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ORIGINAL ARTICLE

Phase I and biodistribution study of recombinant adenovirus vector-mediated herpes simplex virus thymidine kinase gene and ganciclovir administration in patients with head and neck cancer and other malignant tumors

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In this study, we investigated the safety and efficacy in cancer patients of a single intra-tumor injection of recombinant adenovirus vector-mediated herpes simplex virus thymidine kinase gene (AdV/TK) followed by systemic administration of ganciclovir (GCV). In 18 patients with malignant tumors refractory to standard treatment, AdV/TK was injected on day 1 with dose escalation from 2.5×10^{11} to 1×10^{12} virus particles (VP), and GCV (5 mg kg^{-1}) was delivered intravenously every 12 h from days 2 to 15. The most common treatment-related toxicities were transient fever (10/18) and local injection site reaction (10/18), and most adverse events were WHO grade I/II. Anti-adenovirus antibody levels increased continuously during treatment, but anti-HSV antibody levels remained stable. One patient had a PR at the injection site but PD was found in the primary site (lung cancer), one patient with fibrosarcoma of the neck had an MR, five patients had SD, and 10 patients had PD. In conclusion, AdV/TK followed by GCV can be administered safely to Chinese cancer patients, and achieved a local response with few environmental effects. Because the response was localized, single regional tumor relapse, especially after radiation, may be an indication for this suicide gene therapy.
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Keywords: suicide gene therapy; thymidine kinase; ganciclovir; phase I clinical trial

Introduction

The use of gene therapy, including suicide gene therapy, in the treatment of malignancy has been researched for decades. Extensive experience related to toxicity, pharmacology and clinical indications has been gained and reported.[1–3] The concept of suicide gene therapy (also known as prodrug therapy) refers to the delivery into tumor cells of enzymes that metabolize systemically administered nontoxic prodrugs into locally active chemotherapeutic agents. The most commonly used suicide gene in human clinical trials has been the herpes simplex virus thymidine kinase (HSV tk) gene, whose protein product is an enzyme that can convert the nontoxic, clinically used, antiviral drug ganciclovir (GCV) into a highly cytotoxic phosphorylated form.[4,5] One attractive feature of the HSV tk/GCV system is the presence of a 'bystander effect' and the induction of antitumor immune responses.[6–8]

On account of shortages of the retrovirus vector, researchers have recently begun to use recombinant replication-incompetent adenoviruses (Ad) as vectors to transfer different target genes including HSV tk. Ad-expressing HSV tk (AdV/TK)/GCV has shown evidence of *in vitro* and *in vivo* efficacy in many different animal models of malignancy. Phase I/II trials have subsequently been conducted using AdV/TK/GCV in a number of malignant tumors, including ovarian carcinoma, brain tumors, prostate cancer, hepatocellular carcinoma and so on.[9–14] All of these trials have shown that the approach is relatively safe, but efficacy has been localized to the injection site, which has limited its use. However, this treatment may be particularly suitable for some tumors that have the tendency to relapse locally, such as head and neck cancer or nasopharyngeal carcinoma (NPC). Such malignancies are common among Chinese people in whom relapse is usually local with no distant metastasis. Systemic treatment often fails because of poor regional blood supply; these cancers are also resistant or intolerant to radiotherapy. There have been no earlier reports of suicide therapy for these types of tumors.

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For this study, we selected NPC and head and neck cancer patients, and also included some other solid tumors. Most patients had received radiotherapy and/or chemotherapy and some had relapsed in the region that had been treated with full-dose radiation. Our goal was to determine the safety profile, humoral immune response and biologic activity of a single intra-tumor injection of AdV/TK followed by systemic administration of GCV and to characterize the pharmacokinetics of AdV/TK in cancer patients.

Patients and methods

Patients

Patients with histologically confirmed malignant tumors refractory to standard treatment were eligible. All patients had superficial tumors that were easily accessible. Other inclusion criteria were: age $\geqslant 18$ years and $\leqslant 70$ years; life-expectancy $\geqslant 12$ weeks; an Eastern Cooperative Oncology Group performance status of 0 to 2; chemotherapy completion $\geqslant 4$ weeks prior and recovery from drug-induced toxicities; hemoglobin $\geqslant 9$ g per 100 ml; ANC $\geqslant 2000\,\mu l^{-1}$; platelet count $\geqslant 100\,000\,\mu l^{-1}$; bilirubin $\leqslant 1.5\times$ institutional upper limit of normal; AST and ALT $\leqslant 2.5\times$ institutional upper limit of normal; creatinine clearance rate $>50\,ml\,min^{-1}$; and no coexisting medical problems that would limit compliance. Patients with CNS metastases, prior gene therapy, second primary tumor, gravidity, lactation, hypersensitivity to antiviral drugs, immunologic deficit or active infections were excluded. All patients provided written informed consent for all clinical and research aspects of the study according to institutional guidelines before treatment. The trial was conducted in accordance with the Declaration of Helsinki and approved by the appropriate institutional review board.

Study design

In this phase I, single center, open label study a single injection of AdV/TK (Shenzhen Tiandakang Gene Engineering Co., Ltd, High Technology Industrial Park Central, Shen Zhen, P.R. China) was given intra-tumor on day 1, and the dose escalation depended on preclinical studies and the literature. We planned to enroll six patients at each of three dose levels of AdV/TK. Starting at 2.5×10^{11} virus particles (VP)/patient, the AdV/TK dose was doubled until three of nine patients at the same level experienced dose-limited toxicity, which was defined as any grade $\geqslant 3$ hematologic or nonhematologic toxicity (excluding fever). The highest dose was 1.0×10^{12} VP/patient. GCV (HU BEI KEYI Pharmaceutic Co., Ltd, Donghu New Technology Development District, Wuhan, P.R. China) was delivered at a dose of $5\,mg\,kg^{-1}$ i.v. every 12 h from days 2 to 15. The maximal toxicity dose was defined as the highest dose at which fewer than three of nine patients experienced dose-limited toxicity. No other cancer therapy was permitted while patients were on study. Any other medications were recorded. All patients completed treatment in the absence of progressive disease or intolerable toxicity.

Safety and tumor response

Full physical examination, tumor assessment, CBC, liver and renal function evaluation and $CD4^+/CD8^+$ ratio measurements were performed at enrollment and at periodic intervals until 30 days after the last GCV dose. Adverse events (AEs) were recorded throughout the study and graded according to the WHO Common Toxicity Criteria. Responses of the target tumor and all lesions were assessed according to WHO Response Criteria every week, and were confirmed at least 4 weeks after the initial observation.

Vector dissemination and biodistribution

Peripheral venous blood, injection site swabs, throat swabs and urine and stool samples in relation to treatment were collected in drying tubes at predetermined intervals. A probe (Da An Gene Co., Ltd. of Sun Yat-sen University, High and New Technology Development District, Guangzhou, P.R. China) was used to detect the adenoviral vector in the patient samples by real-time polymerase chain reaction assay. The forward primer was 5′-CATTGGTGTGCACCTCCAAG-3; the reverse primer was 5′-CGCAGACGCGTGCTGAT-3′. The probe was 5′-FAM-AGCTCGGATCTTGGTGGCGTGAAAC T-TAMRA-3′. Briefly, a DNA extraction kit was used to extract DNA and compare it with a standard curve constructed using a series of dilutions of control DNA extract. The PCR cycling program consisted of 1 cycle at 93 °C for 10 min, 40 cycles at 93 °C for 45 s (denaturation) and 55 °C for 45 s (annealing and extension).

Detection of neutralizing antibodies directed to adenovirus 5 and herpes simplex virus

Plasma samples were taken at pretreatment and on days 7, 14, 21 and 28 after treatment. ELISA was used to quantify IgM and IgG against HSV I/II and IgG against adenovirus 5 (Ad5). Neutralizing antibodies against Ad5 were detected using an E1-deleted replication defective Ad5 virus encoding β-galactosidase in 293 cells *in vitro*.[15]

Detection of plasma concentrations of GCV

Plasma samples were taken at pretreatment and on days 1, 3, 7, 10 and 18 after treatment. HPLC (Waters 2487, Waters Co., Ltd, http://www.waters.com) was used to detect the plasma concentration of GCV based on a standard curve constructed using a prepared series of concentrations of GCV.

Results

Patient characteristics and treatment

Eighteen patients with advanced malignancies were enrolled in this trial. The median age was 43 years (range 29–65 years), and 10 patients were men. The diagnoses included NPC ($n=9$), head and neck cancer ($n=3$), breast cancer ($n=3$), fibrosarcoma of the neck ($n=1$),

non-small cell lung cancer ($n=1$) and angioimmunoblastic T-cell lymphoma ($n=1$). All patients had received anti-tumor therapy and progressed before enrollment. The target tumors of 13 patients (NPC = 8, head and neck cancer = 2, other tumors = 3), into which AdV/TK was injected, were in the region that received full-dose radiation. There were three dose levels of AdV/TK, 2.5×10^{11} VP, 5.0×10^{11} VP and 1.0×10^{12} VP, and each was given to six patients. All patients completed treatment and were assessed for toxicity, and 17 patients were evaluated for response. Five patients withdrew during the study (one for voluntary reasons, four because of disease progression).

Safety and tumor response

Table 1 summarizes the treatment-related AEs according to the AdV/TK dose level for each schedule. Most events were mild or modest. There were no instances of dose-limited toxicity or treatment-related severe AEs. The major events were fever (10/18) and injection site reactions (10/18). Fever was often present at day 1 and the median duration was 2 days (1–6 days). Four patients who experienced fever complained of chills, one had transient hypertension, one had grade II fever that recurred on days 2 and 3, and one had repetitive low-grade fever from days 1 to 6. The fever could be controlled by paracetamol or physical hypothermy methods. Injection site reactions often occurred on day 2 and included increases in skin temperature, rubescence or swelling of the skin and pain. Most reactions were mild and did not need special treatment. The median duration was 5 days (2–13 days). One patient on a dose of 1.0×10^{12} VP who had prior target tumor region irradiation 40 days before enrollment experienced injection site ulceration and infection on day 3 and received antibiotic therapy and recovered by day 12. Leukopenia often occurred on day 7 but was not accompanied by infection, and the median duration was 9 days (3–35 days). Gastrointestinal reactions included loss of appetite (33.33%), nausea (16.67%) and vomiting (16.67%). These symptoms were self-limiting. Five patients with flu symptoms experienced nasal occlusion and nasal discharge; the median time for appearance of symptoms was day 8 (days 5–28) and the median duration was 14 days (6–24 days). Two patients treated at the 1.0×10^{12} VP dose complained of dizziness and fatigue and needed fluid replacement. The median time to hepatic transaminase elevation was 7 days, and in three patients this was accompanied by grade 1 alkaline phosphatase elevation. Two patients needed liver aids. There was no significant difference in the $CD4^+/CD8^+$ ratios before and after treatment.

Table 1 Observed treatment-emergent adverse events

Adverse event	*Grade (WHO-CTC)*	*AdV/TK dose, 2.5×10^{11} VP*	*AdV/TK dose, 5.0×10^{11} VP*	*AdV/TK dose, 1.0×10^{12} VP*
Fever	I	1	1	0
	II	2	3	2
	III	0	1[a]	0
Injection site reaction	I	6	1	2
	II	0	0	1
Loss of appetite	I	3	1	1
	II	0	0	1
Nausea	I	1	0	2
Vomiting	I	1	0	2
Hypertension	I	1	0	0
Hypotension	I	0	0	1
Abdominal pain	II	0	1	0
Chills	I	2	0	0
	II	2	1	0
Flu-like symptoms	I	2	0	1
	II	0	0	2
Leukopenia	I	1	2	2
	II	1	1	0
Anemia	I	3	3	2
Thrombocytopenia	I	1	0	0
Granulocytopenia	I	1	0	1
	II	1	0	0
Hyponatremia	I	0	0	1
AST elevation	I	1	2	0
ALT elevation	I	1	2	0
	II	1	1	0
Alkaline phosphatase elevation	I	1	2	0
Hypoproteinemia	I	0	1	0
Proteinuria	I	3	2	0

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; VP, virus particles; WHO-CTC, WHO common toxicity criteria.
[a]The patient's temperature reached 40.5°C after AdV/TK injection at day 1 and the fever lasted for 30 min.

Seventeen patients were suitable for efficacy assessment and the responses according to AdV/TK dose level are given in Table 2. No patients had complete response. With regard to target tumor response, one patient had partial response (PR; NSCLC) and six had minor response (MR; 2 NPC, 2 breast cancer, 1 fibrosarcoma of the neck, 1 angioimmunoblastic T-cell lymphoma). For target tumors in the region that had received full-dose radiotherapy, three patients had PR or MR. An NPC patient who had been given radiotherapy for metastasis in a submental lymph node experienced a reduction of 41% in the target tumor volume after treatment with 2.5×10^{11} VP. Ultrasound showed the liquefaction and necrosis of the lesion, but a new metastasis appeared in the axillary region at day 21. After treatment with 1.0×10^{12} VP, an NSCLC patient who had received chemotherapy and radiotherapy at the primary site in the lung and bilateral supraclavicular region, had a PR at the injection site (metastasis in the supraclavicular lymph node) but progressive disease was found in the opposite site. At the same dose level, a patient who had fibrosarcoma of the neck and relapsed locally after two operations had an MR in the target tumor (Figure 1).

Vector dissemination and biodistribution

Real-time polymerase chain reaction using an AdV/TK-specific primer and probe combination found the vector

Table 2 Tumor response at different dose levels of AdV/TK

AdV/TK dose	*No of patients (N = 17)*	*Patients with target tumor response*				*Patients with total response*		
		PR	*MR*	*SD*	*PD*	*MR*	*SD*	*PD*
2.5×10^{11} VP	5	0	2	2	1	0	1	4
5.0×10^{11} VP	6	0	3	1	2	0	1	5
1.0×10^{12} VP	6	1	1	3	1	1	3	2
Total	17	1	6	6	4	1	5	11

Abbreviations: MR, minor response; PD, progressive disease; PR, partial response; SD, stable disease; VP, virus particles.

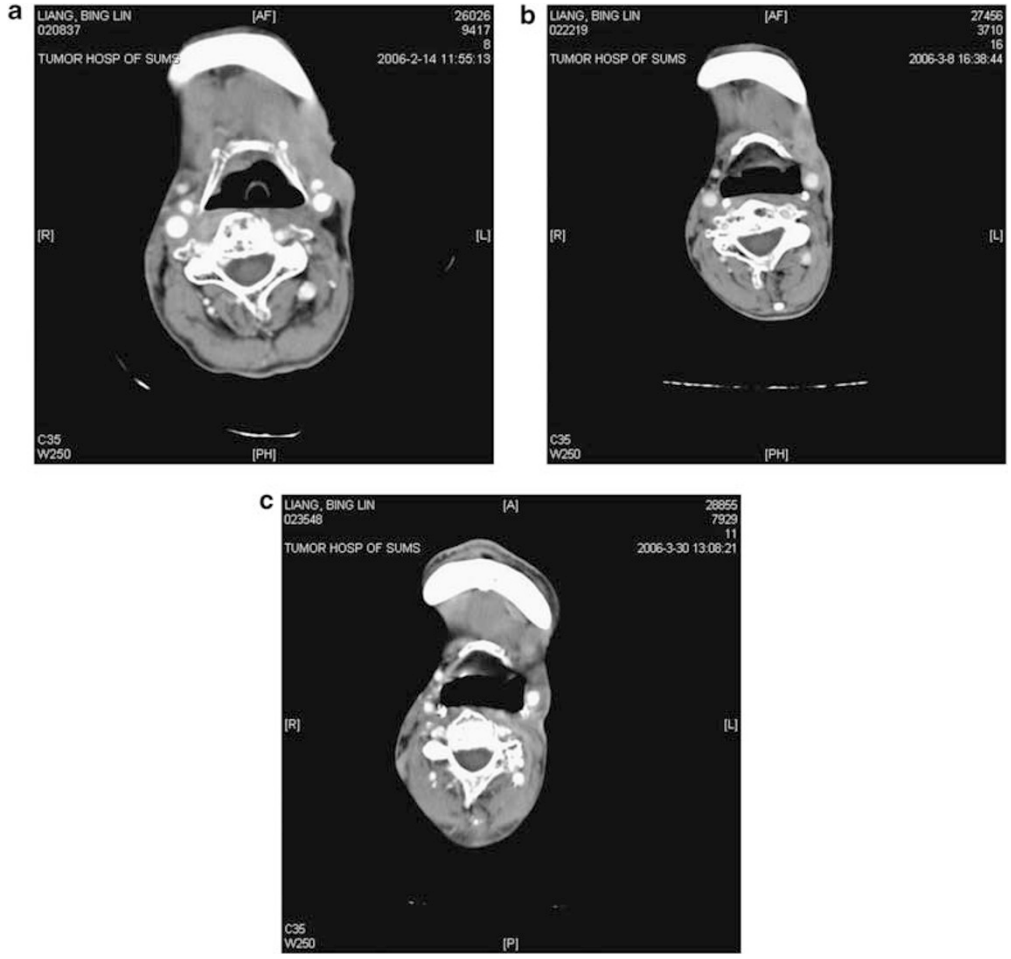

Figure 1 The patient was diagnosed with fibrosarcoma in the neck region and had received full-dose radiotherapy because of NPC 5 years earlier. The fibrosarcoma relapsed twice after two operations. After treatment with AdV/TK injection, a minor response (MR) was recorded in the target lesion. (**a**) CT scan before treatment, and further scans on (**b**) day 22 and (**c**) day 44 after injection.

in the plasma, throat, injection site, urine and stool (Tables 3–5). No vector was detected in the air in the injection room. In the plasma, vector was detected 2–8 h after AdV/TK injection and the mean peak plasma concentration (C_{max}) occurred approximately 16 h after injection. After 72 h most of the AdV/TK DNA in the plasma had been eliminated. The highest C_{max} values were 4.2×10^6 VP, 2.7×10^7 VP and 2.4×10^7 VP at the 2.5×10^{11} VP, 5×10^{11} VP and 1×10^{12} VP dose levels, respectively. The amount of AdV/TK absorbed into the blood circulation was no more than 0.0054%. AdV/TK DNA was detected in the throats and injection sites of all patients and had disappeared by day 7. AdV/TK DNA was also detected in urine specimens and rectal/stool swab specimens. At a dose of 2.5×10^{11} VP, two of six patients were found to be positive in the urine and stool at day 1. At 5.0×10^{11} VP and 1.0×10^{12} VP, all patients were found to be positive until days 3 to 7. The highest numbers of copies in the urine and stool were 4.7×10^7 VP and 6.7×10^6 VP, respectively, and the percentage of

Table 3 Vector shedding into the plasma after AdV/TK injection

AdV/TK dose	*Patient no*	*Time after AdV/TK injection*									
		Pre	*4 h*	*8 h*	*12 h*	*16 h*	*24 h*	*32 h*	*D3*	*D7*	*D14*
2.5×10^{11} VP	1	−	+	+	++	++	++	#	+	−	−
	2	−	+	+	++	++	+	#	+	−	−
	3	−	+	+	++	++	+	#	+	−	−
	4	−	+	+	++	++	+	#	+	−	−
	5	−	+	+	++	++	+	+	+	−	−
	6	−	+	+	++	+	+	+	+	−	−
5.0×10^{11} VP	7	−	+	+	+	−	+	+	+	−	−
	8	−	+	+	+	++	+	−	−	−	−
	9	−	+	+	+	++	+	+	−	−	−
	10	−	+	+	+	−	−	+	−	−	−
	11	−	+	+	+	+	+	+	−/+	+	−
	12	−	+	+	+	+	+	+	−/+	−	−
1.0×10^{12} VP	13	−	−	+	−/+	+	+	+	+	−	−
	14	−	+	+	+	+	+	+	+	+	−
	15	−	+	+	+	+	+	+	-/+	−	−
	16	−	+	+	+	+	+	#	+	−	−
	17	−	+	+	+	+	++	#	+	−	−
	18	−	−	+	+	++	+	−	−	−	−

Note: −, −/+, +, ++, +++ represent AdV/TK vector copies 0–10^2, 10^2–10^4, 10^4–10^6, 10^6–10^8, 10^8–10^{10} copies, respectively, #, sample absent.

Table 4 Vector shedding into the throat after AdV/TK injection

AdV/TK dose	*Patient no.*	*Time after AdV/TK injection*								
		Pre	*Immediate*	*2 h*	*4 h*	*12 h*	*24 h*	*D3*	*D7*	*D14*
2.5×10^{11} VP	01	−	+	+	+	++	++	++	++	−
	02	−	+	+	+	+	+	+	+	−
	03	−	+	+	+	+	+	+	+	−
	04	−	+	++	+	+	+	+	+	−
	05	−	+	+++	++	+	+	−	−	−
	06	−	++	++	+	−	++	+	−	−
5.0×10^{11} VP	07	−	++	+	+	+	−/+	+	+	−
	08	−	+	+	+	+	+	+	−	−
	09	−	++	+	++	++	++	+	−	−
	10	−	++	++	++	++	++	++	+	−
	11	−	++	+	+	+	+	+	++	−
	12	−	+	+	++	+	+	+	++	−
1.0×10^{12} VP	13	−	++	+	++	+	+	+	+	−
	14	−	++	+	++	+	+	+	++	−
	15	−	++	++	++	+++	++	++	++	−
	16	−	+++	+	++	++	++	+	++	−
	17	−	+	+	+	+	+	−	−	−
	18	−	+	+	++	+	+	++	−	−

AdV/TK discharge was no more than 0.0084 and 0.0067%, respectively.

Detection of neutralizing antibodies directed to adenovirus 5 and herpes simplex virus

Before the AdV/TK injection, many patients had detectable IgG antibodies to adenovirus 5 (see Figure 2a). In all, 12 patients showed an increase in titer of anti-adenovirus 5 IgG between 7 and 14 days post-treatment. There was a direct correlation between the AdV/TK injection dose and the extent of antibody response, but there was no relationship between the amount of vector in the plasma and the antibody response. Compared with the value before treatment, anti-HSV I/II antibody levels remained stable after injection (see Figures 2b and c).

Table 5 Vector shedding into the injection site after AdV/TK injection

AdV/TK dose	*Patient no*	*Time after AdV/TK injection*								
		Pre	*Immediate*	*2 h*	*4 h*	*12 h*	*24 h*	*D3*	*D7*	*D14*
2.5×10^{11} VP	01	−	++	++	++	++	+	+	++	−
	02	−	+++	++	++	++	++	+	+	−
	03	−	++	++	++	++	+	+	+	−
	04	−	+++	+++	++	++	++	+	+	−
	05	−	+++	++	++	++	++	+	−	−
	06	−	+++	+++	#	+++	++	++	−	−
5.0×10^{11} VP	07	−	++	++	++	++	++	++	++	−
	08	−	++	+	++	++	++	−	−	−
	09	−	++	++	++	+	+	−	−	−
	10	−	+++	+++	++	++	++	+	−	−
	11	−	+++	++	++	++	++	+	+	−
	12	−	++	++	++	+	+	−	−	−
1.0×10^{12} VP	13	−	+++	++	++	+	++	−	−	−
	14	−	+++	++	+	++	#	+	+	−
	15	−	+++	+++	+++	+++	+++	++	+	−
	16	−	+++	++	++	++	++	−	−	−
	17	−	+++	++	+	+	++	++	+	−
	18	−	+++	+++	+++	++	+++	++	+	−

Detection of plasma concentrations of GCV

GCV concentrations in all patients were higher than $100\,\mu g\,l^{-1}$ (see Figure 3), and in seven patients they were higher than $200\,\mu g\,l^{-1}$. There was no correlation between GCV concentration and tumor response. We also found an entity that may be a phosphorylated GCV that persisted in the plasma until 12 days after GCV administration. However, as there is no standard phosphorylated GCV sample, we were unable to verify its identity.

Discussion

To determine the feasibility of TK suicide therapy in human cancer treatment, several clinical trials incorporating regional administration of ADV/TK have been conducted in patients with a variety of malignancies.[8–14] There have been no reports evaluating this agent in NPC or head and neck cancer, both of which are common among Chinese people, often relapse locally and are often resistant to radiotherapy and chemotherapy. In this study, the majority of subjects had been diagnosed with NPC or head and neck cancer. Most of the injection sites

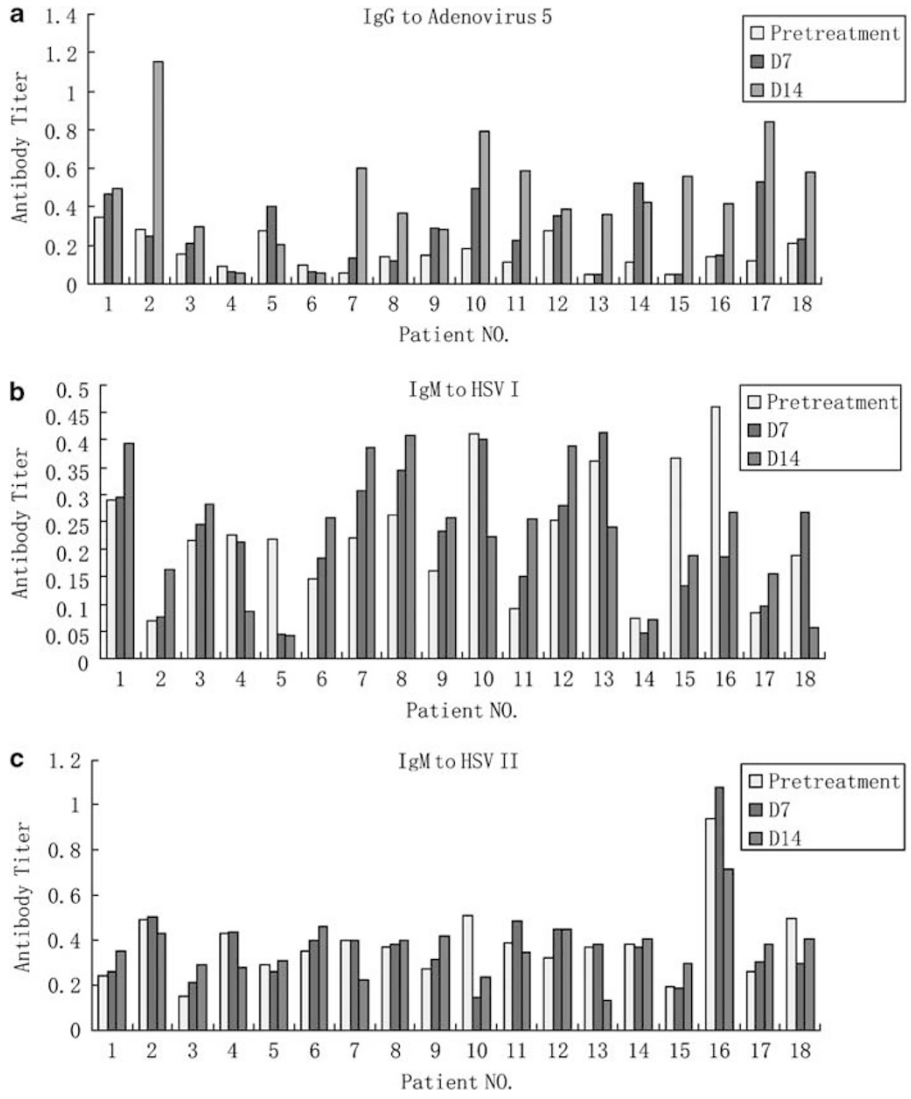

Figure 2 Neutralizing antibodies. Plasma samples were taken before treatment and at weekly intervals after treatment, and tested by enzyme-linked immunosorbent assay for type-specific neutralization antibodies of (**a**) Ad 5 and (**b**/**c**) HSV I/II.

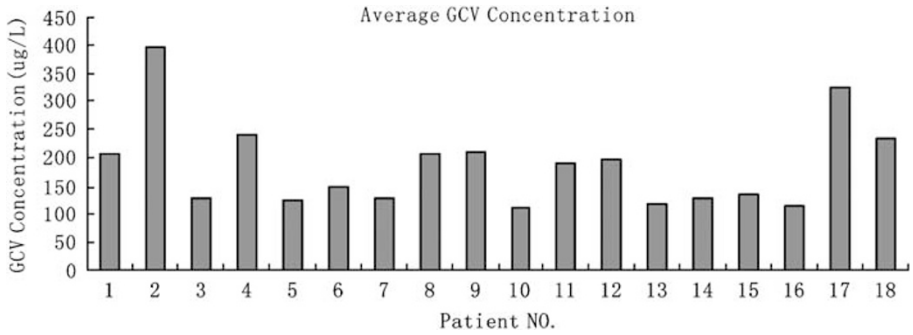

Figure 3 GCV concentrations. Plasma samples were taken before treatment and on days 1, 3, 7 and 10 during GCV treatment.

had previously received local treatment such as radiotherapy and surgery. The study showed that AEs in a Chinese population were similar to those reported in previous studies. Fever was one of the most common treatment-related AEs, and may be due to a physical immune response to the vector as fever was often associated with increasing numbers of neutralizing antibodies directed to Ad5. Although the incidence of fever

(affecting 55.56% of the patients) was high, the symptom was often minor and transient and easily controlled. Injection site reactions were another common AE and most were mild and subsided after several days without treatment, even if the site had previously received radiation treatment. If the patient has been given local treatment such as radiotherapy just before ADV/TK administration, the skin at the local injection site needs to be carefully protected. In this study, some patients experienced other AEs including digestive tract reactions, flu-like symptoms, chills, fatigue, lethargy and diarrhea, which frequently occur in patients who have received adenovirus vector gene therapy. According to literature reports, GCV can cause myelosuppression including leukopenia, anemia and neutropenia. We also found myelosuppression during and after GCV treatment, and these events may be attributed to GCV. Though the effects were modest, it is advisable to carefully monitor the patient's blood cell count during and after GCV treatment, especially in patients whose bone marrow may have been destroyed by chemotherapy. Our study also showed there is no correlation between the ADV/TK dose and AEs, except for the flu-like symptoms. At a dose of 1.0×10^{12} VP, flu-like symptoms were likely to be a little more serious than at the other two dose levels, and their duration was longer.

After local administration, the adenovirus vector remained at the injection sites. The number of AdV/TK copies was more than 10^8 VP in 12 patients, so local site cleaning or protection is important. Our results also suggested AdV/TK has a tendency to migrate to the upper respiratory tract, including pharynx tissue, and the titer depended on the ADV/TK injection dose. AdV/TK was also absorbed into the circulation through the local injection site, and the vector titer in the plasma was related to the blood supply at the injection site, but not the AdV/TK dose. AdV/TK was seldom found in urine and stool specimens, implying a low incidence of central spread of AdV/TK vectors. Our findings also showed that there was some correlation between Ad5 antibody levels and systemic inflammatory reaction, though the reaction was mild.

As to treatment effect, AdV/TK followed by GCV did reduce the local target tumor in part. The effect was independent of the dose of AdV/TK. Moreover, suicide gene therapy appeared to elicit no distant response, which was consistent with the literature. This is the first report of a response to suicide gene therapy among NPC patients ($n = 8$), all of whom had received radiotherapy and chemotherapy before enrollment. We found two patients displayed MR in the target tumor, and two SD. In tumors, which frequently relapse at the local site, such as NPC, AdV/TK followed by GCV may be an appropriate treatment choice.

We monitored GCV concentration in 18 patients, and GCV concentration was $>100\,\mu g\,l^{-1}$ in all patients and $>200\,\mu g\,l^{-1}$ in seven patients. Increasing the dose of GCV increased the toxicity but not the response, so GCV administered at a dose of $5\,mg\,kg^{-1}$ q12h was appropriate.

In summary, our results suggest that AdV/TK /GCV combination suicide gene therapy is safe in Chinese patients and achieved a local response with few environmental effects. Because the response was localized, single regional tumor relapse, especially after radiation, may be an indication for this gene therapy.

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