2-y) -2, 5-diphenyl-tetrazolium bromide (MTT) staining method in a 96-well plate. The HepG2 cells (logarithmic phase) were digested by trypsin and diluted into 2×10^4/mL tumor cell suspension. 200 μL was drew into each well of a 6-well plate and cultured with DMEM and 10% CS at 37 °C and 5% CO_2 overnight. The cells were infected with rClone30-CD at MOI of 0.001, 0.01, 0.1 and 1, respectively. 0.2 mL 5-FC at concentration of 10 mg/mL were added into each well of cells, the total reaction volume was 2 mL. The cell samples treated with rClone30-CD only was considered as a control group. 20 μl (5 mg/mL) MTT were added into each well at 24 h, 48 h and 72 h p.i, and incubated for 4 h, the culture medium was discarded, 100 μl DMSO were added into each well, the samples were shocked for 10 min, the optical density (OD) of every well was detected by micro-plate reader at OD490.

Determination of the maximum safe dose of 5-FC

We proceeded to determine maximum safe dose of 5-FC by tail vein injection to optimize the dose. 8-week-old Kunming mice, 10 in each group, half male and half female, were assigned into five experimental groups (I, II, III, IV, V). Basing on the maximum LD_0 (180 mg·kg-1) dose of the LD_{50}, dose difference between adjacent groups was 10mg·kg-1 as an arithmetic sequence. In I to V group, with this formula, the injecting dose are 190, 200, 210, 220 and 230 mg·kg-1 respectively. The maximum dose of LD_0 in this experiment was considered as the maximum safe dose of 5-FC. All of the mice were treated with the same injecting method in LD_{50} study. After injected, body signs, activities, eating situation and survival of the mice were observed twice a day for 7 days.

Table 1. Maximum Safe Dose of 5-FC in Kunming Mice

Group	Dose mg/kg	Log dose	Daily mortality						Mortality	Death rate%	P
			12 h	1 d	2 d	3 d	4 d	5 d			
D4	180	2.255	0	0	0	0	0	0	0	0	0
I	190	2.279	0	0	0	0	0	0	0	0	0
II	200	2.301	0	0	0	0	0	0	0	0	0
III	210	2.322	0	1	0	0	0	0	1	10	0.1
IV	220	2.342	0	1	0	0	0	0	1	10	0.1
V	230	2.362	1	0	0	0	0	0	1	10	0.1

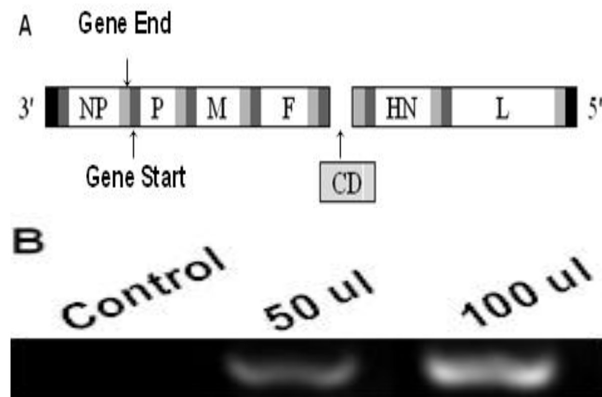

Figure 1. Generation of the NDV rClone30-CD Virus. (A) Schematic diagram of the generated rClone30-CD virus genome and the inserting site of the CD gene. (B) The CD gene was amplified by RT-PCR using RNA template from the allantoic fluid infected with the rClone30-CD virus in two reaction systems (50 μl and 100 μl) and confirmed by DNA sequencing

Tracking observation of the mice in maximum safe dose group

In the end of the maximum safe dose study, the body weight of the mice in maximum safe dose group and in control group (saline) were weighed on the d1, d2, d3, d5, d7, d14 and d180 after injection, the result was analyzed by statistics to identify whether the chronic effects and sequela were existed in the mice.

Treatment of the tumor-bearing model with NDV rClone30-CD/5-FC

rClone30-CD/5-FC was used to inhibit the growth of tumor *in vivo*. The syngeneic murine H22 liver cancer cells were injected into the body of mouse by i.p. The ascites was harvested with 1 mL insulin syringe at sterile condition 7days post-injection (Alabsi et al., 2011). The ascites containing amplified H22 cells was diluted to 106/ mL and 100 μL cells were injected at right groin area of the mice with 105 of cultured H22 cells. After 7 days, solid tumors were formed, mice were treated with NDV rClone30-CD/5-FC. Six mice in each group were assigned as: experimental group (rClone30-CD/5-FC) and 3 control group: rClone30-CD/Saline (rClone30-CD), Saline/5-FC (5-FC), Saline/Saline (control). The word (before the "/") was the substance injected at the tumor-affected area, the word (after the "/") was the substance injected at the same area. In another experiment, the word (after the "/") was the substance injected into the tail vein, The mice were treated every other day for 5 times with 107 PFU of recombinant virus and the injection was 500 μl. Before injected, the shortest diameter and the longest diameter of tumors were measured with calipers. The formula ($V = 4/3\times\pi\times S^2/2\times L/2$, simplified: $V=1.05\times S^2\times L$ (L is the longest diameter, S is the shortest diameter) was used to calculate the volume of tumors (Vigil et al., 2008). The volume of tumors was measured every other day until the mice were sacrificed.

Observation of the mice survival

The survival of the experimental mice was observed for 80 days. The mice was sacrificed when the diameter of tumors reached to 12 mm in length.

Detection of the 5-FU trace

To verify the function of CD gene integrated in the viral genome, 5-FU trace was detected. The standard (Positive) 5-FU sample was diluted to 25 μg/mL, the negative control (Negative) was 100 μl blood sample from mouse in 5-FC group after receiving 5-FC. According to the fact that the half-life of both 5-FC and 5-FU is 6 h in mice body (Patyar et al., 2010).100 μl blood was harvested from the tail vein of the mice injected with NDV-CD/5-FC at 0 h, 3 h, 6 h and 9 h post-injection. The blood samples were centrifuged; the supernatant was collected and diluted to the final volume of 1000 μl with dd H_2O. Concentration of the trace of 5-FU was detected by capillary electrophoresis.

Statistical analysis

All data were expressed as mean±standard deviation. The statistical analysis was performed by software (SPSS version 17.0). A P value less than 0.05 was considered to be statistical significant.

Results

Construction and characterization of the NDV rClone30-CD

CD gene is a wide-studied suicide gene and plays a significant role in the anti-tumor therapy. We obtained the CD gene from E.coli JM109 by PCR method, the correct CD gene was ligated into the pBrClone30 vector between F gene and HN gene (Figure 1A). Then the double-enzyme digest and PCR method were used to verify that the recombinant pBrClone30-CD plasmid was correct. The recombinant virus was rescued with helper plasmids in BHK-21 cell line and the titter of the virus was determined by HA test. The allantoic fluid of egg embryos was harvested, which was used to extract the RNA of rClone30-CD virus. The CD gene was amplified from the viral genome by RT-PCR method (Figure 1B) and confirmed by DNA sequencing.

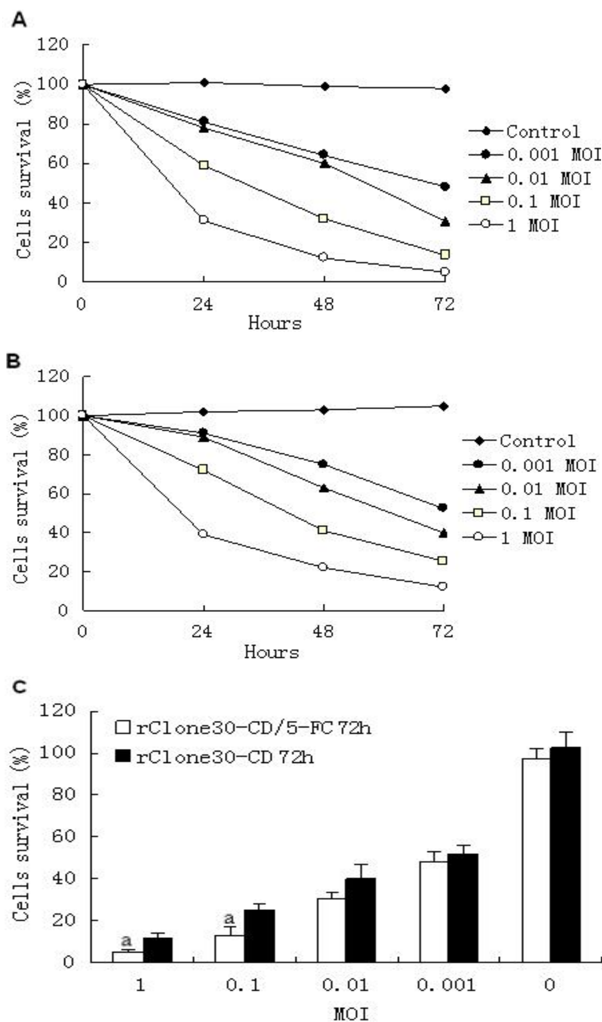

Figure 2. Cytotoxic Effect of 5-FC on rClone30-CD Virus-infected HepG2 Cells. (A) Cytotoxicity of the NDV rClone30-CD in HepG2 cell lines with 5-FC. The HepG2 cell line were infected with NDV rClone30-CD at appropriate MOI, the control was treated with 5-FC. Cytotoxicity was assessed at 24 h, 48 h and 72 h by MTT method. (B) Cytotoxicity of the NDV rClone30-CD in HepG2 cell lines without 5-FC. Cytotoxicity was assessed at 24 h, 48 h and 72 h by MTT method. (C) Cytotoxic comparison of the virus-infected HepG2 cells with or without 5-FC. The data were analyzed by statistics. $^{a}P<0.05$ vs the rClone30-CD group at 0.1 and 1 MOIs after p.i

NDV rClone30-CD kills and efficiently replicates in human liver cancer cells

To investigate the level of cytotoxicity and replication of the NDV rClone30-CD, The HepG2 cell line was selected for this experiment, the NDV and 5-FC were added into each well. The NDV rClone30-CD/5-FC showed significant cytotoxic activity in HepG2 cell line. At 0.001, 0.01, 0.1 and 1 MOIs, the cell survivals were 48.2%, 30.8%, 13.5% and 5.0%, respectively, the control was treated with 5-FC (Figure 2A). In the control group (NDV without 5-FC), the cell survival were 52.3%, 39.9%, 25.4% and 12.1% (Figure 2B), respectively. The NDV viruses showed a significant cytotoxic effect in a dose-dependent manner in HepG2 cell line. At 0.1 and 1 MOIs, the system (rClone30-CD/5-FC) has a significant cytotoxic effect compared to the control (rClone30-CD) at 72 h p.i (Figure 2C).

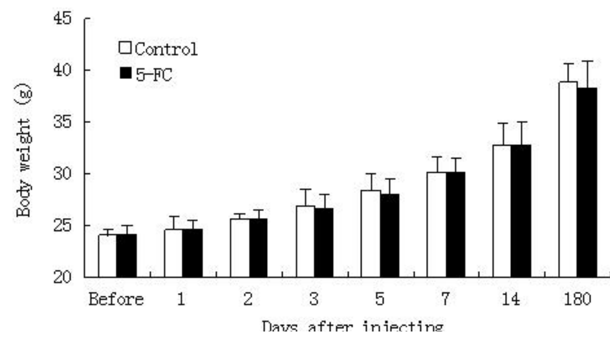

Figure 3. The Mice Were Treated with Indicated Dosages of 5-FC (control with saline), and Observed for 7-day's Survival. Determination of maximum safe dose (MSD) of 5-FC in mice. The mice were treated with indicated dosages of 5-FC, and observed for 7-day's survival. Body weight change of the mice injected with the MSD. The mice was injected with MSD followed by observation of the body weight at indicated time points, the difference between MSD and control groups $P>0.05$

Determination of the maximum safe dose of 5-FC in mice

To ensure the safety of mice in the tumor-bearing model study, the maximum safe dose (MSD) must be measured. 60 Kunming mice, half female and half male, 8-week-old, 25 g were used to determine MSD. Based on the max LD_0 dose in LD_{50}, 180 mg/kg was set and considered as least safe dose, interval dose was 10 mg/kg. The death rates of mice in 180, 190 and 200 mg/kg groups were all 0%, in 210, 220 and 230 mg/kg groups were all 10% (Table 1), maximum LD_0 (no mice dead) in this study was considered as the maximum safe dose, which was 200 mg/kg.

Observation of the mice weight and signs in maximum safe dose study

To investigate whether 5-FC have some sequela in Kunming mice, we measured the body weight and observed the signs of the mice in 5-FC and saline groups. Firstly, before injected with 5-FC, the weight of mice were measured, this step was repeated on each appropriate days after injected with 5-FC. After 180 days, the mice in 5-FC and control groups were all alive. At each time-point, body weights of two groups was compared (Figure 3C), $P>0.05$, which was not significant. The result showed that 5-FC did not induce sequela and significant effect to mice after injected with 5-FC at opportune doses for 180 days.

rClone30-CD/5-FC significantly reduces tumor volume of the tumor-bearing mice

To prove whether the rClone30-CD/5-FC system has superior anti-tumor effect compared to rClone30-CD *in vivo*. H22 groin tumor-bearing mice were treated with 5-FC. When the diameter of tumor grew to 5 mm approximately, the mice were treated with different injections every other day for 5 times. The mice were sacrificed when the tumors reached about 12 mm in length. The volume of tumors in the rClone30-CD/5-FC group was significantly reduced compared with that in the rClone30-CD group. The volume of tumors in NDV group was significantly reduced compared with that in the 5-FC group and control group (Figure 4A and 4B).

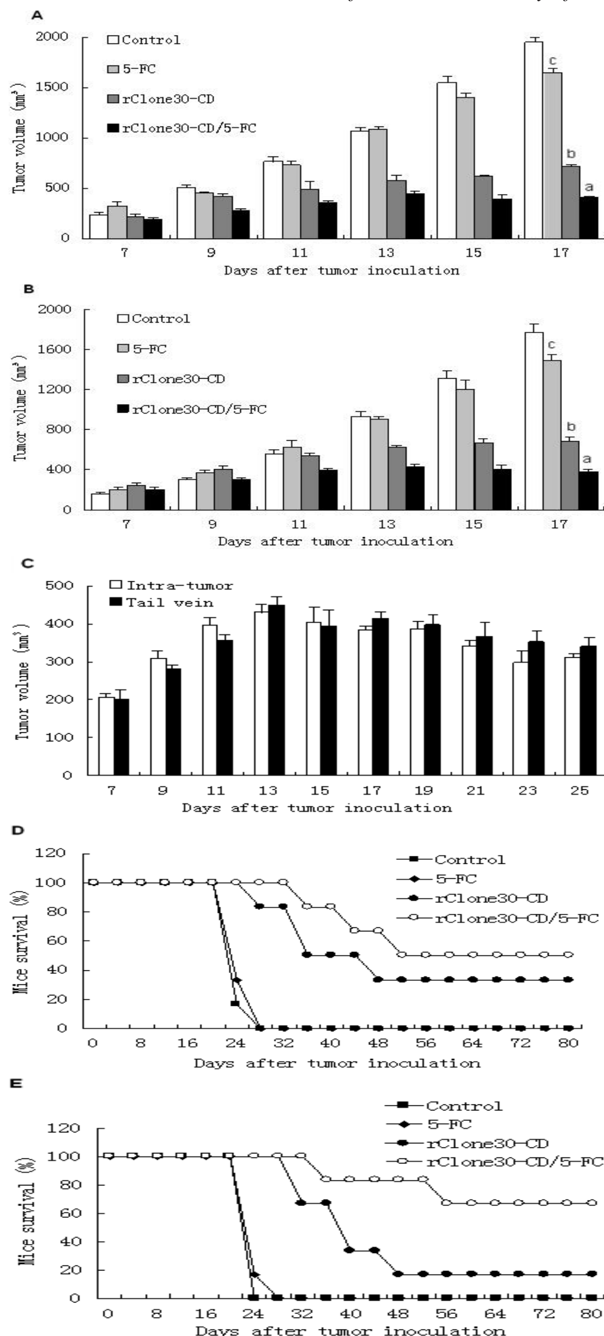

Figure 4. NDV rClone30-CD/5-FC System Inhibits Tumor Growth and Prolongs Mice Survival in H22 Tumor-bearing Models. (A) Tumor volume of the rClone30-CD-treated mice in combination with 5-FC injection by tail vein. Mice (n=6) in each group were injected with 105 of cultured H22 liver cancer cells at the right groin. The tumors were treated with NDV rClone30-CD/5-FC on d 7 after tumor cell inoculation, 107 PFU of NDV and 250 μl (2.5mg) of 5-FC was injected into the tumor area and tail vein, respectively, once every two days for 5 times. $^{a}P<0.05$ vs the NDV group, $^{b}P<0.01$ vs the control group, $^{c}P>0.05$ vs the control group. (B) Tumor volume of the rClone30-CD-treated mice in combination with 5-FC injection by intra-tumor. $^{a}P<0.05$ vs the NDV group, $^{b}P<0.01$ vs the control group, $^{c}P>0.05$ vs the control group. (C) Comparison of the tumor inhibiting effect by different injecting methods for 5-FC. No significant different showed between tail vein and intra-tumor injections. (D) Summary of 80-day survival of the mice treated as A. Mice were sacrificed when the diameters of tumors reached 12 mm. (E) Summary of 80-day survival of the mice treated as B. Mice were also sacrificed when the tumors reached 12 mm in length

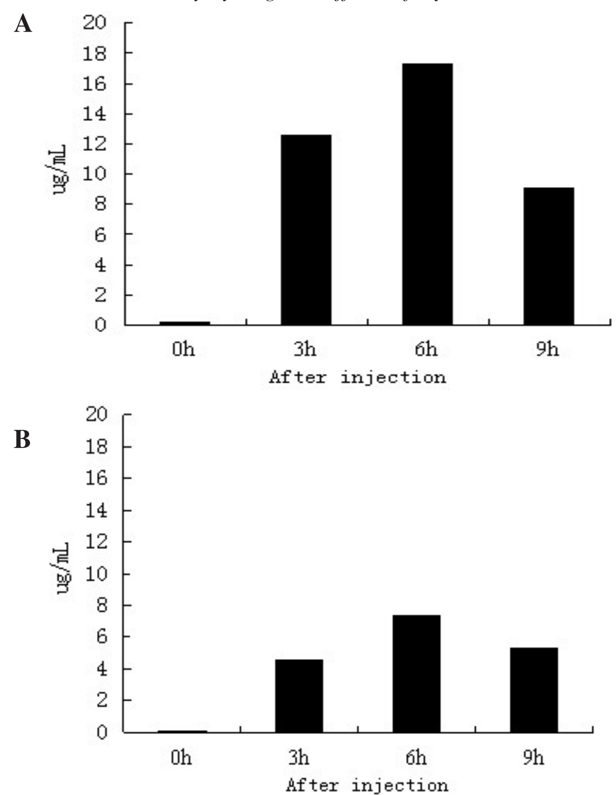

Figure 5. Detection of 5-FU Trace in Blood Sample of rClone30-CD/5-FC Group. (A) The detection of 5-FU trace in the mice infected with rClone30-CD plus 5-FC given by tail vein injection. 5-FU was detected in blood samples of the experimental animals by capillary electrophoresis at the indicated times points after injection of 5-FC. The concentration of 5-FU reached the peak level 17.28 μg/mL at 6 h after injecting. (B) The detection of 5-FU trace in the mice infected with rClone30-CD plus 5-FC given by intra-tumor injection. The concentration of 5-FU reached to the peak level 7.33 μg/mL 6 h after injecting

Comparison of tumor volume of the mice treated with NDV rClone30-CD and 5-FC which was given by different administration routes

To compare the tumor volume of the mice treated with rClone30-CD and 5-FC which was given by tail vein or intra-tumor infection, we measured the tumor volume of the mice once in two days from d 7 to d 25 after tumor inoculation. Although the tumor volume of the mice receiving 5-FC by intra-tumor infection was slightly small, the difference is not statistically significant, suggesting that the rClone30-CD /5-FC enhances the anti-tumor activity of the lentogenic virus by different administration of 5-FC (Figure 4C).

rClone30-CD/5-FC prolongs survival of the tumor-bearing mice

Survivals of the tumor-bearing mice were observed for 80 days after treatment. The treated mice were sacrificed when the diameter of the tumor reached 12mm and considered as death. At d 80 after tumor inoculation, the total survivals for the remaining mice in each groups were 0/6 for control group, 0/6 for 5-FC group, 2/6 for rClone30-CD group and 3/6 for rClone30-CD/5-FC group when 5-FC was given by tail vein injection; 0/6 for control group, 0/6 for 5-FC group, 1/6 for rClone30-CD group and

4/6 for rClone30-CD/5-FC group when 5-FC was given by intra-tumor infection (Figure 4D and 4E).

Detection of 5-FU in the blood of rClone30-CD/5-FC-treated mice

To confirm rClone30-CD can convert the 5-FC into 5-FU in treated tumors, 5-FU was detected by capillary electrophoresis in blood samples of the experimental animals at 3, 6 and 9 h after 5-FC infection. 5-FU concentration in the mice 5-FC given by tail vein injection was 12.54, 17.28 and 9.05 μg/mL and the peak was 6 h post-injection. 5-FU concentration in the mice 5-FC given by intra-tumor injection was 4.59, 7.33 and 5.30 μg/mL, the peak was 6 h post-injection. The concentration of 5-FU by tail vein injection was slightly higher than intra-tumor infection (Figure 5A and 5B). In view of 5-FU was detected in rClone30-CD/5-FC-treated mice, therefore, the ability of CD to convert 5-FC to 5-FU is proved and translation of the CD gene to cytosine deaminase is confirmed.

Discussion

Newcastle disease virus (NDV) is an avian virus, which has been proved to infect some avian species but does not infect human. The virulence of the NDV has been postulated to be a major determinant of anti-tumor efficacy. Several wild NDV velogenic and mesogenic strains (MTH-68, 73-T, Ulster, PV701, HUJ) have been shown to be cytotoxic for a range of classes of human tumor cells. In clinical studies, some have shown promise for treating a variety of tumor types. Strain MTH-68 has been shown to have beneficial effects in glioma, astrocytoma and various advanced cancers; 73-T in sarcomas, carcinomas and melanomas; PV701 in various advanced solid tumors, HUJ in glioblastoma and lung tumors; Ulster strain in melanoma, breast and gastrointestinal tumors (Schirrmacher et al., 1999; Fabian et al., 2007). These viruses are either velogenic or mesogenic strains, which have a better anti-tumor effect than the lentogenic strain, but the clinical application or large scale production of velogenic or mesogenic strains may be harmful to the poultry industries. Although the mesogenic strains are still used as a poultry vaccine to prevent the Newcastle disease, the application has been terminated in some countries. From long run, the mesogenic strains are not conducive to use in clinic. The application of the lentogenic strain is safe, it is more than half a century of history to use the lentogenic LaSota strain as a vaccine to prevent the Newcastle disease in poultry industries. It has been proved that LaSota strain is a safe and steady virus to use in clinic, but its drawback is that absence of trypsin, the virus is hard to infect the host cells repeatedly. Therefore, the anti-tumor effect of the virus is poor. The purpose of our study is to maintain its advantages, and simultaneously enhance its cytotoxic and anti-tumor efficacy though integration of the CD/5-FC system.

5-FC is a broad-spectrum antifungal drug and cytosine deaminase gene (CD gene) is metabolic bypass enzyme that exists in the bacteria or yeast, does not exist in mammal. Normal mammalian cells do not contain CD and are relatively resistant to 5-FC (Bentires-Alj et al., 2000). 5-FU is a common moderately toxic anti-tumor drug for treating the digestive system cancers, liver cancer, breast cancer and other cancers in clinic, 5-FU has low toxicity to the normal cells, tissues and organs (Huber et al., 1994). 5-FU in the cancer cells is converted to 5-FUMP, the substance can combine with reduced tetrahydrofolic acid and thymidylate synthetase (TS) as a trimer to inactivate TS and prevent deoxyuridine acid from changing into deoxythymidine acid, which in turn inhibits DNA synthesis to achieve the goal of tumor inhibition (Springer, 1996; Arica et al., 2002; Springer et al., 2004; Kaliberova et al., 2008). In previous studies, some cytokines were expressed in recombinant NDV vectors, which are uncontrollable and may cause some unexpected side effects. The biggest advantage of CD/5-FC system is controllable, the operate efficacy of this system is prone to control by adding an appropriate dosage of 5-FC. Although CD/5-FC system has many advantages, it is difficult to find an effective way to deliver a suicide gene into mammalian cancer cells, therefore, the barrier limits the application of this system. In previous studies, the delivery methods for CD gene relied on the adenovirus vector or lentivirus vector (Ichikawa et al., 2000), but the adenovirus and lentivirus do not have inherent ability to kill the cancer cells. NDV has many advantages compared to the adenovirus and the lentivirus. Firstly, NDV specially infects the cancer cells due to the sialic acid (SA) receptors that dominately anchored on the surface of cancer cells, therefore, NDV is prone to infect the cancer cells. There are little SA receptors on the surface of normal cells in mammal. Therefore, it is considered that the treatment of patients with NDV is safe. Secondly, the NDV has ability to kill cancer cells by apoptosis effect (Altomonte et al., 2010).

In our study, the lentogenic LaSota Clone30 was chosen as a vector to deliver CD gene into the cancer cells. We evaluated the cytotoxicity of the rClone30-CD/5-FC system in HepG2 cell line. The significant efficiency of this system was demonstrated in the HepG2 cell lines at 1 and 0.1 MOIs on opportune time points by MTT method. However, at 0.01 and 0.001 MOIs, the difference between the two groups was not significant, we infer that at low MOIs, the quantum of CD was insufficient to convert 5-FC to 5-FU.

In our murine H22 tumor-bearing model experiment, CD/5-FC in deed improved the anti-tumor efficacy of the lengtogenic NDV. Although NDV alone can inhibit the tumor growth, rClone30-CD/5-FC significantly increased the tumor repression. The result was confirmed by two different administrations of 5-FC, intratumorous injection and intraveinous injections. In 80 day period observation, none of the animals in the control groups (treated with PBS or 5-FC alone) could live to d 28 after tumor inoculation. rClone30-CD/5-FC significantly improved the survival rate of the treated animal from 17% (1/6, treated by NDV alone) to 67% (4/6, treated by rClone30-CD/5-FC) in one experiment where 5-FC was given by intratumorous injection. The result was confirmed by intraveinous injection of 5-FC. These improvements can be explained

by detection of 5-FU in the blood of the rClone30-CD/5-FC-treated mice, but not in other mice.

To our knowledge, this is the first attempt to use NDV as a shuttle vector to deliver suicide genes into tumors of the xenographic mice for cancer therapy. The CD/5-FC significantly improves the anti-tumor effect of the lengtogenic NDV. The treatment of tumor-bearing mice with rClone30-CD/5-FC leads to an effective tumor inhibition and retention, and raises survival of the treated mice. Currently, we are investigating therapeutic efficiency and safety of the recombinant rClone30-CD/5-FC against other tumor-bearing models.

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