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Original Article

Long-term toxicity, pharmacokinetics and immune effects of a recombinant adenovirus vaccine expressing human papillomavirus 16 E6 and E7 proteins (HPV16 E6E7-Ad5 Vac) in primates

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Abstract: Objective: This study aimed to: evaluate long-term toxicity and pharmacokinetic parameters; to identify the target organ of toxicity of a recombinant adenovirus vaccine expressing human papillomavirus 16 E6 and E7 proteins (HPV16 E6E7-Ad5 Vac) in primates; and to determine the specific immune response of this recombinant adenovirus vaccine. Method: HPV16 E6E7-Ad5 Vac (dose 4.68 × 10^9 IU/bottle) was administered to *Macaca fascicularis* (*M. fascicularis*) to evaluate its long-term toxicity. The Cynomolgus Monkeys were divided into a negative control group (sodium chloride injection group), a low-dose group (4.68 × 10^8 IU/macaque), and, a high-dose group (4.68 × 10^9 IU/macaque). The drugs were administered at intervals of once every three weeks (D1, D21, D42). The macaques were observed until the sixth week of the recovery period (D84) for safety and toxicological indicators and pharmacokinetic indicators. To study the specific immune response in Rhesus Macaque, empty viruses (rAd5-null) and buffer were inoculated as controls, respectively. Two doses of the vaccine were given at 1.0 × 10^8 IU/ml and 1.0 × 10^9 IU/ml and theHPV-16 E6-/HPV-16 E7-specific IFN-γ productions were measured. Results: The macaques of both the high-dose group and the low-dose group did not exhibit any systemic toxic response. The administered safe dose of the vaccine was 4.68 × 10^9 IU per animal. Following vaccination, HPV16 E6/E7-specific antibodies were observed to be generated in both groups, indicating an immune response of the lymphocytes targeting HPV16 E6 and HPV16 E7 epitopes (specific NF-r) was elicited. The peak level of HPV-16 E6-/HPV-16 E7-specific IFN-γ production was observed in the ninth week.

Keywords: HPV16, adenovirus vector, primate, long-term toxicity, pharmacokinetics, immune responses

Introduction

Persistent high-risk human papillomavirus (HPV) infection is associated with cervical lesions: low immune response has been found in cervical intraepithelial neoplasia (CIN) with high-risk HPV infection and cervical cancer [1, 2]. Vaccinating HPV16 E6E ad5 Vac could promote the body to produce enough cytotoxic lymphocytes (CTL) to target virus early proteins (E6 and E7 transforming protein), in which the CTL could kill the cells with integrated DNA and tumor cells, as well as control the proliferation of HPV at early infection. Meanwhile, the body could produce the neutralizing antibody against the virus to decrease the number of infected cells and help the CTL to better clear the virus. This is identified as a potential pathway to treat high-risk HPV infection [3-5].

A recombinant adenovirus vaccine expressing HPV-16 E6 and E7 proteins (HPV16 E6E7-Ad5 Vac) was prepared using replication-defective adenovirus type 5 as the carrier. This vaccine stimulated the production of neutralizing antibodies and specific cellular immune response [6].

We evaluated the long-term toxicity and pharmacokinetic parameters to identify the target

organs of toxicity of HPV16 E6E7-Ad5 Vac in *M. fascicularis*. The specific immune response of this vaccine in the macaques was observed. Moreover, we observed whether the pre-stored anti-adenovirus type 5 antibody interfered with the immune response elicited by the vaccine.

Materials and methods

Materials

Vaccine: HPV16 E6E7-Ad5 Vac was prepared under GMP conditions and used for the pilot test. Dose: 4.68×10^9 IU/bottle, 1.0 ml/bottle. The vaccine was stored at -200°C with a shelf life of 12 months.

To verify the inserted target HPV type 16 E6/E7 gene the virus from work seed bank (WSB) [6] and the final product were tested by PCR, western blot analysis, and, immunofluorescence analysis. The 771-bp codon-optimized HPV type 16 E6/E7 gene was inserted into the adenovirus. The specific bands at a molecular weight of 40,000 Da indicated the final product (vaccine) expressed the HPV 16 E6/E7 fusion proteins: this was re-confirmed by immunofluorescence analysis. The product seed bank (batch 2011-1130) and its production of vaccine (20121-201, 20160301) were directly sequenced with whole genes employed by Shanghai Personalbio Company. This product from its initial working seed bank through to the final vaccine products found no gene mutation phenomenon. No gene mutation phenomenon was found following 15 continuous generations of the working seed bank, indicating that we had established the virus library has a good passaging stability (HPV16 E6E7-Ad5 Vac was approved for clinical trail license No: 2017L04912 by State Food & Drug Administration, China).

Experimental macaques

Eighteen healthy female adult Cynomolgus Monkeys (3-4 years of age) were used to observe the long-term toxicity and pharmacokinetics and to identify the target organs of toxicity of the vaccine. Twelve healthy adult rhesus monkeys (8 females and 4 males, 3-5 years of age) were used to observe the specific immune response of the vaccine. It was ascertained whether the pre-stored anti-adenovirus type 5 antibody affected the immune response of the vaccine.

Grouping

For the long-term toxicity evaluation the Cynomolgus Monkeys were randomly divided into 3 groups (n=6), which were designated as a negative control group (sodium chloride injection group), a low-dose group (4.68×10^8 IU/macaque) and a high-dose group (4.68×10^9 IU/macaque). The vaccine was given via intramuscular injection once every three weeks (D1, D21, D42). Multi-point injections were performed with a dose not above 0.5 ml at each point a total of three times. Euthanasia was performed on 3 macaques at the end of drug administration and at the sixth week of the recovery period (D84), respectively.

Observation indicators: The Cynomolgus Monkeys were monitored for body weight, body temperature, electrocardiogram (ECG), blood cell count, coagulation function, blood biochemistry, urine, ophthalmological indicators, T-cell subgroups ($CD3^+$, $CD4^+$, $CD8^+$, $CD4^+/CD8^+$), adenovirus antibody in serum, specific IgG antibody against HPV16 protein, cytokines (TNF-α, IFN-γ, IL-2, IL-4, IL-6), spot numbers of IFN-γ-secreting T cells in PBMCs, and, distribution in tissues.

Absorption kinetics and distribution in tissues

Whole blood samples were collected before the first and last administrations of drugs and at different time points after drug administration. Euthanasia was performed for three macaques at three days after the last administration (D46) and at the sixth week of the recovery period (D84), respectively. Tissues were collected from each group, and quantitative PCR was performed to determine the contents of viral vectors in the whole blood and tissues. The lowest limit of quantification was 100 copies/reaction. For PCR: the upstream primer was GAAAGGGCAGCCAAAACGTA; the downstream primer was AATGAGTGCCAATATGGAATTTCC; and the probe sequence was [FAM] CACCGCCCCGGTTTTCCCC [TAMRA]. Non-compartmental analysis technique (NCA) was used to calculate the main absorption kinetic parameters from the whole blood samples in each group. Gross total resection was performed for all sacrificed animals. Major organs were harvested and weighed, and the organ coefficient was calculated. Histopathological examination was performed on over 40 organs and tissues.

Table 1. Summary of clinical observations

Group	Dose (IU)	n	Death	Open mouth breathing	Lack of energy	Sleepy	Prone	Abnormal activity	Soft stools	Rash	Coarse sparse	Hair removal	Pale	Sagging skin	Perianal contamination
1	0	6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	5/6	0/6	0/6	0/6	0/6
2	4.68×10^8	6	1/6	1/6	2/6	1/6	1/6	2/6	1/6	4/6	4/6	0/6	1/6	2/6	1/6
3	4.68×10^9	6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	4/6	1/6	0/6	0/6	0/6

Table 2. Body weight, body temperature, electrocardiogram, blood cell count, coagulation function, blood biochemistry, cytokine test results of animals in each dose group (observed till the 84th day)

Group	Dose (IU/only)	n	Weight (kg)	TM (°C)	HR* (times/min)	Blood cell number# RBC ($\times 10^{12}$/L)	Coagulation function$ PT (s)	Blood biochemistry $ ALT(U/L)	Cytokines¢ $CD4^+/CD8^+$
1	0	6	3.64±0.313	38.10±0.00	248.3±8.73	5.68±0.19	8.33±0.26	51.3±2.7	0.99±0.15
2	4.68×10^8	6	3.80±0.38	38.07±0.17	253.7±7.13	5.90±0.31	8.07±0.12	49.3±5.2	1.19±0.51
3	4.68×10^9	6	3.42±0.13	38.50±0.15	246.7±9.23	5.81±0.12	8.83±0.03	50.7±9.7	1.10±0.11

Note: ECG * includes: heart rate (beats/min), P-R interval (s), QRS duration (s), Q-T interval (s). Compared with the first group in the same period, all indexes P>0.05; Blood count # included: RBC ($\times 10^{12}$/L), HGB (g/L), HCT (%), MCV (fL), MCH (Pg), MCHC (g/L), PLT ($\times 10^9$/L), Retic (%), WBC ($\times 10^9$/L), Neut (%), Lymph (%), Mono (%), Eos (%), Baso (%), Compared with the group 1 in the same period, all indexes P>0.05; Blood coagulation function $ including: PT (s), APTT (s); Blood Biochemicals $ including: ALT (U/L), AST (U/L), TBiL (umol/L), ALP (U/L), GGT (U/L), TP (g/L), AlB (g/L), G1b (g/L), A/G, CK (U/L), LDH (U/L), Glu (mmol/L), BUN (mmol/L), Cre (umol/L), TCH (mmol/L), TG (mmol/L), Ca (mmol/L), P (mmol/L), Na (mmol/L), K (mmol/L), Cl (mmol/L); Cytokines ¢ including: $CD3^+$ (%), $CD4^+$ (%), $CD8^+$ (%), $CD4^+/CD8^+$.

Table 3. Detection results of anti-adenovirus Ad5 antibody and anti-HPV16E6E7 protein-specific antibody (IgG) in each dose group

Test time	Group	Dose (IU)	N	Anti-adenovirus Ad5 antibody		Anti-HPV16E6E7 protein-specific antibody (IgG)	
				Positive conversion rate	Antibody titers (1:X)	Positive conversion rate	Antibody titers (1:X)
D15	1	0.0	6	0/6	-	0/6	-
	2	4.68×10^8	6	0/6	-	1/6	100
	3	4.68×10^9	6	3/6	168±33.7	4/6	268±56.7
D21	1	0.0	6	0/6	-	0/6	-
	2	4.68×10^8	6	0/6	-	1/6	100
	3	4.68×10^9	6	2/6	223±66.7	6/6	333±33.7
D42	1	0.0	6	0/6	-	0/6	-
	2	4.68×10^8	5	1/5	100	1/5	400
	3	4.68×10^9	6	4/6	267±33.7	6/6	2678±333.7
D57	1	0.0	3	0/3	-	0/3	-
	2	4.68×10^8	3	1/3	100	3/3	100
	3	4.68×10^9	3	3/3	333±33.7	3/3	1023±126.7
D71	1	0.0	3	0/3	-	0/3	-
	2	4.68×10^8	3	0/3	-	1/3	100
	3	4.68×10^9	3	3/3	333±33.7	3/3	1023±126.7
D85	1	0.0	3	0/3	-	0/3	-
	2	4.68×10^8	3	0/3	-	0/3	-
	3	4.68×10^9	3	3/3	333±33.7	3/3	567±66.7

Note: N indicates the number of animals; "-" indicates that no antibody was detected.

The specific immune response in rhesus macaque

The monkeys were divided into four groups (n=3, 2 females and 1 male). Intramuscular injection was performed in the lower limbs at the dose of 1.0 ml a time. All included monkeys were negative for total anti-adenovirus type 5 (Ad5) antibody two days prior to the commencement of the experiment. In group A, one dose (1×10^8 IU/ml) of empty viruses (rAd5-null) was given at 0 d (D0), the fourth week (D29) and the eighth week (D56), respectively. In group B, empty viruses (rAd5-null) were given at a dose of 1×10^8 IU/ml, followed by one dose of HPV16 E6E7-Ad5 Vac (1.0×10^9 IU/ml) at D28 and D56, respectively. In group C, empty viruses (rAd5-null) were given at a dose of 1×10^8 IU/ml at D0, followed by one dose of HPV16 E6E7-Ad5 Vac (1.0×10^8 IU/ml) at D28 and D56, respectively. In group D, buffer was inoculated, followed by one dose of HPV16 E6E7-Ad5

Table 4. T lymphocyte numbers of PBMCs secreting IFN-[gamma] in peripheral blood of each dose group Individual test data (SFCs/10^6 PBMCs)

Group	Dose (IU)	Sex	Mmonkey number	D7		D13		D28		D46		D85	
				E6	E7	E6	E7	E6	E7	E6	E7	E6	E7
1	0	♀	15-701	0	6	0	0	14	2	0	22	/	/
			15-702	2	0	0	0	6	4	0	0	/	/
			15-703	0	0	0	0	0	0	0	0	/	/
			15-704	0	6	2	0	0	0	0	0	0	0
			15-705	0	0	0	0	2	2	0	4	0	0
			15-706	0	2	0	0	0	0	4	0	0	0
2	4.68×10^8	♀	15-707	2	0	0	0	2	0	0	32	/	/
			15-708	32	0	24	8	x	x	x	x	/	/
			15-709	0	2	0	0	0	0	0	0	/	/
			15-710	0	0	6	0	0	0	0	0	122	2
			15-711	8	2	0	0	0	2	4	0	0	0
			15-712	2	2	36	0	2	0	48	0	6	6
3	4.68×10^9	♀	15-713	2	0	4	4	16	4	64	16	/	/
			15-714	4	0	8	2	6	0	28	0	/	/
			15-715	2	2	0	0	20	0	26	4	/	/
			15-716	18	0	0	0	4	8	10	12	0	8
			15-717	0	8	20	2	12	0	18	0	100	0
			15-718	2	2	0	4	4	32	14	20	24	14

Vac (1.0×10^8 IU/ml) at D28 and D56, respectively. The monkeys were observed until the eleventh week (D77), after which the rhesus monkeys were given euthanasia.

Detection of HPV16 E6/E7-specific IFN-r

From each monkey 2 ml of whole blood samples were collected from each monkey and combined with an anticoagulant to separate the lymphocytes. Elispot detection was performed using the kit (Mabetech, article number 3421-4HPT-10). The results were interpreted based on the experiment. Among approximately 200 thousand cells per well, at least 11 spot-forming cells (SFCs) were observed on average after the addition of the corresponding antigen or stimulation with peptide. Moreover, the number of SFCs should be four times that of the number in the negative control well. The results were considered positive if these two conditions were met [7].

Euthanasia was performed according to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition (the American Veterinary Medical Association, 2013).

The rhesus monkeys experiment was conducted by JOINN laboratories, Inc. (Suzhou) and Institute of Medical Biology, Chinese Academy of Medical Sciences (Kunming). Both were authenticated by AAALAC International.

Statistical analysis

All statistical analyses were conducted using a two-tailed test with the significance level set at 0.05. Levene's test was used for homogeneity of variance test. Also employed were the Kruskal-Wallis test and Mann-Whitney U test.

Results

One macaque died in the low-dose group (4.68×10^8 IU per animal) before the second administration (D22). Based on observed clinical symptoms and a histopathological examination, it was concluded that the death was caused by respiratory failure due to bacterial pneumonia. This phenomenon was only observed in the low-dose group and its incidence was low. The autopsy revealed prominent features of bacterial infection of the lung, thus the death was considered an accident not arising from the use of the vaccine.

Table 5. Results of T lymphocyte speciation assay (SFCs/10^6 PBMCs) of PBMCs secreting IFN--γ

Test time	Group	Dose (IU)	N	E6	E7
D7	1	0.0	6	0.3±0.3	2.3±1.2
	2	4.68×10^8	6	7.3±5.1	1.0±0.4
	3	4.68×10^9	6	4.8±3.3	2.4±1.5
D13	1	0.0	6	0.3±0.3	0.0±0.0
	2	4.68×10^8	6	11.0±6.3	1.3±1.3
	3	4.68×10^9	6	5.3±3.2	2.0±0.7
D28	1	0.0	6	3.7±2.3	1.3±0.7
	2	4.68×10^8	5	0.8±0.5	0.4±0.4
	3	4.68×10^9	6	10.3±2.8	7.3±5.1
D46	1	0.0	3	0.7±0.7	4.3±3.6
	2	4.68×10^8	3	10.4±9.4	6.4±6.4
	3	4.68×10^9	3	26.7±8.0*	8.7±3.5
D85	1	0.0	3	3.7±2.3	0.0±0.0
	2	4.68×10^8	3	42.7±39.7	2.7±1.8
	3	4.68×10^9	3	41.3±30.1	7.3±4.1

Note: N indicates the number of animals; *indicates that $P \leq 0.05$ compared with the first group in the same period.

Except for the single death in the low-dose group (4.68×10^8 IU per animal) noted above, euthanasia was implemented for all other animals as planned. No clinical manifestations associated with the vaccine were observed in the living animals in the low-dose group (4.68×10^8 IU per animal) and high-dose group (4.68×10^9 IU per animal) (**Table 1**).

During the experiment, body weight, body temperature, ECG, blood cell count, coagulation function, blood biochemistry, urine, ophthalmological indicators and cytokines were monitored. No findings of toxicological significance were observed (**Table 2**).

Antibody detection: Four out of six animals in the high-dose group (4.68×10^9 IU per animal) were positive for anti-HPV16 IgG antibody at 14 days after the first administration (D15) with a high titer of 1:100-1:400. Three out of six animals were positive for the anti-adenovirus antibody with a high titer of 1:100-1:400.

As the number of administrations increased, both the number of animals positive for anti-HPV16 IgG antibody and the antibody titer increased in the high-dose group (4.68×10^9 IU per animal). At 42D, antibody titer reached the peak of 1:100-1:6400 (2678±333.7). At 85D, the end of the recovery period, the high-dose group remained positive for anti-HPV16 IgG antibody, with a titer of 1:100-1:1600 (567±66.7), which signified a decrease compared with the measurements taken at previous time points. However, no apparent changes were observed with the anti-adenovirus antibody.

The low-dose group (4.68×10^8 IU per animal) was positive for anti-HPV16 IgG antibody and anti-adenovirus antibody only at a few time points, with an antibody titer of 1:100-1:400. As the number of administrations increased, no apparent changes were observed in the low-dose group compared with the findings in the high-dose group (**Table 3**).

At 3D after the last administration (D46), the spot number of IFN-γ-secreting T cells in PBMCs increased slightly after E6 antigen stimulation for the high-dose group (4.68×10^9 IU per animal). At other time points, the spot number of IFN-γ-secreting T cells in PBMCs did not change significantly for other groups (**Tables 4**, **5**).

Toxicokinetic detection: Within the dose range of 4.68×10^8-4.68×10^9 IU per animal, the drug exposure in the whole blood increased with dose. The dose was administered at a ratio of 1:10 between the low- and high-dose groups. Following the first administration, the average Cmax/AUC (0-24 h) ratio was 1:10.10 and 1:11.60, respectively. Following the last administration, the average Cmax/AUC (0-24 h) ratio was 1:21.77 and 1:21.13, respectively.

The accumulation factors were 1.20 and 1.89 in the low- and high-dose groups, respectively, indicating no apparent accumulation (**Tables 6**-**8**).

Distribution in tissues: At 3D after the last administration (D46), HPV16 genomic DNA could be only detected in the muscles where the vaccine was injected. The detected content was proportional to the dose injected, and the dose ratio was 1:10 between the low- and high-dose groups. The average content ratio of HPV16 genomic DNA was 1:17.06 between the low- and high-dose groups. HPV16 genomic DNA was not detected in any other tissues. No HPV16 genomic DNA could be detected in any

Table 6. Toxicokinetic parameters are as follows

Time	Group		T_{max} h	C_{max} copy/0.5 μg	AUC_{last} h·copy/0.5 μg	$AUC_{(0\text{-}24\ h)}$ h·copy/0.5 μg	AF
D1	Low dose group (4.68 × 10^8 IU)	Mean	24.00	2.32E+03	5.48E+04	2.19E+04	
		SD	0.00	1.08E+03	3.11E+04	9.47E+03	
		n	6	6	6	6	
	High dose group (4.68 × 10^9 IU)	Mean	24.00	2.34E+04	7.18E+05	2.54E+05	
		SD	0.00	1.98E+04	4.83E+05	1.34E+05	
		n	6	6	6	6	
D43	Low dose group (4.68 × 10^8 IU)	Mean	24.00	2.24E+03	6.13E+04	2.45E+04	1.20
		SD	0.00	9.29E+02	1.79E+04	7.87E+03	0.29
		n	6	6	6	6	6
	high dose group (4.68 × 10^9 IU)	Mean	22.00	4.87E+04	1.03E+06	5.18E+05	1.89
		SD	4.90	5.27E+04	6.77E+05	4.91E+05	1.03
		n	6	6	6	6	6

Table 7. Determination of genomic DNA content of human papillomavirus type 16 adenovirus vector vaccine in cynomolgus Monkey whole blood - 4.68 × 10^8 IU-whole blood (copy/0.5 μg)

Time (hour) 15-707		Cynomolgus monkey number						Mean (Copy/0.5 ug)	SD	CV (%)
		15-708	15-709	15-710	15-711	15-712				
D1-D4	0 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	0.5 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	1 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	2 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	4 h	BLQ	BLQ	BLQ	8.22E+01	BLQ	7.24E+01	7.33E+01	6.93E+00	8.96
	6 h	7.75E+01	BLQ	BLQ	9.87E+01	2.02E+02	2.10E+02	1.47E+02	6.87E+01	46.72
	8 h	8.30E+01	1.39E+02	2.99E+02	2.48E+02	4.48E+02	5.75E+02	2.99E+02	1.86E+02	62.32
	12 h	2.45E+02	4.33E+02	1.09E+03	8.73E+02	1.43E+03	8.36E+02	8.19E+02	4.32E+02	52.79
	24 h	1.18E+03	2.69E+03	3.10E+03	2.37E+03	3.67E+03	9.00E+02	2.32E+03	1.08E+03	46.75
	48 h	BLQ	9.19E+02	6.79E+02	3.82E+02	1.37E+03	3.86E+02	7.47E+02	4.14E+02	55.42
	72 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
D43-D46	0 h	BLQ	-	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	0.5 h	BLQ	-	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	1 h	BLQ	-	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	2 h	BLQ	-	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	4 h	BLQ	-	BLQ	6.58E+01	1.72E+02	7.12E+01	1.03E+02	5.98E+01	58.07
	6 h	1.79E+02	-	7.16E+01	3.13E+02	3.40E+02	3.11E+02	2.43E+02	1.14E+02	47.12
	8 h	3.20E+02	-	2.96E+02	7.56E+02	8.28E+02	3.76E+02	5.15E+02	2.56E+02	49.61
	12 h	5.65E+02	-	7.25E+02	1.43E+03	1.82E+03	9.53E+02	1.10E+03	5.16E+02	47.07
	24 h	1.59E+03	-	3.55E+03	2.53E+03	2.39E+03	1.14E+03	2.24E+03	9.29E+02	41.53
	48 h	7.57E+02	-	7.45E+02	5.86E+02	1.16E+03	9.03E+02	8.29E+02	2.14E+02	25.84
	72 h	BLQ	-	BLQ	BLQ	BLQ	BLQ	NA	NA	NA

Note: BLQ: indicates lower than the lower limit of the standard curve; NA indicates that it can not be calculated; - indicates that the sample does not exist.

tissues at the end of the recovery period (**Table 9**).

Pathological examination: The weights of the organs and the organ coefficient did not change after different treatments. Neither the overall examination nor histopathological examination revealed any systemic toxicological and pathological changes caused by drug administration. No abnormal changes associated with drug administration were readily observed locally (**Figure 1**).

Detection of HPV16 E6/E7-specific IFN-r: At 6 W (two weeks after the first dose of vaccine), specific cellular immune response was elicited

Table 8. Determination of Genomic DNA of Human Papillomavirus Type 16 Adenovirus Vector Vaccine in Cynomolgus Monkeys - High-dose (4.68×10^9 IU) - Whole blood (copy/0.5 μg)

TIME (hour) 15-713		Cynomolgus monkey number						Mean (Copy/0.5 ug)	SD	CV (%)
		15-714	15-715	15-716	15-717	15-718				
D1-D4	0 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	0.5 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	1 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	2 h	1.91E+03	BLQ	BLQ	BLQ	BLQ	1.21E+03	1.56E+03	4.94E+02	31.72
	4 h	4.88E+03	BLQ	BLQ	BLQ	4.05E+03	1.80E+03	3.58E+03	1.59E+03	44.48
	6 h	5.85E+03	1.09E+03	3.47E+03	6.75E+03	4.51E+03	3.96E+03	4.27E+03	1.98E+03	46.32
	8 h	7.44E+03	3.31E+03	6.20E+03	8.10E+03	7.64E+03	4.64E+03	6.22E+03	1.90E+03	30.54
	12 h	1.08E+04	6.54E+03	1.16E+04	9.38E+03	1.15E+04	8.48E+03	9.71E+03	1.97E+03	20.34
	24 h	1.12E+04	8.68E+03	1.46E+04	5.70E+04	3.84E+04	1.05E+04	2.34E+04	1.98E+04	84.70
	48 h	1.01E+04	3.10E+03	5.58E+03	1.42E+04	5.70E+03	4.98E+03	7.27E+03	4.08E+03	56.17
	72 h	2.41E+03	BLQ	BLQ	6.07E+03	2.60E+03	1.98E+03	3.27E+03	1.89E+03	57.71
D43-D46	0 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	0.5 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	1 h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA	NA
	2 h	2.54E+03	BLQ	BLQ	BLQ	4.27E+02	BLQ	1.49E+03	1.50E+03	100.77
	4 h	2.93E+03	9.65E+02	3.03E+03	BLQ	2.27E+03	2.49E+03	2.34E+03	8.28E+02	35.40
	6 h	7.36E+03	2.58E+03	6.40E+03	8.14E+03	4.35E+03	2.68E+03	5.25E+03	2.39E+03	45.56
	8 h	9.11E+03	5.31E+03	1.12E+04	1.34E+04	6.30E+03	5.39E+03	8.45E+03	3.35E+03	39.63
	12 h	1.47E+04	7.37E+03	3.15E+04	1.49E+05	1.33E+04	9.72E+03	3.76E+03	5.52E+04	146.86
	24 h	2.37E+04	1.31E+04	6.53E+04	2.95E+04	2.59E+04	1.53E+04	2.88E+04	1.90E+04	65.84
	48 h	8.99E+03	2.96E+03	1.03E+04	1.03E+04	8.10E+03	7.64E+03	8.04E+03	2.71E+03	33.78
	72 h	2.57E+03	BLQ	BLQ	3.51E+03	1.46E+03	BLQ	2.51E+03	1.02E+03	40.75

Note: BLQ: means below the lower limit of the standard curve; NA means it can not be calculated.

in all animals of group B, 2/3 of the animals in group C and 1/3 of the animals in group D in terms of HPV16 E6-specific IFN-r. At 9 w (one week after the second dose of vaccine), specific cellular immune response was elicited in all animals of group B and 2/3 of the animals in group C in terms of HPV16 E6-specific IFN-r. At 11 W (three weeks after the second dose of vaccine), specific cellular immune response was elicited in all animals of group B, 1/3 of the animals in group C and 1/3 of the animals in group D in terms of HPV16 E6-specific IFN-r.

At 6 W (two weeks after the first dose of vaccine), specific cellular immune response was elicited in 1/3 of the animals of group B and 1/3 of the animals in group C in terms of HPV16 E7-specific IFN-r. At 9 w (one week after the second dose of vaccine), specific cellular immune response was elicited in 2/3 of the animals of group B, 2/3 of the animals in group C and 2/3 of the animals in group D in terms of HPV16 E7-specific IFN-r. At 11 W (three weeks after the second dose of vaccine), specific cellular immune response was elicited in all animals of group B and 2/3 of the animals in group D in terms of HPV16 E7-specific IFN-r (**Table 10**; **Figures 2**, **3**).

Discussion

We prepared a recombinant adenovirus vaccine expressing human papillomavirus 16 E6 and E7 proteins (HPV16 E6E7-Ad5 Vac) in accordance with the specifications in Guidelines for Researches on Human Gene Therapy and Formulation Quality Control Technology (released by the State Food and Drug Administration dated March 20th, 2003) and Research and Development and Quality Control of Biotechnology Medicines (edited by Wang Zhijun) [8].

We have demonstrated in previous studies that this vaccine elicited specific immune response and had an anti-tumor effect in C57BL/6 mice. Subcutaneous inoculation of 1×10^4 TC-1 tumor cells was performed to the groin area

Table 9. Determination of genomic DNA content of human papillomavirus type 16 adenovirus vector vaccine (copy/0.5 μg) in cynomolgus monkey tissues (D46, D85)

Test time	Groups	Different organization of Cynomolgus monkeys											
		Brain	Heart	Liver	Spleen	Lung	Kidney	The muscle of administration site	The muscle of no-administration site	Gonads	Stomach	Jejunum	Whole blood
D46	Negative control	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	Low dose group (4.68 × 10^8 IU)	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	Mean: 8.35E+04, SD: 4.74E+04, CV (%): 56.83	BLQ	BLQ	BLQ	BLQ	BLQ
	High dose group (4.68 × 10^9 IU)	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	Mean: 1.42E+06, SD: 1.27E+06, CV (%): 89.34	BLQ	BLQ	BLQ	BLQ	BLQ
D85	Negative control	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	Low dose group (4.68 × 10^8 IU)	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	High dose group (4.68 × 10^9 IU)	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ

Note: BLQ: means lower than the lower limit of the standard curve.

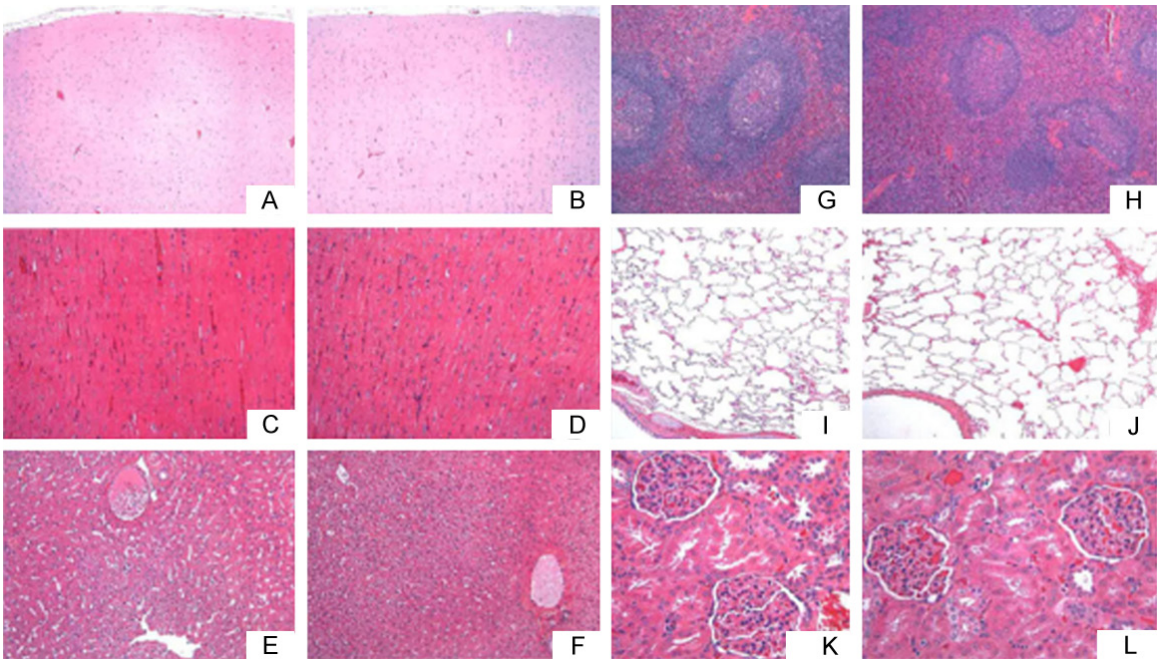

Figure 1. Pathological examination of experimental cynomolgus monkey after euthanasia (D46) 3 days after the last administration (HE staining, 50 ×). Note: Section staining (A and B) Brain tissue (control, high dose); (C and D) Heart (control, high dose); (E and F) liver (control, high dose); (G and H) Spleen (control, high dose); (I and J) Lung (control, high dose); (K and L) Kidney (control, high dose). All compared groups were no different observations.

Table 10. Elispot detection points (SFCs/2 × 10^5 PBMCs) in each group after rhesus monkeys vaccination

Stimulating peptides	Test time	Group A			Group B			Group C			Group D		
E6	6 W	2	4	0	**59**	**32**	**55.5**	**22**	8.5	**22**	5.5	10.5	**41**
	9 W	0	3.5	1	**21.5**	**81**	**88**	**46**	0	**21**	0	0	2
	11 W	4.5	1.5	1	**27.5**	**20**	**12**	**11.5**	8.5	0	0	0	**11**
E7	6 W	0	0.5	0	13.5	0	**28.5**	**31**	0.5	2	0	2.5	1.5
	9 W	0	2	3.5	6	**66.5**	**215**	**63**	0	**89.5**	17	**66**	**89**
	11 W	0	0	1	**45**	**63.5**	**28.5**	6	3	8	0	**18**	**24.5**

Note: Data in black and crossed in the table is a positive response to the criterion. Elispot Test Points: Schematic.

on the medial side of the left leg of the mice. The vaccine was concurrently injected intramuscularly to the right leg (2.36 × 10^7 IU per mouse), with a control group in place. The results showed that the HPV16 E6/E7-specific IFN-r levels increased significantly. When the dose was 2.36 × 10^7 IU per mouse, the protection rate in the anti-tumor test was over 80%. Furthermore tumor occurrence was delayed in mice in which the tumor was not effectively inhibited and the tumor size was much smaller compared with the control group. The experiment had a high repeatability [9, 10].

To further study the pharmacological features of the vaccine before clinical use, we administered the vaccine to primates. As TC-1 tumor cells cannot form tumors in primates, the immune response was measured by detecting antibody production and specific IFN-γ [11, 12].

Cynomolgus Monkeys were used for the pharmacokinetic experiments. We observed no systemic toxicological response after injection of HPV16 E6E7-Ad5 Vac either at a low dose (4.68 × 10^8 IU per animal) or at a high dose (4.68 × 10^9 IU per animal). The safety dose of repeated intramuscular injection (a total of 3 administra-

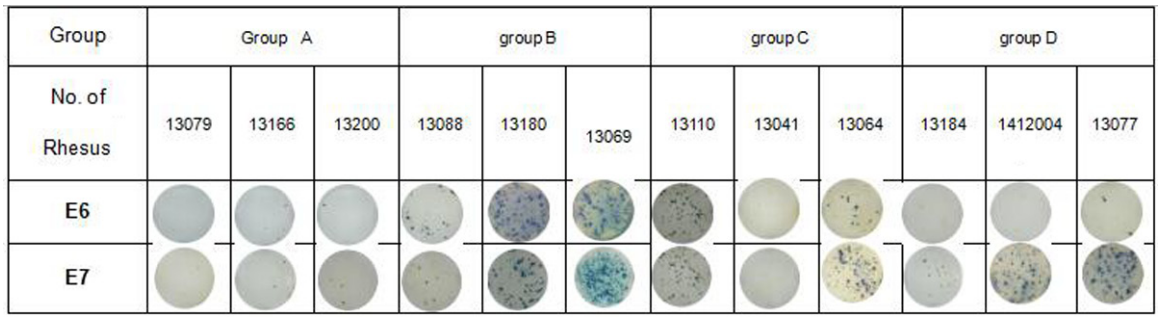

Figure 2. Elispot test read plate results after the vaccination in each group of rhesus (at 9 w). Positive points were elicited in 3/3 of group B, 2/3 group C and 0/3 group D in terms of HPV16 E6-specific IFN-r. Positive points were elicited in 2/3 of group B, 2/3 group C and 2/3 group D in terms HPV16 E7-specific IFN-r. (group A: control).

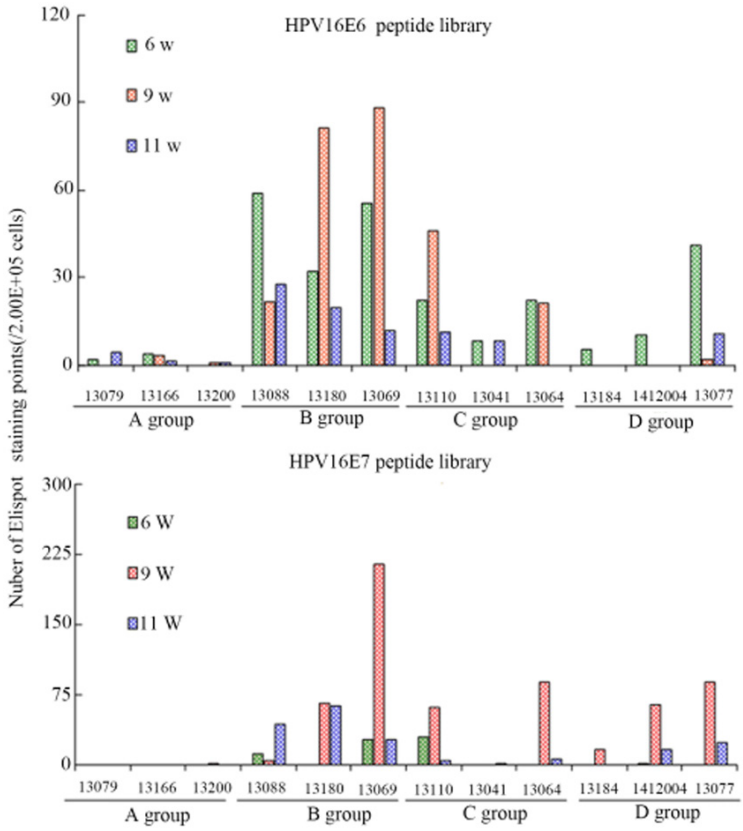

Figure 3. Diagramatic sketch of ELISPOT in different groups of Rhesus macaque (at date of 6 w, 9 w, and 11 w). Positive results for HPV16 E6-specific IFN-r were elicited at 6 w, 9 w, 11 w in 3/3,3/3,3/3 in group B, and 2/3, 2/3, 1/3 in group C, and 1/3, 0/3, 1/3 in group D respectively. Positive results for HPV16 E7-specific IFN-r were elicited at 6 w, 9 w, 11 w in 1/3, 2/3, 3/3 in group B, and 1/3, 2/3, 0/3 in group C, and 0/3, 2/3, 2/3 in group D respectively. (group A: control).

tions for 6 weeks consecutively) in the Cynomolgus Monkeys was 4.68×10^9 IU per animal.

HPV16 E6/E7-specific IFN-r levels were measured to assess the long-term toxicity of the vaccine in the Cynomolgus Monkeys. An increase in the HPV16 E6-specific IFN-r level was only observed at D46, whilst the changes in the HPV16 E7-specific IFN-r level were not significant (**Table 5**). This may be explained by noting Cynomolgus Monkeys are mainly used for safety evaluation and thus are less sensitive. Another possible reason may be the improper choice of E7 peptide library. Elispot assay in macaques' peripheral blood lymphocytes is concerned with the stimulation duration of peptide library for lymphocytes and the sensitivity to TMB-based color development.

Rhesus Monkeys were used for the further experiments and the quality control was optimized by extending the stimulation duration of E7 peptide to approximately 42 h (the recommended duration in the instruction manual is 12-48 h, but the stimulation effect is better at 42 h than at 24 h). Phytohemagglutinin (FHA) was employed as a positive stimulus.

Following the vaccination of the Rhesus Monkeys, specific antibodies against HPV16 E6/E7 could be detected in all treatments, indicating that an immune response of the lymphocytes targeting HPV16 E6 and HPV16 E7 epitopes

(specific NF-r) was elicited. Peak levels were reached at 9 W. As the dose of the vaccine increased, the number of monkeys with a rise in the HPV16 E6/E7-specific IFN-γ level also increased. The HPV16 E6/E7-specific IFN-γ could be still detected at 11 W (e.g group B, **Table 10**) [13]. Where the vaccine dose was 1×10^9 IU/ml, which was higher than the dose of empty viruses (1×10^8 IU/ml, e.g group B, **Table 10**), the level of pre-stored adenovirus type 5 antibody had little impact on the immune response.

Many studies have reported the use of adenoviral vectors in the preparation of therapeutic HPV vaccine to prevent cervical cancer [14-16]. Ahn WS et al. prepared the recombinant adenoviral vector expressing IL-12 and E7 that could enhance the anti-tumor immunity against HPV16-associated tumors. The effect of combined infection with IL-12 and AdE7 was better than that of either alone, and the tumor size was 9 mm in 80% of the animals [17, 18]. Lee DW et al. studied the adenoviral vaccine expressing E7 and E7 proteins which prevented the growth of HPV-positive tumors. It was found that the splenocytes in mice vaccinated with Ad5-E6/E7 only produced E6/E7-specific IFN-γ. At two weeks post vaccination, the production of E6/E7-specific IFN-γ increased significantly and this trend persisted until day 70 [19]. Our results also demonstrated that HPV16 E6/E7-specific IFN-γ was produced post vaccination.

Adenoviruses have been used in anti-tumor treatments as carriers, and many clinical trials are now under way [20-23]. China is the first country to approve the use of recombinant adenoviruses in anti-tumor gene therapy. Following many years of clinical application, the safety of recombinant adenoviruses has been proved and the quality and testing standards have been established [8]. Adenoviral vectors are considered the most suitable carriers for gene delivery in the preparation of therapeutic HPV vaccine.

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Disclosure of conflict of interest

None.

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References

[1] Zur Hausen H. Papillomavirus and cancer: from basic studies to clinical applications. Nat Rev Cancer 2002; 2: 342-350.

[2] Faridi R, Zahra A, Khan K and Idrees M. Oncogenic potential of human papillomavirus and its relation with cervical cancer. Virol J 2011; 8: 269.

[3] Padilla-Paz LA. Human papillomavirus vaccine: history, immunology, current status, and future prospects. Clin Obstet Gynecol 2005; 48: 226-240.

[4] Roden RB, Ling M and Wu TC. Variation to prevent and treat cervical cancer. Hum Pathol 2004; 35: 971-982.

[5] Riezebos-Brilman A, Regts J, Freyschmidt EJ, Dontje B, Wilschut J and Daemen T. Induction of human papillomavirus E6/E7-specific cytotoxic T-lymphocyte activity in immunetolerant, E6/E7-transgenic mice. Gene Ther 2005; 12: 1410-1414.

[6] Wu J, Chen KD, Gao M, Chen G, Jin SF, Zhuang FC, Wu XH, Jiang YS and Li JB. Genetic stability of a recombinant adenovirus vaccine vector seed library expressing human papillomavirus type 16 E6 and E7 proteins. Exp Ther Med 2015; 9: 1161-1165.

[7] Moodie Z, Huang Y, Gu L, Hural J and Self SG. Statistical positivity criteria for the analysis of ELISpot assay data in HIV-1 vaccine trials. J Immunol Methods 2006; 315: 121-132.

[8] Wang J. Biological engineering drugs research development and quality control. Beijing: Science Press; 2002. pp. 606-612.

[9] Wu J, Chen G, Jin SF, Gao M and Zhuang FC. Immune responses of recombinant adenovirus-5 vector vaccine of human papillomavirus type 16 E6E7 and its anti-tumor effects in mice. Inter J Epidemiol Infect Dis 2014; 41: 368-373.

[10] Zhuang FC, Chen G, Wu J, Jin SF, Jiang YS, Gao M, Li JB, Zhao L, Mao ZA and Tian HW. Evalua-

tion of Pre-clinical efficacy to HPV16 L2E6E7 vaccine and HPV16 E6E7 adenovirus-5 vector vaccine with different dosages and prime-booster regiments in mouse model. Vaccine Vaccination 2013; 5.1000189.

[11] Tindle RW and Frazer IH. Immune response to human papillomavirus-specific immunization. Curr Top Microbiol Immunol 1994; 186: 217-253.

[12] Bosch FX, Rohan T, Schneider A, Frazer I, Pfister H, Castellsagué X, de Sanjosé S, Moreno V, Puig-Tintore LM, Smith PG, Muñoz N and zur Hausen H. Papillomavirus research update: high lights of the Barcelona HPV 2000 international papillomavirus conference. J Clin Pathol 2001; 54: 163-175.

[13] Chu NR. Therapeutic vaccination for the treatment of mucosotropic human papillomavirus-associated disease. Expert Opin Biol Ther 2003; 3: 477-486.

[14] Gomez-Gutierrez JG, Elpek KG, Montes de Oca-Luna R, Shirwan H, Sam Zhou H and McMasters KM. Vaccination with an adenoviral vector expressing calreticulin-human papillomavirus 16 E7 fusion protein eradicates E7 expressing established tumors in mice. Cancer Immunol Immunother 2007; 56: 997-1007.

[15] Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, Drijfhout JW, Wafelman AR, Oostendorp J, Fleuren GJ, Offringa R, van der Burg SH and Melief CJ. Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clin Cancer Res 2008; 14: 169-177.

[16] Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, Essahsah F, Fathers LM, Offringa R, Drijfhout JW, Wafelman AR, Oostendorp J, Fleuren GJ, van der Burg SH and Melief CJ. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med 2009; 361: 1838-1847.

[17] Ahn WS, Bae SM, Kim TY, Kim TG, Lee JM, Namkoong SE, Kim CK and Sin JI. A therapy modality using recombinant IL-12 adenovirus plus E7 protein in a human papillomavirus 16 E6/E7-associated cervical cancer animal model. Hum Gene Ther 2003; 14: 1389-1399.

[18] Jin HS, Park EK, Lee JM, NamKoong SE, Kim DG, Lee YJ, Jun HJ, Han BD, Bae SM and Ahn WS. Immunization with adenoviral vectors carrying recombinant IL-12 and E7 enhanced the antitumor immunity to human papillomavirus 16-associated tumor. Gynecol Oncol 2005; 97: 559-567.

[19] Lee DW, Anderson ME, Wu S, Lee JH. Development of an adenoviral vaccine against E6 and E7 oncoproteins to prevent growth of human papillomavirus-positive cancer. Arch Otolaryngol Head Neck Surg 2008; 134: 1316-1323.

[20] Lubaroff DM, Konety BR, Link B, Gerstbrein J, Madsen T, Shannon M, Howard J, Paisley J, Boeglin D, Ratliff TL and Williams RD. Phase I clinical trial of an adenovirus/prostate-specific antigen vaccine for prostate cancer: safety and immunologic results. Clin Cancer Res 2009; 15: 7375-7380.

[21] Smith C, Tsang J, Beagley L, Chua D, Lee V, Li V, Moss DJ, Coman W, Chan KH, Nicholls J, Kwong D and Khanna R. Effective treatment of metastatic forms of Epstein-Barr virus-associated nasopharyngeal carcinoma with a novel adenovirus-based adoptive immunotherapy. Cancer Res 2012; 72: 1116-1125.

[22] Kim KH, Dmitriev I, O'Malley JP, Wang M, Saddekni S, You Z, Preuss MA, Harris RD, Aurigemma R, Siegal GP, Zinn KR, Curiel DT and Alvarez RD. A phase I clinical trial of Ad5.SSTR/TK.RGD, a novel infectivity-enhanced bicistronic adenovirus, in patients with recurrent gynecologic cancer. Clin Cancer Res 2012; 18: 3440-3451.

[23] Tamminga C, Sedegah M, Regis D, Chuang I, Epstein JE, Spring M, Mendoza-Silveiras J, McGrath S, Maiolatesi S, Reyes S, Steinbeiss V, Fedders C, Smith K, House B, Ganeshan H, Lejano J, Abot E, Banania GJ, Sayo R, Farooq F, Belmonte M, Murphy J, Komisar J, Williams J, Shi M, Brambilla D, Manohar N, Richie NO, Wood C, Limbach K, Patterson NB, Bruder JT, Doolan DL, King CR, Diggs C, Soisson L, Carucci D, Levine G, Dutta S, Hollingdale MR, Ockenhouse CF and Richie TL. Adenovirus-5-vectored P. falciparum vaccine expressing CSP and AMA1. Part B: safety, immunogenicity and protective efficacy of the CSP component. PLoS One 2011; 6: e25868.