行政院國家科學委員會專題研究計畫　期中進度報告

正常人、子宮頸內皮細胞赘生瘤和子宮頸癌病人對人類乳突病毒第十六型的 E7 特異免疫反應之建立及比較(1/2)

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計畫主持人：陳祈安
共同主持人：謝長堯，鄭文芳
計畫參與人員：李建南 蘇怡寧 林怡君

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行政院國家科學委員會補助專題研究計畫 ■期中進度報告

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The establishment and comparison on the HPV type 16 E7-specific immunologic responses between normal population, patients with CIN and patients with cervical cancer

計畫類別：■ 個別型計畫　□ 整合型計畫

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一、計畫中文摘要

關鍵詞：子宮頸癌、人類乳突病毒、癌症疫苗、免疫治療

子宮頸癌是台灣女性最常好發和死亡率排名第三位的惡性腫瘤。此外每年全世界有大約二十萬的婦女死於子宮頸癌。目前可利用的子宮頸癌治療方法諸如手術、放射線治療或化學治療都會在殺害癌細胞的同時，也會破壞健康的細胞。我們極需要不只降低子宮頸癌的發生率，也需要有比目前治療方式較好的治療模式。免疫療法能選擇性攻擊腫瘤細胞而不會破壞正常細胞，它看起來將提供一個令人期待地新的治療策略。

理想的癌症治療方式必須能夠清除體內多處全身性的腫瘤細胞並且具有分辨腫瘤細胞和非腫瘤細胞此特異性。以此觀點看來抗原特異的癌症免疫治療是一種吸引人的癌症治療方式。目前很清楚地了解MHC class I CD8毒殺T細胞在抗腫瘤免疫上扮演著重要的角色。

經由與約翰霍普金斯吳子丑教授合作，我們最近已經發展出多種策略的E7專一的癌症疫苗，這些策略包括DNA型及病毒型疫苗。我們發現這些針對E7的DNA疫苗在動物實驗上可以避免和治療腫瘤的形成和生長。這些從前臨床動物實驗得到的好結果，鼓勵我們持續癌症疫苗和免疫治療的研發並進一步應用到人體上。然而建立人體內E7特異的免疫評估法對未來臨床試驗評估癌症疫苗和免疫治療的效果是非常重要的。因此我們設計這個計畫來研究人體內E7特異的免疫評估法。1）分析及比較正常人、受人類乳突病毒感染而無子宮頸病變的人，子宮頸內皮細胞病變的病人和子宮頸癌患者對人類乳突病毒第16型 E7抗原免疫反應的差異性。2）比較子宮頸癌患者期疾病嚴重度和其對人類乳突病毒第16型 E7抗原免疫反應的差異性。

二、計畫英文摘要

Keywords: cervical cancer, human papillomavirus, cancer vaccine, immunotherapy

Cervical cancer the most frequent neoplasm and the third mortality rate of malignancies of the women in the world. It results in about 200,000 women dying of cervical cancer each year worldwide. The available forms of treatment-surgery, radiation therapy, and chemotherapy are all cytoreductive treatment modalities, so in addition to killing cancerous cells, healthy cells are also destroyed in the process. Indeed, there is a need to decrease the incidence of cervical cancer and develop better forms of its treatment.

The 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 nonneoplastic cells. In this regard, antigen-specific cancer immunotherapy represent an attractive approach for cancer treatment. It is now clear that major histocompatibility complex (MHC) class I restricted $CD8^+$ T cytotoxic cells are critical to the generation of antitumor immunity. Cell-mediated responses are critical in anti-tumor immunity.

By cooperating with Dr. TC Wu in Johns Hopkins Medical Institutes, we have recently developed some E7-specific cancer vaccines of different strategies such as DNA, or replication-defective SINrep5 virus. We found that these E7-chimeric DNA vaccines are capable of preventing and treating the growth of murine model tumors expressing E7. These positive results from the preclinical murine models have encouraged us to focus on the development of cancer vaccine and immunotherapy and apply these vaccines to human subjects. However, it is very important to set up various E7-specific immunologic assays of human being to evaluate the effect of cancer vaccine or immunotherapy in the future clinical trials. So we would like to provide this proposal to address on the development of HPV 16 E7-specific immunologic assays in human being. There are two main goals in this study. First, we would like to establish and compare the differences of HPV type 16 E7-specific immunologic responses between normal population, people with HPV infection, patients with CIN lesions and patients of cervical cancer. Second, we would like to correlate the disease severity of cervical cancer with the immunologic responses to HPV type 16 E7 antigen.

三、研究計畫之原由及目的：

HPV Oncogenic Proteins, E6 and E7, as Ideal Targets for the Development of Antigen-Specific Immunotherapies or Vaccines for HPV-Associated Cervical Malignancies

E6 and E7 represent ideal targets for the development of antigen-specific immunotherapies or vaccines for HPV-associated malignancies. First, more than 90% of cervical cancers have been associated with HPVs, particularly type 16, and E6 and E7 are consistently expressed in most cervical cancers. Second, while most tumor specific antigens are derived from normal proteins or mutated protein, E6 and E7 are completely foreign viral proteins, and potentially may harbor more antigenic peptides/epitopes than a mutant protein (i.e. p53) or a reactivated embryonic protein (i.e. MAGE-1). Third, since E6 and E7 are required for the induction and maintenance of malignant phenotype of cancer cells [1], cells of cervical cancer cannot evade an immune response through antigen loss. Without functional E6 and E7, these cells would cease to be tumorigenic. Therefore, E6 and E7 proteins represent ideal targets for developing antigen-specific immunotherapies or vaccines for cervical cancer.

Cellular Immune Responses to HPV

The understanding of T-cell mediated immunity to HPV infections was facilitated by identification of MHC class I and class II epitopes of HPV proteins. Several groups have attempted to map murine [2-4] and human [5,6] T helper (Th) cell epitopes on HPV proteins. Several groups have also tried to map murine [7-11] as well as human [12-15] cytotoxic T-lymphocyte (CTL) epitopes on HPV proteins. Kast *et al.* have identified several high affinity binding peptides of HPV-16 E6 and E7 proteins for human HLA-A alleles[13]. Furthermore, HPV-specific CTLs recognizing HPV E6 and E7 proteins have been demonstrated in peripheral blood

of cervical cancer patients [16,17], in healthy donors [14,18] and in patients with CIN lesions [15,19,20]. Furthermore, infiltration of cervical cancer tissue with HPV-specific CTLs has been recently described[21].

Cell-mediated immune responses in HPV-infected lesions can be demonstrated by *in vivo* skin tests [22,23], *in vitro* CTL assays [17,19,21] and *in vitro* lymphoproliferative response [5,6,24-30]. For instance, Hopfl *et al.* have used bacterially-expressed HPV-16 proteins for skin tests in patients with CIN lesions and have found specific skin responses to the virion protein L1 and not the E4 protein [22]. In patients with CIN lesions, HPV-specific CTLs have been identified in PBMC [17,19] and in cervical tissues [21]. The *in vitro* lymphoproliferative responses in patients with CIN lesions has been actively investigated. For example, de Gruijl et al. reported that T cell proliferative responses against HPV-16 E7 oncogenic protein were most prominent in CIN patients with a persistent HPV infection [27]. However, Kadish et al. reported that lymphoproliferative responses to specific HPV-16 E6 and E7 peptides appeared to be associated with the clearance of HPV infection and the regression of CIN lesions [28].

Role of Cytokines in Cell-Mediated Immunity

Cell mediated immunity is regulated by cytokines which are secreted by T helper cells. In general, T helper cells can be classified as Th1 and/or Th2 cells based on the different types of cytokines they secrete. Th1 cells secrete interleukin (IL) 2 and interferon gamma (IFN-γ). Th2 cells produce IL-4, IL-5, IL-10 and IL-13. The Th1 lymphocytes are the most important effector cells in inflammatory reactions associated with vigorous delay-type hypersensitivity but low antibody production, as occurs in contact dermatitis and in viral or intracellular bacterial infections (for review, see [31,32]). The functional phenotype of most Th2 cells may account for both the persistent production of certain antibody isotypes, particularly IgG1 and IgE, and the eosinophilia observed in human helminthic infections and allergic disorders. Lymphocyte mediated protection from viral infections as well as control of tumors is thought to be mediated by Th1 cytokine responses and impaired by Th2 cytokine responses. The IL-2 and IFN-γ producing Th1 response is likely to be the major component that contributes to the development of cell mediated immunity against HPV infections and HPV-associated neoplasms.

Chimeric E7-specific vaccines can control the HPV16 E7-expressing tumor model

With cooperating with Prof. TC Wu in Johns Hopkins Medical Institutes, we have successfully developed several chimeric DNA, RNA, and virus-vector vaccines to prevent and treat HPV16 E7-expressing tumor in the animal model [33-35]. We found that these E7-chimeric DNA vaccines are capable of preventing and treating the growth of murine model tumors expressing E7. These positive results from the preclinical murine models have encouraged us to focus on the development of cancer vaccine and immunotherapy and apply these vaccines to human subjects.

However, it is very important to set up various E7-specific immunologic assays of

human being to evaluate the effect of cancer vaccine or immunotherapy in the future clinical trials. So we would like to provide this proposal to address on the development of HPV 16 E7-specific immunologic assays in human being. There are several aims in this project: 1) to develop and utilize assays to measure CTLs to HPV 16 E7 proteins, 2) to develop and utilize assays to measure T helper (Th) responses to HPV 16 E7 antigens.

四、研究方法、進行步驟及執行進度：

Human subjects

Freshly drawn blood was diluted 1:1 with Hanks' balanced salt solution. The samples were collected from healthy volunteers, people infected with HPV type 16 but without CIN lesions, patients with CIN lesions, patients of cervical cancer from National Taiwan University Hospital after informed consent was obtained, and the protocols were reviewed and approved by the appropriate Investigative Review Boards. The diagnosis of CIN lesion or cervical carcinoma will be approved and reviewed by two of the pathologists. Patients' age, International Federation of Gynecology and Obstetrics (FIGO) stage, histology of tumors, and status of lymph-node metastasis were recorded after thorough clinical investigations if these data are available.

HPV detection and genotyping

HPV detection and genotyping will be performed from the specimen of cervical swab. Degenerative primers of MY11 and MY09, designed from a highly polymorphic sequence of the L1 region of HPV [36], were used to detect genital HPV DNA by polymerase chain reaction (PCR). The amplification products (about 450 bp in length)will be visualized by ethidium-bromide staining under UV light, after agarose (1.0%)-gel electrophoresis. For HPV genotyping, the PCR products will be further digested with a panel of restriction enzymes, including BamHI, PstI, HinfI, HaeIII, DdeI, and RsaI, and again detected by agarose-gel electrophoresis and ethidium-bromide staining. The HPV types will be determined according to the published restriction patterns of 44 types of genital HPV [37]. The minimal HPV DNA that can be amplified and typed by this restriction-fragment-length polymorphism(RFLP) assay was determined to be 1 pg of pBR322-cloned HPV 16 DNA, or 10^5 HPV copies. The beta-actin gene was PCR-amplified for samples without detectable HPV DNA, to rule out false-negative results.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were prepared with Ficoll-Paque solution (Amersham Biosciences, Uppsala, Sweeden) for separation of lymphocytes according to the method described previously with some modification [38]. After centrifuging at 600 x *g* for 30 min, the layer containing lymphocytes was transferred to another centrifuge tube, then washed twice in 10 ml of Hanks' balanced salt solution and then centrifuged at 1200 x *g* for 10 min.

Identification of human lymphocyte antigen haloptype

The PBMCs of the volunteers were collected and prepared as described earlier. Human HLA haplotype was identified by microlymphocytotoxicity test by the commercial HLA tissue typing trays (One

Lambda Inc., Canoga Park, CA) as described earlier [39].

Synthetic peptides

Two peptides used were chosen from the identified HLA class I-restricted epitopes from HPV type 16 E7 as reported previously [15]. The synthesized peptides (Kelowna Inc., Taipei, Taiwan) included HLA A2-restricted peptides aa 11-20 [YMLDLQPETT] and aa 86-93 [TLGIVCPI].

Reagents

The recombinant human cytokines used were IL-2 (R&D Systems, Minneapolis, MN), GM-CSF (Peprotech Inc., Rocky Hill, NJ), IL-7 (R&D Systems, Minneapolis, MN), and IL-4 (Peprotech Inc., Rocky Hill, NJ). The tumor cell lines used were HeLa and Caski cells, which were both purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM+ 10% FBS and 1% antibiotics.

Cell culture and DC generation

Immature monocyte-derived DCs were generated from the adherent fraction of peripheral blood PBMCs as described earlier [40]. Briefly, PBMCs were suspended in medium and allowed to adhere to plastic dishes. After 2-h incubation at 37°C, the nonadherent cells were removed, and the adherent cells were cultured with 800 IU/ml GM-CSF and 500 U/ml IL-4 for 6 days in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 1% human sera. The cytokines were added to the DCs at days 0, 2, or 4. On days 6, nonadherent cells were collected for analysis of DC markers.

The DCs were collected and washed with FACScan buffer (PBS+0.2% FBS and 0.5% sodium azide). The cells were further stained with FITC- or PE-conjugated anti-human Abs (CD1a, CD3, CD56, CD80, HLA-A,B,C and HLA-DR) (PharMingen, San Diego, CA) incubated on ice for 30 min followed by washing with FACScan buffer. Data acquisition and analysis were performed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software.

Generation of peptide-specific CTLs

E7 peptide-specific CTLs were generated from the peripheral blood of four HLA-A2-positive donors. DCs ($2x10^5$ cells) isolated as described above were pulsed for 2 h with the peptide (10 mM), washed, and incubated with $2x10^6$ PBMC cells in 24-well plates with human T cell medium (RMPI + 10% human serum + 5% HEPES + 1% L-glutamine + 1% penicillin) in the presence of IL-7 (15 U/ml) (R&D Systems, Minneapolis, MN). IL-2 (15 U/ml) (R&D Systems, Minneapolis, MN) was added 2 days later. T cells were restimulated with peptide-pulsed DC on days 7, 14, or 21. IL-7 (15 U/ml) was added immediately after restimulation, and IL-2 (15 U/ml) was added 2 days later. CTLs were harvested on day 7, 14, 21 or 28 and either used immediately or cryopreserved in liquid nitrogen until being tested in the other immunologic assays.

IFN-γ ELISPOT Assay

The ELISPOT assay described by Miyahira *et al.* [41] and Murali-Krishna *et al.* [42] was modified to detect HPV-16 E7-specific CD8+ T cells. The 96-well filtration plates (Millipore, Bedford, MA) were coated with 10 μg/ml anti-human IFN-γ antibody (PharMingen,

San Diego, CA) in 50 µl of PBS. After overnight incubation at 4°C, the wells were washed and blocked with culture medium containing 10% fetal bovine serum. Different concentrations of fresh or cultured PBMCs from each volunteer, starting from 1 x 10^6/well, mixed with autologous DCs (ratio 10:1), were added to the well. The autologous DCs have been cultured for six days as described earlier and pulsed with or without 10 mM E7-specific HLA-A2 CTL epitopes (E7, amino acids 11-20 or 86-93) for 2 hours before cocultured with the PBMCs. After culturing, the plate was washed and then followed by incubation with 5 µg/ml biotinylated rat anti-human IFN-γ antibody (PharMingen, San Diego, CA) in 50 µl in PBS at 4°C overnight. After washing six times, 1.25 µg/ml avidin-alkaline phosphatase (Sigma, St. Louis, MO) in 50 µl of PBS were added and incubated for 2 h at room temperature. Afterwards, spots were developed by adding 50 µl of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Boehringer Mannheim, Indianapolis, IN) and incubated at room temperature for 20 minutes. The reactions were stopped by discarding the substrate and washing the plates under tap water. The plates were then air-dried, and colored spots were counted using a dissecting microscope.

E7 peptide-specific INF-γ secretion by enzyme-linked immunosorbent assay (ELISA)

E7-specific CD8+ T cells of HLA-A2 haplotype were cultured as described earlier. At day 7, 14, 21 and 28, the supernatants of cultured media were collected to detect the concentrations of INF-γ with sandwich ELISA kits as described previously [43,44] (R&D Systems, Minneapolis, MN). A standard curve using purified protein for each cytokine was generated, and the protein levels in the experimental groups were calculated.

五、結果與討論

E7 peptide-specific INF-γ secretions from the cultured PBMCs of the HPV 16-infected populations were higher than those from normal populations

The concentrations of IFN-γ of the PBMCs from each sample pulsed with E7 peptide (aa 11-20) in normal and HPV 16-infected populations are shown in **Figure 1A**. The median concentration of IFN-γ from twelve HPV 16-infected populations was higher than that from 4 normal populations. We further examined the concentrations of IFN-γ of the PBMCs in different subgroups of HPV 16-infected populations. As shown in **Figure 1A**, none of the PBMCs from the normal populations or cervical cancer patients, when pulsed with peptide (aa 11-20), secreted more than 400 pg/ml of IFN-γ. The PBMCs of all four persons with HPV 16 infection, and three out of four patients with CIN lesions, could secrete more than 400 pg/ml of IFN-γ.

The concentrations of IFN-γ of the PBMCs from each sample stimulated with E7 peptide (aa 86-93) in normal and HPV 16-infected populations are shown in **Figure 1B**. The median concentration of IFN-γ from twelve HPV 16-infected populations was also higher than that from 4 normal populations. The concentrations of IFN-γ of the PBMCs from each sample stimulated with E7 peptide (aa 86-93) are shown in **Figure 1B**. None of the PBMCs from the normal populations and

only one of four cervical cancer patients, when pulsed with peptide (aa 86-93), secreted more than 400 pg/ml of IFN-γ. The PBMCs from 3 out of four persons with HPV 16 infection and two out of four patients with CIN lesions could secrete more than 400 pg/ml of IFN-γ. Whereas, the concentrations of IFN-γ of the PBMCs from each sample stimulated with matrix peptide of influenza virus are similar between the normal and HPV 16-infected populations.

Our data showed that PBMCs of HPV 16-infected populations, when pulsed with respective E7-specific peptide, secreted higher amounts of IFN-γ than those of normal populations did. Besides, according to the hisotopathologic abnormalities, the PBMCs of persons with HPV 16 infection only, or patients with CIN lesions had higher percentages to secrete higher concentrations of IFN-γ, when pulsed with E7-specific peptides, than those of cervical cancer patients did.

PBMCs from HPV 16-infected populations generated higher numbers of E7 peptide-specific IFN-γ spots than those from normal populations

The numbers of IFN-γ spots, stimulated with E7 peptide (aa 11-20), of twelve HPV 16-infected patients and those from four normal volunteers are shown in **Figure 2A and 2B** (**Figure 2A** for fresh PBMCs without *in vitro* culture and **Figure 2B** for PBMCs pulsed with E7 aa1-20 and cultured for 7 days). The median numbers of IFN-γ spots of twelve HPV 16-infected patients were higher than those from four normal volunteers. None of the PBMCs of the 4 normal volunteers generated more than 50 IFN-γ spots/1x10^6 PBMCs. However, the PBMCs in three out of four persons with HPV 16 infection only, 2 out of four patients with CIN lesions, and 3 out of four cervical cancer patients generated more than 50 IFN-γ spots/1x10^6 PBMCs. When evaluating the numbers of IFN-γ spots from PBMCs cultured for 7 days, none of the PBMCs from the normal populations and only one out of four cervical cancer patients generated more than 200 IFN-γ spots/1x10^6 PBMCs **(Figure 2B)**. However, the PBMCs in 3 out of four persons with HPV 16 infection only, and 3 out of four patients with CIN lesions generated more than 200 IFN-γ spots/1x10^6 PBMCs.

The numbers of IFN-γ spots, stimulated with E7 peptide (aa 86-93), of twelve HPV 16-infected patients and those of four normal volunteers are shown in **Figure 2C and 2D** (**Figure 2C** for fresh PBMCs without *in vitro* culture and **Figure 2D** for PBMCs pulsed with E7 aa 86-93 and cultured for 7 days**)**. The median numbers of IFN-γ spots of twelve HPV 16-infected populations were higher than those of the four normal volunteers. We further examined the numbers of IFN-γ spot of the PBMCs in different subgroups of HPV 16-infected populations. As shown in **Figure 2C**, the PBMCs in three out of four persons with HPV 16 infection only, 2 out of four patients with CIN lesions, and 3 out of four cervical cancer patients, when pulsed with peptide (aa 86-93), could generate more than 50 IFN-γ spots/1x10^6 PBMCs. When evaluating the numbers of IFN-γ spots of PBMCs cultured for 7 days, none of the PBMCs from the normal populations generated more than 200 IFN-γ spots/1x10^6 PBMCs and

the PBMCs in all of four persons with HPV 16 infection only, 3 out of four patients with CIN lesions, and 2 out of four cervical cancer patients generated more than 200 IFN-γ spots/1x10^6 PBMCs **(Figure 2D)**. Whereas, the numbers of IFN-γ spots of PBMCs without *in vitro* culture or cultured for 7 days, when pulsed with the matrix peptide of influenza virus, were similar between the normal and HPV 16-infected populations.

Our data showed that HPV 16-infected populations generated stronger E7-specific immunologic responses than normal populations did. Besides, higher percentages of persons with HPV 16 infection only, or patients with CIN lesions produced higher immunologic response to the E7 specific epitopes of HPV 16 than the cervical cancer patients did.

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Figure 1

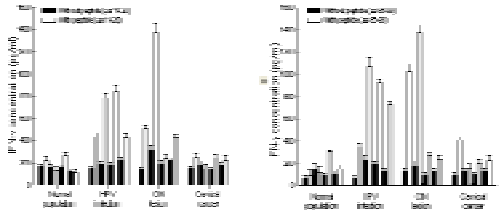

Figure 2