

Original Article

De-oncogenic HPV E6/E7 vaccine gets enhanced antigenicity and promotes tumoricidal synergy with cisplatin

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In order to develop more effective therapeutic vaccines against cancers with high-risk human papillomavirus (HPV) infection, it is crucial to enhance the immunogenicity, eliminate the oncogenicity of oncoproteins, and take a combination of E7- and E6-containing vaccines. It has been shown recently that PE(Δ III)-E7-KDEL3 (E7), a fusion protein containing the HPV16 oncoprotein E7 and the translocation domain of *Pseudomonas aeruginosa* exotoxin A, is effective against TC-1 tumor cells inoculated in mice, therefore, we engineered PE(Δ III)-E6-CRL-KDEL3 (E6), the de-oncogenic versions of the E7 and E6 fusion proteins [i.e. PE(Δ III)-E7(d)-KDEL3, E7(d), and PE(Δ III)-E6(d)-CRL-KDEL3, E6(d)] and tested the immunoefficacies of these fusion proteins as mono- and bivalent vaccines. Results indicated that the E7(d) get higher immunogenicity than its wild type and the E6 fusion proteins augmented the immunogenicity and antitumor effects of their E7 counterparts. Furthermore, the bivalent vaccine system E7(d) plus E6(d), in the presence of cisplatin, showed the best tumorigenic and tumoricidal effects against established tumors *in vivo*. Therefore, it can be concluded that this novel therapeutic vaccine system, upon further optimization, may shed new light on clinical management of HPV-related carcinomas.

Keywords cancer vaccine; papillomavirus E7 protein; papillomavirus E6 protein; cisplatin; uterine cervical neoplasms

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Introduction

The persistent infection with high-risk human papillomavirus (HR-HPV) such as HPV16 and 18 is a major risk

factor associated with the development of cervical cancer, the second leading cause of cancer deaths in the female population worldwide [1,2]. The prognosis of this disease is far from satisfactory since up to 35% of cervical cancer patients undergoing standard therapy suffer a relapse or metastasis. In addition, the HR-HPVs were also found etiologically to be associated with head and neck squamous cell carcinoma (~20%–25%), mostly in the oropharynx [3,4].

The relationship between the expression of HR-HPV oncogenes, e.g. HPV16 E6 and E7, and the carcinogenesis of HPV-associated cancers is well established [5,6] and therefore many approaches directly against the viral oncogenes have been reported [7,8]. For instance, E6 can enhance the degradation of p53 and disrupt the function of PTPN13, a cellular phosphatase tumor suppressor; E7 can disable pRb and activates Mi2 β [9,10] and Rb can control the activity of E2F transcription factors, which are key regulators of S phase genes. Inactivation of Rb is important for the differentiation-dependent productive viral lifecycle and for tumor progression. In addition, E6 can activate telomerase and then immortalize human primary epithelial cells by co-operating with E7 [6]. Therefore, the E7 and E6 oncoproteins are considered as potential tumor-specific target antigens for the immunotherapy of HPV-associated cervical as well as head and neck cancer [11]. To date, two particle-based prophylactic vaccines, Gardasil (Merck, USA) and Cervarix (GlaxoSmithKline, USA), are shown to have a high effect for blocking initial infection by HPVs 16 and 18, thus reducing the risk for cervical cancer development [12,13]. Unfortunately, these vaccines can not prevent cancer transformation and progression in patients already infected by HPV.

A number of therapeutic vaccine types targeted to E7 and/or E6, including peptide and recombinant protein vaccines

[14–16], as well as DNA vaccines [17–20], have been tested in the hope of blocking the cancer transformation and eradicating the systemic tumor cells instead of merely the HPV infection. Although all of these vaccine systems offer high levels of protection of cervical cancer development, there are still such unresolved concerns as oncogenicity of native proteins, degradation of peptide/protein vaccines, and integration into host genome of DNA vaccines. Furthermore, both of E6 and E7 proteins and DNA vaccines show low antigenicity, thus greatly hinder their clinical developments.

The retrograde-delivery domains of *Pseudomonas aeruginosa* exotoxin A (PE) appear to be a good option to enhance the antigenicity of a given antigen, since these domains are able to facilitate cytosolic localization and antigen presentation [21]. We have reported that the addition of PE domains to the E7 antigen would enhance the antigen-specific immunological response and improve the vaccine potency in DNA or fusion protein formats [21,22] and the fusion protein, PE(Δ III)-E7-KDEL3 (E7), would be able to enhance the antigen-specific immunological responses and exhibit antitumor effects in vaccinated mice challenged with TC-1 cells [22]. We also found the linking of the PE domains with the E6 protein, i.e. PE(Δ III)-E6-CRL-KDEL3 (E6), would elicit similar but less potent effects (CRL is a segment of calreticulin, integrated in the E6 recombinant fusion protein in order to augment the antigenicity of E6, data not shown).

For safety reasons, the use of wild transforming oncoproteins for vaccination is not feasible in humans [23]. In order to alleviate the concerns of the oncogenicities of E6 and E7, the disulfide bonds of these oncoproteins were eliminated by site-directed mutagenesis. The aim of this study is to test the antigenicities of the de-oncogenic fusion proteins and their therapeutic effects on tumor-bearing mice in chemo-immunotherapy regimens. In the past, most HPV researchers had focused on E7, but E6 is another important oncoprotein which may result in HPV-associated lesions. Since it is crucial to develop vaccines targeting E6 [24], we also attempt to investigate the immunological response of simultaneous vaccination with the de-oncogenic formats of E6 and E7, and the synergistic tumor-eradicating efficacy by combining the bivalent immunization with the chemotherapy.

Materials and Methods

Mice and cells

The C57BL/6 mice were maintained in the Laboratory Animal Center of the Chinese University of Hong Kong (CUHK) and used at ages of 4–6 weeks. All these animals studied were given prior approval by the Laboratory Animal Research Committee of the CUHK. The TC-1 cell line is a well-characterized lung epithelial cell line immortalized with both HPV16 E6 and E7 and transformed with the *c-Ha-ras* oncogene [25]. The cells were maintained in RPMI 1640 medium

supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 100 μ mol/l non-essential amino acids (AAs), and 1% sodium pyruvate in a 37°C incubator with 5% CO₂. On the day of tumor graft, the cells were harvested by trypsinization, washed twice with phosphate-buffered saline (PBS), and finally resuspended in PBS to the designated concentration for injection.

Plasmid constructions and preparations of the recombinant proteins

The plasmids pPE(Δ III)-KDEL3 and pPE(Δ III)-E7-KDEL3 were from our previous experiments and their constructions have been reported in details [22]. The expression vector of modified E6, pPE(Δ III)-E6-CRL-KDEL3 was constructed by following the similar procedures, using the DNA and AA sequence of HPV16 E6 gene obtained from the National Center Biotechnology Information Website. Furthermore, the disulfide bonds in E6 in PE(Δ III)-E6-CRL-KDEL3 and E7 in PE(Δ III)-E7-KDEL3 were eliminated by site-directed mutagenesis, i.e. C70G-C115G and C24G-E26G, respectively. The resultant plasmids were designated as pPE(Δ III)-E6(d)-CRL-KDEL3 and pPE(Δ III)-E7(d)-KDEL3, respectively. The accuracy of all of the constructs was confirmed by DNA sequencing. The plasmids, under the control of T7 promoter, were transformed into *Escherichia coli* BL21 (DE3) for expression. After the expression of recombinant proteins was induced, the inclusion bodies in the lysates were recovered; inclusion granules were harvested from the insoluble fraction by centrifugation at 1450 g for 10 min. The pellet was then homogenized in TNE buffer (Tris sodium chloride EDTA buffer, pH 7.5, 10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 50 mM NaCl) containing 100 mM phenylmethanesulfonylfluoride and 1 mg/ml deoxycholate, and supernatant was collected by centrifugation at 1450 g for 10 min. After three harvests, the pellets were collected from the combined supernatants by centrifugation at 27,000 g for 20 min. Urea soluble contaminants were removed by resuspending in three washes of 0.1 M Tris-HCl, pH 8.5, containing 1 M urea, followed by centrifugation at 27,000 g for 20 min. The final granule preparation was solubilized in 20 \times volumes of 8 M urea in TNE buffer with gentle stirring for overnight at room temperature [26]. The proteins were then purified by S200 gel filtration chromatography in denatured and reduced condition (10 mM dithiothreitol) with 6 M urea in TNE buffer. Protein elution fractions were renatured by dialysis against TNE buffers containing 4–0 M urea in a Pellicon device (Millipore, Billerica, USA).

Vaccines and vaccination

Recombinant proteins as shown in Fig. 1A were diluted with PBS to a final concentration of 100 μ g/ml and the samples were incubated at 37°C for 2 h for activation and then thoroughly mixed with 40% ISA51 (Seppic Inc., Paris, France).

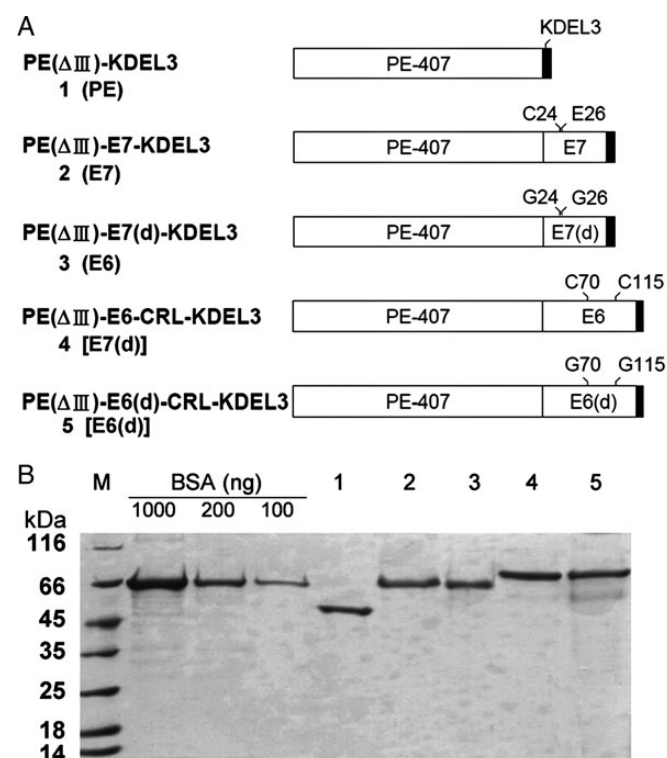


Figure 1. Chimeric DNA construct and characterization of the purified recombinant proteins (A) schematic diagram showing the constructs of DNAs for expressing various recombinant proteins. (B) The SDS–PAGE of various purified protein samples stained with coomassie blue. M, protein markers; BSA, bovine serum albumin with 1000, 200, and 100 ng per well; lane 1, PE(Δ III)-KDEL3; lane 2, PE(Δ III)-E7-KDEL3; lane 3, PE(Δ III)-E7(d)-KDEL3; lane 4, PE(Δ III)-E6-CRL-KDEL3; lane 5, PE(Δ III)-E6(d)-CRL-KDEL3.

In addition to the PE(Δ III)-KDEL3 (PE) control, six kinds of vaccines including PE(Δ III)-E6-CRL-KDEL3 (E6), PE(Δ III)-E6(d)-CRL-KDEL3 [E6(d)], PE(Δ III)-E7-KDEL3(E7), PE(Δ III)-E7(d)-KDEL3 [E7(d)], E6 plus E7, and E6(d) plus E7(d) were thus formulated and then subcutaneously (s.c.) injected into the right flanks of the animals. For each vaccination, each mouse received 100 μ g proteins per dose at days 0, 7, and 14. The immunogenicity of the vaccination regimens were analyzed 1 or 2 weeks after the last immunization as specified in the following.

Cell surface marker staining and cytometric analysis

Cell surface marker staining of CD4 and CD8, intracellular cytokine staining for interferon- γ (IFN- γ), as well as flow cytometric analysis were performed using conditions described previously [27]. Splenocytes (3.5×10^5) from different vaccinated groups of mice were collected (1 week after the last vaccination), pooled, and incubated for 16 h with corresponding peptides prior to FACScan [i.e. 1 μ g/ml E6-MHC-I, AA 49–58, E7-MHC-I, AA 49–57, for detecting E6- or E7-specific CD8 $^+$ T-cell precursors, respectively; 10 μ g/ml E6-MHC-II, AA 43–57, E7-MHC-II, AA 30–67,

for detecting E6- or E7-specific CD4 $^+$ T-cell precursors respectively; all of the peptides were from Invitrogen (Carlsbad, USA)]. The stimulated splenocytes were then washed twice with FACScan buffer and subjected to FACScan analysis. The number of IFN- γ -secreting CD8 $^+$ and CD4 $^+$ T cells were calculated. Analysis was performed on a Becton-Dickinson FACSCalibur with CELLQuest software (Becton-Dickinson Immunocytometry System, Mountain View, USA).

Measurements of E6- and E7-reactive antibodies by enzyme-linked immunosorbent assay

Sera were harvested from the mice 2 weeks after the last immunization. E6- or E7-specific antibody titers were determined by direct enzyme-linked immunosorbent assay (ELISA) as previously reported [28].

Cytotoxic T lymphocyte assays

One week after the last vaccination, the mice were sacrificed to obtain splenocytes, which were stimulated *in vitro* with 25 U recombinant interleukin-2 (Invitrogen) and 1 μ g/ml E6-MHC-I peptide and E7-MHC-I peptide for 5 days. Cytotoxic T lymphocyte (CTL) assay was performed in 96-well round-bottomed plates. Cytolysis was detected by Cyto Tox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA). In brief, CTL assay was performed with effector cells (corresponding splenocytes from various vaccinated group at 3×10^5 per well, E) and target cells (TC-1 cells, 1×10^4 per well, T) mixed at E:T = 30:1 in a final volume of 200 μ l. After a 4 h incubation at 37°C, 50 μ l of culture supernatant was collected to test the amount of lactate dehydrogenase according to manufacturer's instruction.

Tumor treatment *in vivo* with combined immuno- and chemotherapy

TC-1 cells (2×10^5 /mouse) were grafted s.c. into the left flank of C57BL/6 mice. When tumors were ~ 4 –6 mm in mean diameter, mice ($n \geq 5$) were injected with various vaccines s.c. into the right flank in a total of three times at 1 week interval (on days 0, 7, and 14). One day after the injection of vaccines, mice were injected intraperitoneally with cisplatin (5 mg/kg), boosted 1 week later, and monitored twice weekly for tumor growth. Tumor size was measured by using a caliper and recorded as mean diameter in $(a + b)/2$, where a is the longest surface length and b is the width [29]. The survival period of the tumor-bearing mice was monitored and recorded for 70 days after the first treatment.

Statistical analysis

Differences among the treatment groups were assessed by the analysis of variance and the repeated measures define factors by using SPSS software (version 11.5; SPSS Inc., Chicago, USA). $P < 0.05$ was considered statistically significant.

Results

Generation and characterization of various HPV E6 and E7 recombinant proteins

The various kinds of recombinant genes were expressed in *E. coli* host strain and the recombinant proteins, PE, E7, E7(d), E6, and E6(d), were shown schematically in Fig. 1A. The purity of these proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B) before mixing with ISA51 singularly or in combination for the formulations of monovalent and bivalent vaccines.

The de-oncogenic formats E6(d)/E7(d) eliciting similar CD8⁺ and CD4⁺ response as their wild types E6/E7 both in monovalent and bivalent vaccinations

Both the amounts of antigen-specific CD8⁺ and CD4⁺ precursor cells in the splenocytes of the vaccinated mice were determined by immunostaining followed by FACScan analysis. It was found that the number of E6- or E7-specific IFN- γ -secreting CD8⁺ cells remained unchanged [between the modified (de-oncogenic) forms and their wild types] regardless, whether the mice were immunized by the protein

vaccines in monovalent or bivalent form (Fig. 2). On the other hand, the number of E6- or E7-specific IFN- γ -secreting CD4⁺ cells from mice immunized with modified vaccines was significantly higher than that of their corresponding wild types in monovalent form ($P < 0.05$); but the enhancing effect disappeared when these two proteins, E6(d) and E7(d), were used as a bivalent vaccine (Fig. 3).

A higher rate of E7-specific seroconversion in mice vaccinated by bivalent vaccine of E6(d) plus E7(d)

As expected, E7-specific antibodies were significantly augmented when mice were vaccinated by vaccines containing E7 in monovalent and biovalent forms (Fig. 4). When the sera concentration was diluted to 1 : 500 in the assays, results showed that the levels of E7-specific antibodies in E7(d) monovalent-vaccinated mice were significantly higher than those in E7 monovalent-vaccinated mice ($P < 0.01$). The levels of E7-specific antibodies could be further augmented in the biovalent vaccine in the presence of E6-based vaccine (Fig. 4, middle panel). When further diluted sera (1 : 1000) were used, results revealed that E7-specific antibodies titer in bivalent E6(d) plus E7(d)-vaccinated mice was significantly higher than that in bivalent E6 plus E7-vaccinated

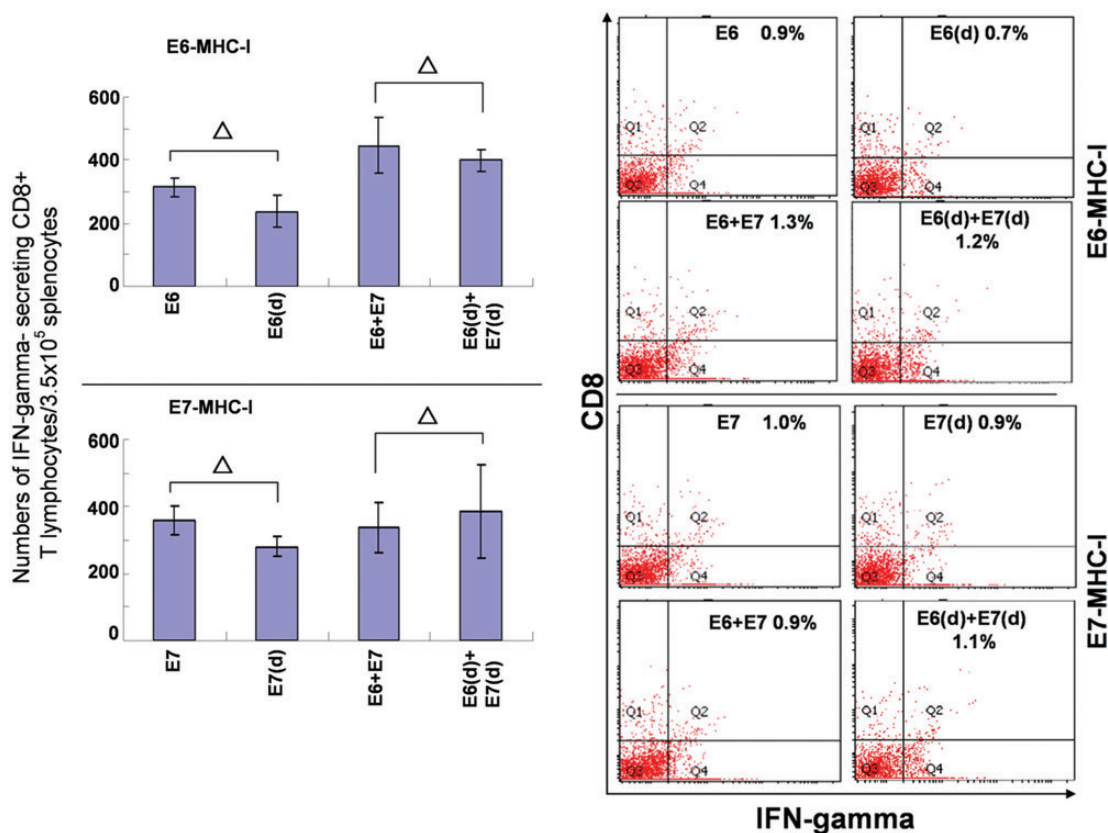


Figure 2. Number of IFN- γ -secreting CD8⁺ T cells in vaccinated mice Number of IFN- γ -secreting CD8⁺ T cells/3.5 × 10⁵ splenocytes in the presence of corresponding E6-MHC-I (AAs 49–58) or E7-MHC-I (AAs 49–57) peptide. Mice were immunized with monovalent or bivalent vaccines and the splenocytes were prepared as described in the 'Materials and Methods' Section. Data were expressed as mean \pm SD. $\Delta P > 0.05$, $n = 3$. Experiments were repeated three times.

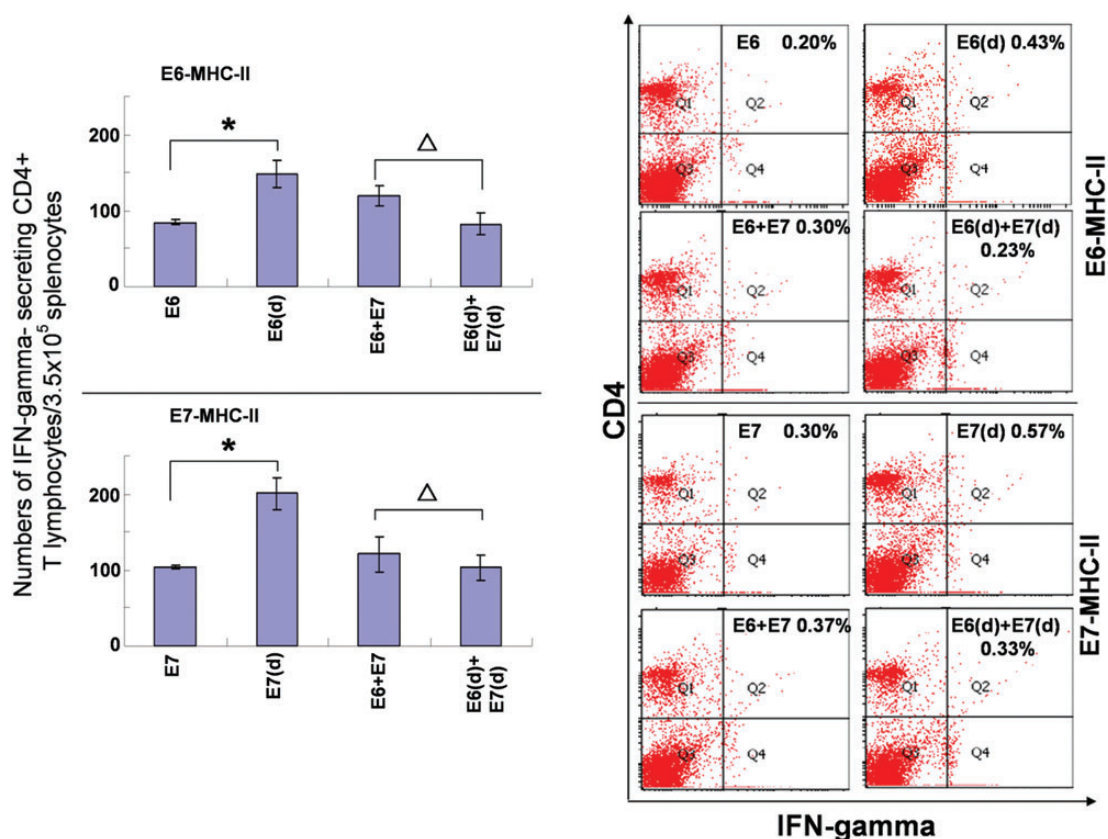


Figure 3. Number of IFN- γ -secreting CD4⁺ T cells in vaccinated mice Number of IFN- γ -secreting CD4⁺ T cells/ 3.5×10^5 splenocytes in the presence of corresponding E6-MHC-II (AAs 43–57) or E7-MHC-II (AAs 30–67) peptide. Mice were immunized with monovalent or bivalent vaccines and the splenocytes were prepared as it was described in the Materials and Methods Section. Data were expressed as mean \pm SD. $^{\Delta}P > 0.05$, $*P < 0.05$, $n = 3$. Experiments were repeated three times.

mice ($P < 0.05$) (Fig. 4, lower panel). However, E6-specific antibodies remained at a low level in all groups (data not shown).

Vaccination by the bivalent vaccines stimulates stronger CTL activities than that by monovalent vaccines

As shown in Fig. 5, at each reacting ratio of E:T, splenocytes from mice vaccinated with monovalent E7 or E7(d) showed a greater increase in CTL activities than those from mice vaccinated with monovalent E6 or E6(d) ($P < 0.05$). Furthermore, splenocytes from mice vaccinated with bivalent (E6 + E7) or E6(d) + E7(d) showed a significantly more increase in CTL activities than those from mice vaccinated with monovalent E7 or E7(d) only ($P < 0.05$). However, there was no significant difference in the CTL activity between E7 and E7(d) vaccines, or between (E6 + E7) and E6(d) + E7(d) bivalent vaccines ($P < 0.05$).

Enhanced antitumor effects of bivalent vaccine E6(d) + E7(d) together with cisplatin in tumor-bearing mice

As shown in Fig. 6A, the treatment with cisplatin alone offered moderate protection since the progression of tumor size was significantly slower in this treatment group, when

compared with that of the untreated (naïve) or vaccinated with PE. There was neither additional protection when only E6 or E6(d) monovalent vaccine was used in the vaccination regimen; however, using E7 or E7(d) monovalent vaccine in the treatment regimen would result in significant inhibitions of tumor growths (Fig. 6B). In bivalent vaccines, E6 + E7 and E6(d) + E7(d) groups, only E6(d) + E7(d) vaccination could further enhance the antitumor effects, that is, the treatment with bivalent vaccine E6(d) + E7(d) plus cisplatin intraperitoneal injection could achieve maximal antitumor synergy (Fig. 6C). It was also worthwhile to note that 5 of 10 mice in this treatment group appeared to be tumor-free after treatment for 10 days, but the recurrence was detected 38 days later.

The significant extension of survival period of the mice bearing tumors with the treatment of E6(d) + E7(d) vaccination plus cisplatin intraperitoneal injection

Corresponding to previous observations, treatment with cisplatin alone, when compared with that with PE alone or the untreated group (naïve), could extend survival period slightly (Fig. 7A), while vaccination with monovalent E6 or E6(d), together with cisplatin treatment, could not extend the

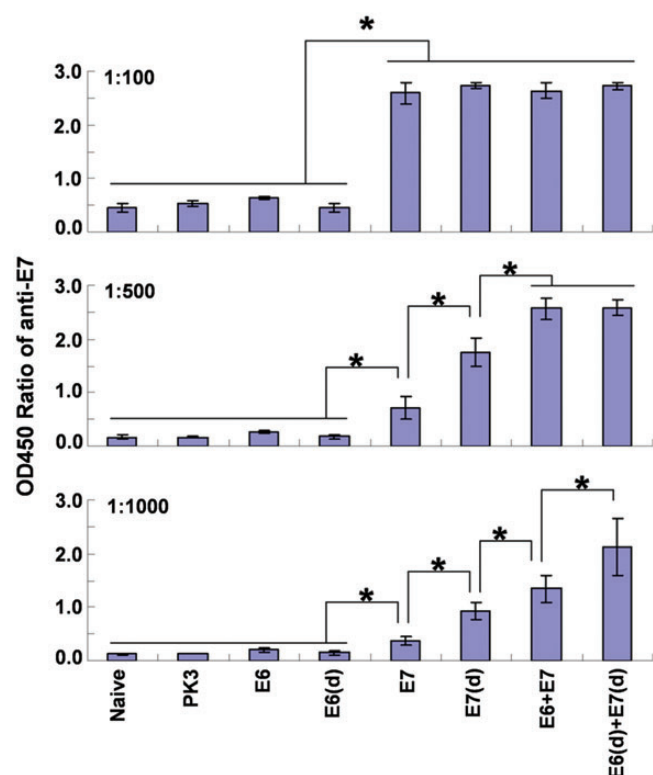


Figure 4. Productions of the anti-E7 antibodies in vaccinated mice Mice were immunized with various monovalent or bivalent vaccines and the sera were prepared as it was described in the Materials and Methods Section. ELISA results show the E7-specific antibodies in mice vaccinated with various proteins. Data were expressed as mean \pm SD. * $P < 0.05$. PK3, PE(Δ III)-KDEL3.

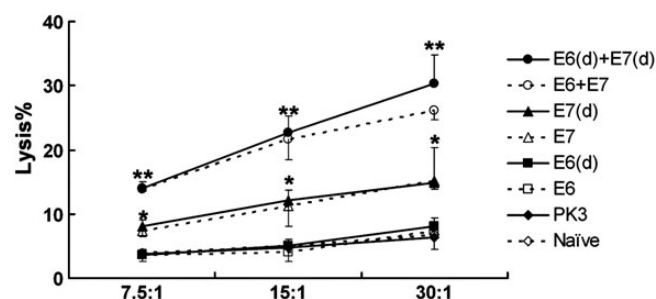


Figure 5. An analysis of CTL activities after vaccinations Animals were sacrificed at 21 days after the first immunization to evaluate *in vitro* CTL activities. Splenocytes were stimulated *in vitro* for 5 days to obtain effector cells, which were then reacted at different E:T (effector cells: target cells) ratio with TC-1 tumor cells (target cells). Data were expressed as mean \pm SD. * $P < 0.05$, E7 or E7(d) vs. E6 or E6(d); ** $P < 0.05$, E6 + E7 or E6(d) + E7(d) vs. E7 or E7(d); $n = 3$. Experiments were repeated three times.

survival period at all (Fig. 7B). However, either the monovalent E7 or the monovalent E7(d) could, by contrast, significantly extend the survival period of tumor-bearing mice (Fig. 7B). Finally, corresponding to the antitumor effect that was observed in mice treated with bivalent vaccine E6(d) + E7(d) plus cisplatin, the eight animals, although still tumor-

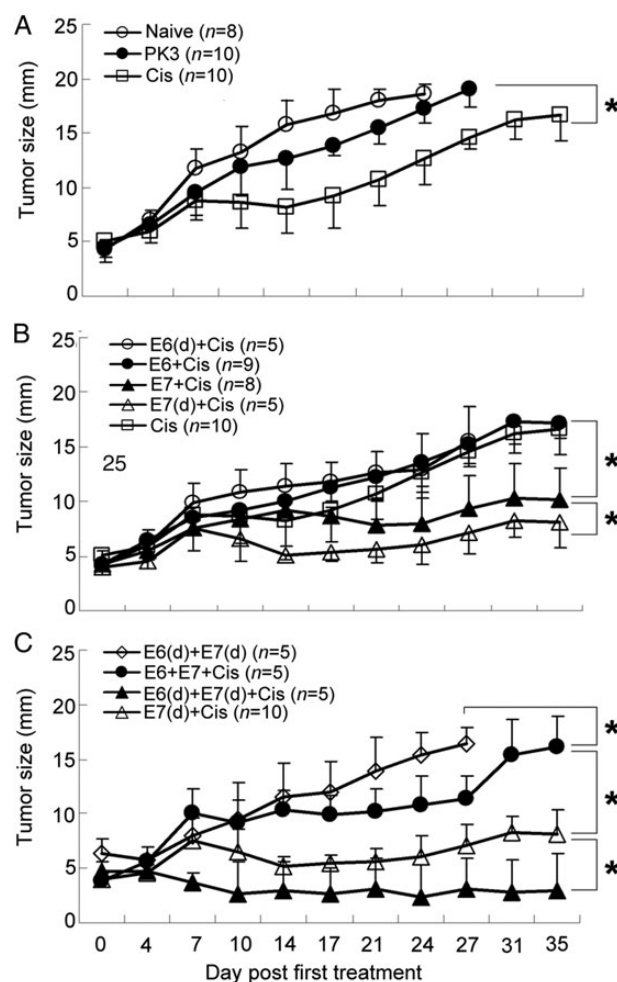


Figure 6. The suppression of tumor growth by combined treatments of vaccination and cisplatin Each group (A–C) of mice with established tumors of 4–6 mm in mean diameter was injected subcutaneously with indicated proteins thrice at 1 week intervals. One day after the first vaccination, the mice were injected intraperitoneally with cisplatin (5 mg/kg body weight) twice at 1 week intervals. At time indicated, tumor volumes in TC-1 tumor-bearing mice treated with the different treatment regimens were measured. Data were expressed as mean \pm SD. * $P < 0.05$.

bearing, were still alive 70 days after the first treatment (Fig. 7C).

Discussion

We have reported previously that the PE- and E7-based fusion protein, PE(Δ III)-E7-KDEL3, can enhance major histocompatibility complex (MHC) class I and II presentation of E7, leading to dramatic increases in the number of E7-specific CD8⁺ and CD4⁺ T-cell precursors and marked raises of the titers of E7-specific antibodies [22]. The fusion protein vaccine could also generate the antitumor effects against s.c. E7-expressing tumors. In spite of this, it is still a concern to the potential oncogenicity of wild E6 and E7 proteins [30]. It has been demonstrated that a mutation at the

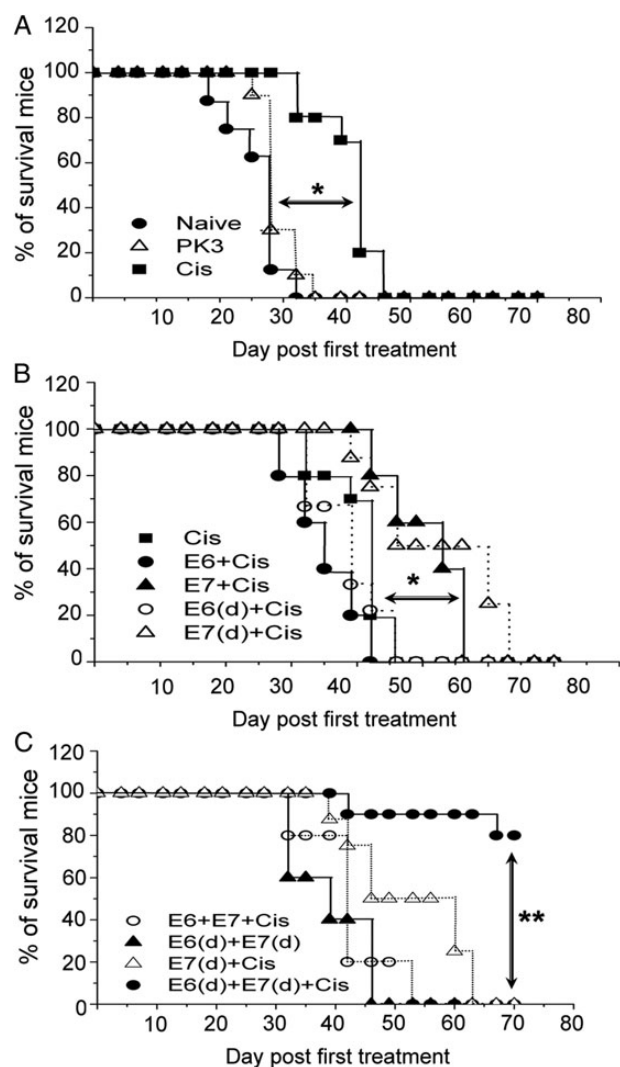


Figure 7. Kaplan–Meier survival analysis of TC-1 tumor-bearing mice treated with various vaccines (A–C) together with cisplatin. The treatment regimens were identical to those in Fig. 6. The survival period of tumor-bearing mice was significantly extended by the E6(d) + E7(d) bivalent vaccine in the presence of cisplatin. * $P < 0.05$ and ** $P < 0.01$.

24th and/or 26th AA of E7 will disrupt the Rb binding site of E7, disabling the capability of E7 to transform cells [31,32]. Furthermore, it has been shown that the mutation at the 50rd/63rd/106th AA [32,33], or the mutation of the LxxLL, and the ETQL binding pocket [34] of E6 could destroy several HPV16 E6 functions, preventing the mutated E6 protein from immortalizing human epithelial cells. In order to alleviate the concern for carcinogenicity of wild protein vaccines, we designed and generated the de-oncogenic formats E7(d)-containing mutagenesis C24G-E26G, and E6(d)-containing mutagenesis C70G-C115G.

We found that both E7(d) and E6(d) could generate similar number of CD8⁺ IFN- γ -secreting T cells (Fig. 2) and similar CTL activity (Fig. 5) and could significantly enhance CD4⁺ IFN- γ -secreting T cells (Fig. 3), when compared with corresponding wild fusion proteins. In addition, E7(d) could elicit

higher levels of anti-E7 production (Fig. 4) and promote synergistic antitumor efficacy in chemo-immunotherapy (Fig. 6B), when compared with its wild format. It was an interesting finding that the de-oncogenic E7 fusion proteins could partially augment the immune response, as the point mutations were not part of the epitopes for CD8⁺ or CD4⁺ T cells. Smahel *et al.* [35] also found the similar phenomenon and the probable mechanisms: (i) the E7 peptide consisting of AAs 21–28, which contains the two AAs substituted in E7(d), has the ability to bind H-2K^b molecules and activate CD8 + CTL response [36]. (ii) The increased antigenicity might be associated with the disability of the E7(d) protein to bind to pRb. It has been demonstrated that the wild-type E7 suppresses dendritic cells (DCs) to present antigen by the inhibition of DCs differentiation [37]. As for the modified E7(d), since its capacity of binding to pRB had been abolished and its inhibition to DCs should be free, thus it could enhance the presentative ability of DCs and augments the immune response.

Our results showed that vaccination with E7 or E7(d) alone could elicit better immune (Figs. 4 and 5) and antitumor responses (Figs. 6B and 7B) in C57BL/6 mice than that with E6 or E6(d) alone. Interestingly, similar findings have also been reported in previous studies [30], but the true causes for this phenomenon remain unclear. One possible reason for this would be that the immunogenicity of the E6 protein is lower than that of the E7 protein. In addition, some other factors unrelated to specific immune interaction between T cells and tumor cells (e.g. different cytokines generated by vaccinations of E6- and E7-fusion proteins, respectively) might also be involved in the different immune and antitumor effects of these vaccines [30]. Recently, it has been found that in the population with HPV⁺ Papanicolaou smear, the CD4⁺ T-cell responses to E6 peptides, but not to E7 peptides, and it is significantly higher in normal women than in women with cervical intraepithelial neoplasia [38], indicating that the E6-specific immune plays a pivotal role in preventing cell transformation. Therefore, it is desirable to develop more efficient vaccines targeting E6 in the future.

The present results showed that the use of bivalent vaccines, i.e. E6 + E7 or E6(d) + E7(d), could produce the synergistic effects on CTL activation and antitumor activity. When it was used as a monovalent vaccine, neither E6 nor E6(d) would offer distinct advantage in all aspects. Interestingly, these E6-based fusion proteins were found to be able to augment the immunogenic and antitumor effects on their E7 counterparts. Both of the bivalent vaccine, E6 + E7 and E6(d) + E7(d), offered higher production of anti-E7 (Fig. 4) and higher CTL activity (Fig. 5), when compared with all monovalent vaccinations. The bivalent vaccine systems also showed better antitumor effects on mono-immunotherapeutic approaches (data not shown). As mentioned previously, the E6 and E7 oncoproteins targeted different key tumor suppressors and worked in concert in the carcinogenesis of

HPV-associated cancers [1,3]. With the data we obtained currently and the increasing important roles of E6 and E7 in most HPV-associated carcinogenesis [39–41], the combination of E6 and E7 as target tumor antigens would prove to be an important therapeutic strategy for designing HPV vaccine in the future.

With the anticancer drug cisplatin being added into the treatment regimen, i.e. in a chemo-immunotherapeutic approach presented in the present study, the bivalent vaccine E6(d) + E7(d) could realize the full potential in tumor regression (Fig. 6C) and could extend the survival duration (Fig. 7C). The effects of the cisplatin-vaccine treatment on the synergistic antitumor might be the result of the increased sensitivity of the cisplatin-treated tumor cells to the CTL activities evoked by the vaccination [29,42]. The enhanced antitumor efficacy of the chemo-immunotherapy by using the bivalent de-oncogenic vaccine may benefit from two aspects. First, the mutation technique eliminated the probable inhibition of the wild E7 fusion protein to DC differentiation [35]. Secondly, E6 augmented the immune response and tumor regression of E7 as it has been shown in the present study.

On the basis of the present results, we conclude that the E7 and E6 fusion proteins act synergistically in the augmentation of immunoresponses in mice vaccinated with a bivalent vaccine regimen. More importantly, with the removal of oncogenicity of both E6 and E7, this vaccine system exhibits better tumoricidal effects on tumor-bearing mice in the presence of cisplatin. This novel therapeutic treatment regimen, upon further optimization, may shed new light on the clinical management of HPV-associated cancers.

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