Fusion Protein Vaccines Targeting Two Tumor Antigens Generate Synergistic Anti-Tumor Effects

Wen-Fang Cheng¹,²,³, Ming-Cheng Chang¹, Wei-Zen Sun⁴, Yu-Wei Jen¹, Chao-Wei Liao⁵, Yun-Yuan Chen¹, Chi-An Chen¹*

¹ Departments of Obstetrics and Gynecology, College of Medicine, National Taiwan University, Taipei, Taiwan, 2 Graduate Institute of Oncology, College of Medicine, National Taiwan University, Taipei, Taiwan, 3 Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan, 4 Department of Anesthesiology, College of Medicine, National Taiwan University, Taipei, Taiwan, 5 Animal Technology Institute Taiwan, Miaoli, Taiwan

Abstract

Introduction: Human papillomavirus (HPV) has been consistently implicated in causing several kinds of malignancies, and two HPV oncoproteins, E6 and E7, represent two potential target antigens for cancer vaccines. We developed two fusion protein vaccines, PE(ΔIII)/E6 and PE(ΔIII)/E7 by targeting these two tumor antigens to test whether a combination of two fusion proteins can generate more potent anti-tumor effects than a single fusion protein.

Materials and Methods: In vivo antitumor effects including preventive, therapeutic, and antibody depletion experiments were performed. In vitro assays including intracellular cytokine staining and ELISA for Ab responses were also performed.

Results: PE(ΔIII)/E6+PE(ΔIII)/E7 generated both stronger E6 and E7-specific immunity. Only 60% of the tumor protective effect was observed in the PE(ΔIII)/E6 group compared to 100% in the PE(ΔIII)/E7 and PE(ΔIII)/E6+PE(ΔIII)/E7 groups. Mice vaccinated with the PE(ΔIII)/E6+PE(ΔIII)/E7 fusion proteins had a smaller subcutaneous tumor size than those vaccinated with PE(ΔIII)/E6 or PE(ΔIII)/E7 fusion proteins alone.

Conclusion: Fusion protein vaccines targeting both E6 and E7 tumor antigens generated more potent immunotherapeutic effects than E6 or E7 tumor antigens alone. This novel strategy of targeting two tumor antigens together can promote the development of cancer vaccines and immunotherapy in HPV-related malignancies.

Citation: Cheng W-F, Chang M-C, Sun W-Z, Jen Y-W, Liao C-W, et al. (2013) Fusion Protein Vaccines Targeting Two Tumor Antigens Generate Synergistic Anti-Tumor Effects. PLoS ONE 8(9): e71216. doi:10.1371/journal.pone.0071216

Editor: Mauricio Martins Rodrigues, Federal University of São Paulo, Brazil

Received April 6, 2013; Accepted June 27, 2013; Published September 13, 2013

Copyright: © 2013 Cheng et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This was a cooperative study sponsored by HealthBanks Biotech Company (NTUH97A126). This study received funding from the HealthBanks Biotech Company. The funder had no role in the study design, collection, analyses and interpretation of data, writing of the report, and the decision to submit the paper for publication.

Competing interests: This was a cooperative study sponsored by HealthBanks Biotech Company (NTUH97A126), and this study received funding from HealthBanks Biotech Company. This does not alter the authors’ adherence to all of the PLOS ONE policies on sharing data and materials.

* E-mail: chianchen@ntu.edu.tw

Introduction

Cervical cancer is the second leading cause of cancer death in women, with approximately 500,000 cases worldwide each year, of which about one-third are fatal [1]. Human papillomavirus (HPV) is recognized as the primary cause of cervical cancer as HPV DNA can be detected in about 99% of all cervical cancers [2]. HPV 16, in particular, is the most prevalent type and is detected in over 50% of patients [3]. The HPV types found in cancer cells have been shown to have the ability to transform in in vitro studies [4], and the viral transforming proteins, E6 and E7, have consistently been shown to be expressed in cervical cancer cell lines [5] and in HPV-associated cancers [6]. In HPV-associated malignant transformation, viral DNA may be integrated into the cellular DNA, often resulting in the deletion of large sectors of the viral genome. Expressions of E6 and E7 are likely to overcome the regulation of cell proliferation normally mediated by proteins such as p53 and Rb, allowing for uncontrolled growth and providing the potential for malignant transformation [7]. Thus, E6 and E7 are two oncogenic proteins that represent ideal target antigens for developing vaccines and immunotherapeutic strategies against HPV-associated neoplasia.

Pre-clinical and clinical studies have targeted E6 or E7 for the development of vaccines to control HPV-associated lesions [8]. Most HPV researchers have focused on E7 such that E7 as the immuno-dominant epitope and the associated immune responses have been well characterized [9,10]. Since E6
represents another important target for vaccines to control HPV-associated lesions, it is crucial to develop vaccines targeting E6.

Ideal cancer treatment should be able to eradicate systemic tumors at multiple sites in the body while having the specificity to discriminate between neoplastic and non-neoplastic cells. As such, the activation of antigen-specific T cell-mediated immune responses allows for the killing of tumors associated with a specific antigen [10,11]. Recently, many strategies such as peptide-based vaccine [12,13], protein-based vaccine [14,15], DNA-based vaccine [16,17], naked RNA vaccine [18,19], and recombinant viruses [20,21] have emerged. Protein-based vaccines are reportedly capable of generating CD8+ T cell responses in vaccinated humans [22,23]. However, one limitation of peptide and protein-based vaccines is their poor immunogenicity, especially with some tumor antigens such as E6 and E7. Novel strategies that enhance protein vaccine potency need to be applied in the development of more effective cancer vaccines and immunotherapy.

A previous study revealed that a fusion protein vaccine with the translocation features of exotoxin A of Pseudomonas aeruginosa (PE(ΔIII)-KDEL3) linked with the model antigen-human papillomavirus (HPV) E6 and E7 enhanced the MHC class I presentation of antigens to CD8+ T cells and thereby enhanced vaccine potency [15]. Another previous study revealed that a DNA vaccine encoding HSP60 linked to E6 and/or E7 generated significantly enhanced E6 or E7-specific CD8+ T cell responses in vaccinated mice, and prevented and controlled lethal E6 and E7-expressing tumors [24]. The chimeric HSP60/E6/E7 DNA vaccine also generated more potent immunotherapeutic effects than the chimeric HSP60/E6 or HSP60/E7 DNA vaccines. However, few studies have focused on E6 as the target antigen [25]. The present study aimed to determine whether retrograde-delivery domains, when linked to the E6 antigen in the fusion protein format, could also enhance E6-specific immunologic responses and anti-tumor effects, and whether the combination of E6 and E7 fusion protein vaccines could generate a more potent antigen-specific immunotherapy than E6 or E7 fusion protein vaccines alone.

Materials and Methods

Preparation of various DNA constructs

The E6, E7, PE(ΔIII)/E6, and PE(ΔIII)/E7 preparations were done as described previously with some modifications [15,24]. Briefly, the wild-type E7 construct was removed and then the wild-type E6 construct was inserted.

Generation and Preparation of Various Protein Vaccines

The induction of expression, production, and purification of various recombinant proteins were also done as described previously [15]. After purification, protein elution fractions were analyzed for purity and quantified by SDS-PAGE analysis.

Preparation and vaccination of protein vaccines

Stock protein vaccines such as E6, E7, PE(ΔIII)/E6, and PE(ΔIII)/E7 were diluted with PBS (1:10) 10 times and incubated for 2 hours at 37°C. The activated proteins were further mixed with 10% ISA206 (SEPPIC Inc., Paris, France) for the in vitro and in vivo experiments. For the in vivo experiments, various ISA206 mixed protein vaccines were subcutaneously injected into the back of the study mice.

Cell lines

The production and maintenance of TC-1 tumor cells, E6- and E7-specific CD8+ T cell lines, were done as previously described [26,27]. On the day of tumor challenge, tumor cells were harvested by trypsinization, washed twice with 1X Hanks buffered salt solution (HBSS) and re-suspended in 1X HBSS to the designated concentration for injection.

Mice

Six- to eight-week-old female C57BL/6J mice were purchased from the National Taiwan University (Taipei, Taiwan) and bred in the animal facility of the National Taiwan University. The Institutional Animal Care and Use Committee of National Taiwan University approved this study. All animal procedures were performed according to approved protocols and strictly adhered to the guidelines set by the committee for the proper use and care of laboratory animals.

Intracellular cytokine staining and flow cytometry analysis

For the first experiment, mice (5 per group) were immunized with a total of 0.1 mg of various protein vaccines mixed with 10% ISA206 adjuvant. The mice were then boosted subcutaneously one and two weeks later with the same regimen. One week after the last immunization, the mice were sacrificed and splenocytes were prepared as described previously [28]. To detect E6-specific immunologic responses, E6-specific peptide (aa 50-57) containing an MHC class I epitope [25] to detect E6-specific CD8+ T cell precursors or 50 µg/ml E6 recombinant protein [15] to detect E6-specific CD4+ T cell precursors were used. To detect E7-specific immunological responses, E7-specific peptide (aa 49-57) containing an MHC class I epitope [29] to detect E7-specific CD8+ T cell precursors or 10 µg/ml E7 peptide (aa 30-67) containing an MHC class II epitope [17] to detect E7-specific CD4+ T cell precursors were used.

Before intracellular cytokine staining, 3.5x10⁵ pooled splenocytes from each vaccinated group were incubated for 16 hours with either peptide (or recombinant protein) to detect E6- or E7-specific CD4+ or CD8+ T cell precursors. Cell surface marker staining for CD8 or CD4, and intracellular cytokine staining for IFN-γ, as well as FACScan analysis were performed [16].

In the second experiment, the mice were immunized at various times with PE(ΔIII)/E6, PE(ΔIII)/E7, or PE(ΔIII)/E6+PE(ΔIII)/E7 fusion proteins as described earlier. The mice were sacrificed 7 days after the last vaccination. Splenocytes were harvested, prepared, stained, and analyzed.
Enzyme-linked immuno-absorbent assay (ELISA) for anti-E6 or E7 antibodies

Mice (5 per group) were vaccinated with various protein vaccines three times as described earlier. Sera were prepared from the mice 14 days after the last immunization. E6- or E7-specific antibody titers were detected by direct ELISA as previously described [17,30].

In vivo tumor protection experiments

For the first experiment, mice (5 per group) were subcutaneously vaccinated with a total of 0.1 mg of various protein vaccines and boosted one or two weeks later with the same regimen. One week after the last vaccination, the mice were challenged with 5x10⁴ TC-1 tumor cells by subcutaneous injection in the right leg. Naïve mice received the same amount of TC-1 cells to assess natural tumor growth and served as the controls. Tumor growth was monitored by visual inspection and palpation twice weekly until 60 days after tumor challenge. Tumor-free mice were defined as those with no grossly visible or palpable tumor nodules.

For the second experiment, mice (5 per group) were vaccinated with 0.1 mg PE(ΔIII)/E6, PE(ΔIII)/E7, or PE(ΔIII)/E6+PE(ΔIII)/E7 fusion proteins once or twice. One week after the last vaccination, the mice were challenged with 5x10⁴ TC-1 tumor cells subcutaneously and tumor growth was monitored.

In vivo Ab depletion experiments

In vivo Ab depletion experiments were performed as described previously with some modifications [16,17]. Mice (5 per group) were first injected intraperitoneally with the respective Abs on day 0, challenged with 5x10⁴ cells/mouse TC-1 tumor cells via intravenous tail vein injection on day 3, and immunized subcutaneously with 0.1 mg PE(ΔIII)/E6, 0.1 mg PE(ΔIII)/E7, or 0.05 mg PE(ΔIII)/E6 + 0.05 mg PE(ΔIII)/E7 protein vaccines boosted once and two weeks after day 7 to determine the effects of lymphocyte subsets on the potency of the PE(ΔIII)/E6 and/or PE(ΔIII)/E7 protein vaccines. The mAb GK1.5 was used for CD4 depletion, mAb 2.43 for CD8 depletion, and mAb PK136 for NK1.1 depletion. Depletion was terminated on the day of sacrifice 28 days after TC-1 tumor challenge.

In vivo tumor treatment experiments

In vivo tumor treatment experiments were performed using a previously described lung hematogenous spread model [16,18]. In the first experiments, mice (5 per group) were challenged with 5x10⁴ TC-1 tumor cells/mouse via tail vein injection. Three or seven days after tumor challenge, the mice received a total of 0.1 mg/mouse of various protein vaccines subcutaneously every 7 days for 2 weeks (total of 0.3 mg protein). The mice that were not vaccinated were used as negative controls. The mice were sacrificed and their lungs were explanted on day 30. Pulmonary tumor nodules in each mouse were evaluated and counted by investigators blinded to the sample identity.

The second experiments were the subcutaneous therapeutic experiments. C57BL/6 mice (5 per group) were challenged with 5x10⁴ TC-1 tumor cells/mouse subcutaneously on the right leg to generate subcutaneous tumor nodules in the morning of day 0. In the morning of day 3 after tumor challenge, the mice received various protein vaccines and every week thereafter. Perpendicular tumor diameters were measured using Vernier scale calipers, while tumor volume was calculated using the formula \([x^2y/2]\) for an ellipsoid. The mice were monitored twice a week and sacrificed if the tumor volume became > 1,200 mm³.

Statistical analysis

All data were expressed as mean±SEM. Experimental groups were compared using analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) software (SPSS 9.0, SPSS Inc., Chicago, IL). Statistical significance was set at \(p<0.05\).

Results

Generation and characterization of the E6 and PE(ΔIII)/E6 protein vaccines

The generation and characterization of the PE(ΔIII)/E7 protein were done as described in a previous study [15]. A schematic diagram showing the domains of full-length PE and the construct of E6, and chimeric PE(ΔIII)/E6 is shown in Figure 1A, and the SDS-PAGE findings of PE(ΔIII), E6, and PE(ΔIII)/E6 are shown in Figure 1B.
Combination of PE(ΔIII)/E6 and PE(ΔIII)/E7 fusion proteins generated potent E6- and E7-specific immune responses in vivo

The numbers of E6-specific, IFN-γ-secreting CD4+ T cells in the PE(ΔIII)/E6 (219.5±18.9) and PE(ΔIII)/E6+PE(ΔIII)/E7 (260.0±22.4) groups were significantly higher than those in the E6 (12.0±2.8), E6+E7 (14.5±2.1), and PE(ΔIII)/E7 (38.5±4.9) groups (p<0.01, one-way ANOVA) (Figure 2A). Similarly, the numbers of E7-specific, IFN-γ-secreting CD4+ T cells in the PE(ΔIII)/E7 (258.0±18.2) and PE(ΔIII)/E6+PE(ΔIII)/E7 (239.5±17.5) groups were significantly higher than those in the E6 (15.0±2.8), E6+E7 (18.5±2.1), and PE(ΔIII)/E7 (77.5±7.0) groups (p<0.01, one-way ANOVA) (Figure 2B).

The PE(ΔIII)/E6 (247.5±18.2) and PE(ΔIII)/E6+PE(ΔIII)/E7 (392.5±19.6) groups generated higher numbers of E6-specific, IFN-γ-secreting CD8+ T precursors than the E7 (14.5±2.1), E6+E7 (18.0±2.8), and PE(ΔIII)/E7 (33.5±2.8) groups (p<0.01, one-way ANOVA) (Figure 2C). The PE(ΔIII)/E7 (588.5±18.6) and PE(ΔIII)/E6+PE(ΔIII)/E7 (778.0±18.9) groups also generated higher numbers of E7-specific, IFN-γ-secreting CD8+ T precursors than the E7 (12.5±2.1), E6+E7 (16.5±2.8), and PE(ΔIII)/E6 (89.0±7.7) groups (p<0.01, one-way ANOVA) (Figure 2D).

The titers of E6-specific Abs were higher in the PE(ΔIII)/E6 (3.0±0.180 1: 100 dilution) and PE(ΔIII)/E6+PE(ΔIII)/E7 (3.6±0.195 1: 100 dilution) groups than those in the other groups (Figure 2E) (p<0.01, one-way ANOVA). The PE(ΔIII)/E7 (3.6±0.215 1: 100 dilution) and PE(ΔIII)/E6+PE(ΔIII)/E7 (4.0±0.215 1: 100 dilution) groups also generated significantly higher titers of E7-specific Abs than the other groups (Figure 2F) (p<0.01, one-way ANOVA).

Thus, the PE(ΔIII)/E6 and PE(ΔIII)/E7 proteins generated E6- and E7-specific potent immune responses, respectively. Moreover, PE(ΔIII)/E6+PE(ΔIII)/E7 generated both E6- and E7-specific immune responses.

Vaccination with PE(ΔIII)/E6 and/or PE(ΔIII)/E7 fusion proteins generated potent tumor protection in mice challenged with E6- and E7-expressing tumor cells

In the in vivo tumor protection experiments, 100% of the mice that received the PE(ΔIII)/E6 fusion protein vaccine remained tumor-free for 60 days after TC-1 challenge (Figure 3A). In contrast, all of the unvaccinated mice and mice receiving E6, PE(ΔIII), PE(ΔIII)+E6 developed tumors within 20 days after tumor challenge. Furthermore, 100% of the mice vaccinated with PE(ΔIII)/E7 or PE(ΔIII)/E6+PE(ΔIII)/E7 fusion protein vaccines were also tumor-free 60 days after TC-1 tumor challenge (Figure 3B). These results indicated that PE(ΔIII)/E6, PE(ΔIII)/E7, or a combination of PE(ΔIII)/E6 and PE(ΔIII)/E7 fusion proteins generated the same potent tumor protective effects.

CD4 T cells, CD8 T cells, and NK cells were essential for the anti-tumor effect generated by PE(ΔIII)/E6 and/or PE(ΔIII)/E7 fusion protein vaccines

In the in vivo antibody depletion experiments, all naive mice and all PE(ΔIII)/E6 protein-vaccinated mice depleted of CD8+ T cells grew tumors within 14 days after tumor challenge (Figure 3C). In contrast, 80% of the CD4+ T cell-depleted mice and 40% of the NK1.1 cell-depleted mice grew tumors within 30 days after tumor challenge. In the PE(ΔIII)/E7 protein-vaccinated mice, 100%, 60%, and 40% of the mice depleted of CD8+ T cells, CD4+ T cells, and NK1.1 cells, respectively, grew tumors within 30 days after tumor challenge (Figure 3D), whereas in the mice vaccinated with PE(ΔIII)/E6+PE(ΔIII)/E7 protein vaccines, tumor growth occurred in 100%, 60%, and
60%, respectively (Figure 3E). Taken together, CD4 T cells, CD8 T cells, and NK cells were essential for the anti-tumor immunity generated by the E6 and E7 fusion protein vaccines.

Treatment with E6 and/or E7 fusion proteins led to a significant reduction in pulmonary tumor nodules

The representative figures of pulmonary tumor nodules in various protein vaccinated groups are shown in Figure 4A. The mean numbers of nodules in the mice treated with PE(ΔIII)/E6 (30.6±4.1), PE(ΔIII)/E7 (1.2±0.6), and PE(ΔIII)/E6+PE(ΔIII)/E7 (0.8±0.4) fusion proteins were significantly lower than those in the mice treated with E6, E7, and E6+E7 proteins, when starting treatment 3 days after TC-1 tumor challenge (p<0.001, one-way ANOVA) (Figure 4B). There was no significant difference in the number of pulmonary tumor nodules between the mice treated with PE(ΔIII)/E7 and those treated with PE(ΔIII)/E6+PE(ΔIII)/E7 (p>0.05, one-way ANOVA). However, when starting treatment 7 days after TC-1 tumor challenge, the PE(ΔIII)/E6+PE(ΔIII)/E7 group (10.8±2.5) had the lowest number of pulmonary tumor nodules compared to the PE(ΔIII)/E6 (60.4±10.6) and PE(ΔIII)/E7 (20.8±3.6) groups (p<0.01, one-way ANOVA). (C) The mean pulmonary tumor nodules of the mice treated with various protein vaccines 7 days after TC-1 tumor injection. Note: The mean number of nodules in the mice treated with PE(ΔIII)/E6 (30.6±4.1), PE(ΔIII)/E7 (1.2±0.6), and PE(ΔIII)/E6+PE(ΔIII)/E7 (0.8±0.4) fusion proteins were significantly lower than in the other groups of mice (p<0.001, one-way ANOVA).

Protein Vaccines Generate Strong Anti-Tumor Effect

The mice vaccinated with PE(ΔIII)/E6+PE(ΔIII)/E7 protein vaccines had higher numbers of E6-specific CD8+ T precursors than those vaccinated with PE(ΔIII)/E6 protein vaccine (Figure 5B). Moreover, the mice vaccinated more times with PE(ΔIII)/E6+PE(ΔIII)/E7 protein vaccines generated higher numbers of E6- and E7-specific CD8+ T precursors (Figure 5A and 5B).

For the preventive anti-tumor experiments, all of the naïve mice and mice vaccinated once with PE(ΔIII)/E6 and/or PE(ΔIII)/E7 protein vaccines had tumorigenesis within 14 days after TC-1 tumor challenge (Figure 5C), whereas 20%, 60%, and 100% of the mice vaccinated twice with PE(ΔIII)/E6, PE(ΔIII)/E7, and PE(ΔIII)/E6+PE(ΔIII)/E7 protein vaccines, respectively, were tumor-free 60 days after TC-1 tumor challenge (Figure 5D).

Comparing the vaccine potency in the mice given three vaccinations of each respective protein vaccine through the therapeutic subcutaneous tumor injection model, the mice vaccinated with PE(ΔIII)/E6, PE(ΔIII)/E7, and PE(ΔIII)/E6+PE(ΔIII)/E7 fusion protein vaccines had small tumor volumes than those vaccinated with E6+E7 (p<0.001, one-way ANOVA) (Figure 5E).
Protein vaccines generate strong anti-tumor effect

Discussion

Protein vaccines represent another successful example of using the translocation domains of a bacterial toxin for the development of cancer vaccines and immunotherapy. The translocation domains of a bacterial toxin linked to the tumor antigen E6 in a protein format generated potent E6-specific immunity and E6-specific anti-tumor effects in this study. A previous study demonstrated that the translocation domain of *Pseudomonas aeruginosa* exotoxin A with HPV type 16 E7 tumor antigen, regardless of in a DNA or protein vaccine, enhanced vaccine potency. This suggests that the bacterial domain may serve as a useful tool for introducing exogenous protein into the cytosol. Fominaya et al. demonstrated that the truncated forms of the chimeric protein of a bacterial toxin linked with an antigen did not facilitate efficient protein or DNA transfer when lacking KDEL, the signaling transducer of the translocation domain [31]. However, domain II of the exotoxin without KDEL, when linked to the tumor antigen, generated potent immunity in the DNA vaccine.

The same strategy to enhance antigen-specific immunity can be applied to different tumor antigens. The PE(ΔIII)-E6-KDEL and PE(ΔIII)-E7-KDEL fusion protein vaccines generated potent E6- and E7-specific immunity in the present study. The number of E6-specific, IFN-γ-secreting CD4+ T cells in the PE(ΔIII)/E6 (219.5±18.9) group was significantly higher than that of the E6 (12.0±2.8) group (Figure 2A). The number of E6-specific, IFN-γ-secreting CD8+ T cells in the PE(ΔIII)/E6 group (258.0±18.2) was also significantly higher than that of the E6 (15.0±2.8) group (Figure 2B). In addition, there were more E7-specific, IFN-γ-secreting CD4+ T cells (247.5±18.2) (Figure 2C) and CD8+ T cells (588.5±16.8) (Figure 2D) in the PE(ΔIII)/E7 group than in the E7 group (14.5±2.1 for CD4+ T cells; 12.5±2.1 for CD8+ T cells). Moreover, E6- and E7-specific Abs titers were higher in the PE(ΔIII)/E6 (Figure 2E) and PE(ΔIII)/E7 (Figure 2F) groups than those in the E6 and E7 groups, respectively. Further studies investigating whether translocation domains of a bacterial toxin can be linked to other tumor antigens against other types of cancer are warranted.

A combination of fusion protein vaccines with PE(ΔIII)/E6 and PE(ΔIII)/E7 generated more potent immunotherapeutic effects than PE(ΔIII)/E6 or PE(ΔIII)/E7 fusion proteins alone. Walter et al. reported that the cancer vaccine MA901, consisting of multiple tumor-associated peptides combined with cyclophosphamide, generated immune responses to multiple tumor-associated peptides and provided longer overall survival [32]. A previous study also demonstrated that heat shock protein 60 co-linked to HPV16 E6 and E7 tumor antigens generated more potent immunotherapeutic effects than E6 or E7 tumor antigens alone in the DNA format [24]. The present study again demonstrated that PE(ΔIII)/E6 combined with PE(ΔIII)/E7 fusion protein vaccines generated more potent immune responses (Figure 2) and anti-tumor effects (Figures 4 and 5) than the respective fusion protein vaccines alone. This suggests that HPV E6 and E7 can be utilized together as target antigens in cancer vaccines and immunotherapy.

The mechanisms of the anti-tumor effects using the translocation domains of a bacterial toxin are via similar immuno-effector cells regardless of the targeted antigen. The bacterial toxin (PE(ΔIII)) strategy has been previously tested in a mammalian expressing DNA vector (pcDNA3) [33], and the CD8+ cytotoxic T lymphocytes were found to be the only essential immuno-effector cells for the anti-tumor mechanisms of the PE(ΔIII)/E7 naked DNA vaccine. However, the immuno-effector cells in the anti-tumor mechanism of the PE(ΔIII)/E7 fusion protein vaccine include CD4+ helper T lymphocytes,
Protein vaccines have some advantages over naked DNA vaccines. First, protein vaccines can induce various kinds of antigen-specific effector T cells such as CD4+ helper and CD8+ cytotoxic lymphocytes, as shown in this study. However, naked DNA vaccines only induce CD8+ cytotoxic lymphocytes [17,35]. Protein vaccines are also more convenient to prepare using the present techniques compared to naked DNA vaccines. Furthermore, naked DNA vaccines carry the risk of DNA integration into the host genome, although it is estimated that the frequency of such integration is low [36]. Nevertheless, such concerns of DNA integration are avoided with protein vaccines. The two PE(ΔIII)/E6 and PE(ΔIII)/E7 fusion protein vaccines can be used in future human clinical trials for the prevention of HPV infection and as therapy for HPV-related cancers.

In summary, targeting two tumor antigens, HPV E6 and E7, together enhanced the potency of protein fusion vaccines. The mechanisms of the fusion protein vaccines were found to be through effector cells including antigen-specific CD4+ and CD8+ T cells, and NK cells. Because most cervical cancers express HPV E6 and E7, the proposed vaccine has the potential for use in the prevention and treatment of HPV-associated tumors. The strategy of using the translocation properties of bacterial toxins and two target antigens may hold promise in the future development of vaccines for the control of cancers and infectious diseases.

Author Contributions
Conceived and designed the experiments: WFC CAC. Performed the experiments: WFC MCC YWJ. Analyzed the data: WFC WZS CAC YYC. Contributed reagents/materials/analysis tools: CWL. Wrote the manuscript: WFC CAC.

References
1. International Agency for Research on Cancer (1995) Monograph on cancer Human papillomavirus. Geneva, Switzerland: World Health Organization.
2. zur Hausen H (1991) Human papillomaviruses in the pathogenesis of anogenital cancer. Virology 184: 9-13. doi: 10.1016/0042-6822(91)90816-T. PubMed: 1851807.
3. Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W et al. (2007) Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. Obstet Gynecol 79: 328-337. PubMed: 1310805.
4. Yasumoto S, Burkhardt AL, Doniger J, DiPaolo JA (1986) Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J Virol 57: 572-577. PubMed: 3003388.
5. Nasserri M, Gage JR, Lorincz A, Wettstein FO (1991) Human papillomavirus type 16 immortalized cervical keratinocytes contain transcripts encoding E6, E7, and E2 initiated at the P97 promoter and express high levels of E7. Virology 184: 131-140. doi: 10.1016/0042-6822(91)90829-Z. PubMed: 1651587.
6. Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B et al. (1985) Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature 314: 111-114. doi: 10.1038/314111a0. PubMed: 2983228.
7. Howley PM (1991) Role of the human papillomaviruses in human cancer. Cancer Res 51: 5019-5022. PubMed: 1653110.
8. Cheng WF, Lee CN, Chang MC, SuYN, Chen CA et al. (2005) Antigen-Specific CD8+ T Lymphocytes Generated from a DNA Vaccine Control Tumors Through the Fas-FasL Pathway. Mol Ther 12: 960-968. doi: 10.1038/ymntm.2005.04.020. PubMed: 15979942.
9. King CA, Spellerberg MB, Zhu D, Rice J, Sahota SS et al. (1998) DNA vaccines with single-chain Fv fused to fragment C of tetanus toxoid induce protective immunity against lymphoma and myeloma. Nat Med 4: 1281-1286. doi: 10.1038/3266. PubMed: 9809552.
10. Cheng WF, Lee CN, SuYN, Chang MC, Hsiao WC et al. (2005) Induction of human papillomavirus type 16-specific immunologic responses in a normal and an human papillomavirus-infected populations. Immunolgy 115: 136-149. doi: 10.1111/j.1365-2966.2005.01216.x. PubMed: 15819706.
11. Chen CH, Wu TC (1998) Experimental vaccine strategies for cancer immunotherapy. J Biomed Sci 5: 231-252. doi: 10.1007/BF02255885. PubMed: 9891216.
12. Brossart P, Heinrich KS, Stuhler G, Behnke L, Reichardt VL et al. (1999) Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. Blood 93: 4309-4317. PubMed: 10361129.
13. Vonderheide RH, Hahn WC, Schultz JL, Nadler LM (1999) The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunity 10: 673-679. doi: 10.1016/S1074-7613(00)80686-7. PubMed: 10403642.
14. Marchand M, Punt CJ, Aaralnd S, Escudier B, Kruit VH et al. (2005) Immunisation of metastatic cancer patients with MAGE-3 protein combined with adjuvant SBAS-2: a clinical report. Eur J Cancer 39: 70-77. doi: 10.1016/S0959-8049(02)00479-3. PubMed: 12504861.
15. Liao CW, Chen CA, Lee CN, SuYN, Chang MC et al. (2005) Fusion protein vaccine by domains of bacterial exotoxin linked with a tumor antigen generates potent immunologic responses and antitumor effects. Cancer Res 65: 9089-9098. doi: 10.1158/0008-5472.CAN-05-0958. PubMed: 16204084.
16. Hsieh CY, Chen CA, Huang CY, Chang MC, Lee CN et al. (2007) IL-6-encoding tumor antigen generates potent cancer immunotherapy.
through antigen processing and anti-apoptotic pathways. Mol Ther 15: 1789-1797. doi:10.1038/gt.2006.80. PubMed: 17630258.

17. Cheng WF, Chang MC, Sun WZ, Lee CN, Lin HW et al. (2008) Connective tissue growth factor linked to the E7 tumor antigen generates potent antitumor immune responses mediated by an antia apoptotic mechanism. Gene Ther 15: 1007-1016. doi:10.1038/gt. 2008.25. PubMed: 18356819.

18. Cheng WF, Hung CF, Lee CN, Su YN, Chang MC et al. (2004) Naked RNA vaccine controls tumors with down-regulated MHC class I expression through NK cells and perforin-dependent pathways. Eur J Immunol 34: 1892-1900. doi:10.1002/eji.200424877. PubMed: 15214037.

19. Cheng WF, Hung CF, Hsu KF, Chai CY, He L et al. (2001) Enhancement of sindbis virus self-replicating RNA vaccine potency by targeting antigen to endosomal/lysosomal compartments. Hum Gene Ther 12: 235-252. doi:10.1089/10430340150218387. PubMed: 11177561.

20. Cheng WF, Hung CF, Hsu KF, Chai CY, He L et al. (2002) Cancer immunotherapy using Sindbis virus replicon particles encoding a VP22-antigen fusion. Hum Gene Ther 13: 553-568. doi: 10.1089/10430340252809847. PubMed: 11874633.

21. Marshall JL, Hoyer RJ, Toomey MA, Faraguna K et al. (2000) Phase I study in advanced cancer patients of a diversified prime-and-boost vaccination protocol using recombinant vaccinia virus and recombinant nonreplicating avipox virus to elicit anti-carcinoembryonic antigen immune responses. J Clin Oncol 18: 3964-3973. PubMed: 11099326.

22. Vantomme V, Dantinne C, Amrani N, Permanne P, Gheysen D et al. (2004) Immunologic analysis of a phase I/II study of vaccination with MAGE-3 protein combined with the AS02B adjuvant in patients with MAGE-3-positive tumors. J Immunother 27: 124-135. doi: 10.1097/00002371-200403000-00006. PubMed: 14770084.

23. Zhang Y, Chaux P, Stroobant V, Eggemont AM, Corthals J et al. (2003) A MAGE-3 peptide presented by HLA-DR1 to CD4+ T cells that were isolated from a melanoma patient vaccinated with a MAGE-3 vaccine supports antitumor immunity. J Immunol 171: 219-225. PubMed: 12917001.

24. Huang CY, Chen CA, Liy CA, Chang MC, Su YN et al. (2007) DNA vaccine encoding heat shock protein 60 co-linked to HPV16 E6 and E7 tumor antigens generates more potent immunotherapeutic effects than respective E6 or E7 tumor antigens. Gynecol Oncol 107: 404-412. doi: 10.1016/j.ygyno.2007.06.031. PubMed: 17905417.

25. Peng S, Ji H, Trimble C, He L, Tsai YC et al. (2004) Development of a DNA vaccine targeting human papillomavirus type 16 oncoprotein E6. J Virol 78: 8468-8476. doi:10.1128/JVI.78.16.8468-8476.2004. PubMed: 15280455.

26. Lin KY, Guarnieri FG, Staveley-O’Carroll KF, Levitsky HI, August JT et al. (1996) Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 56: 21-26. PubMed: 848765.

27. Chen CA, Chang MC, Sun WZ, Chen YL, Chiang YC et al. (2009) Noncarrier naked antigen-specific DNA vaccine generates potent antigen-specific immunologic responses and antitumor effects. Gene Ther 16: 776-787. doi:10.1038/gt.2009.31. PubMed: 19357714.

28. Cheng WF, Hung CF, Chai CY, Hsu KF, He L et al. (2001) Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. J Clin Invest 108: 669-678. doi:10.1172/JCI12346. PubMed: 11544272.

29. Feltkamp MC, Smits HL, Vierboom MP, Minnaar RP, de Jongh BM et al. (1993) Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. Eur J Immunol 23: 2242-2249. doi:10.1002/eji.1830230929. PubMed: 7690326.

30. Cheng WF, Chen LK, Chen CA, Chang MC, Hsiao PN et al. (2006) Chimeric DNA vaccine reverses morphine-induced immunosuppression and tumorigenesis. Mol Ther 13: 203-210. doi:10.1016/j.mther. 2006.08.601. PubMed: 16140583.

31. Fominaya J, Uherek C, Wels W (1998) A chimeric fusion protein containing transforming growth factor-alpha mediates gene transfer via binding to the EGF receptor. Gene Ther 5: 521-530. doi:10.1038/sj.gt.33006514. PubMed: 9614577.

32. Walter S, Weinschenk T, Stenzl A, Zdrojowy R et al. (2002) Multiplepeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med, 18: ([MedlinePgn:]). PubMed: 22842478.

33. Hung CF, Hsu KF, Cheng WF, Chai CY, He L et al. (2001) Enhancement of DNA vaccine potency by linkage of antigen gene to a gene encoding the extracellular domain of Fms-like tyrosine kinase 3-ligand. Cancer Res 61: 1080-1088. PubMed: 11221836.

34. Feltkamp MC, Vierboom MP, Toes RE, Ossendorp F, ter Schegget J et al. (1995) Competition inhibition of cytotoxic T-lymphocyte (CTL) lysis, a more sensitive method to identify candidate CTL epitopes than induction of antibody-detected MHC class I stabilization. Immunol Lett 47: 1-8. doi:10.1016/0165-2478(95)00052-7. PubMed: 8537084.

35. Chen CA, Ho CM, Chang MC, Sun WZ, Chen YL et al. (2010) Metronomic chemotherapy enhances antitumor effects of cancer vaccine by depleting regulatory T lymphocytes and inhibiting tumor angiogenesis. Mol Ther 18: 1233-1243. doi:10.1038/mt.2010.34. PubMed: 20372107.

36. Nichols WW, Ledwith BJ, Manam SV, Troilo PJ (1995) Potential DNA vaccine integration into host cell genome. Ann N Y Acad Sci 772: 30-39. doi:10.1111/j.1749-6632.1995.tb44720.x. PubMed: 8546411.