Elevated titer and seropositivity of the antibody to human papillomavirus E6 protein in cervical cancer patients

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Abstract

Development of cervical cancer is associated with human papillomavirus (HPV) infection. However, data on comparison among the antibodies of anti-E6, anti-E7, and anti-L1 in the sera of patients with cervical cancer or HPV infection are very limited. In this study, we examined the expression of HPV-18 nonstructural proteins E6, E7, and the major capsid L1 protein in tissues from 25 patients with cervical cancer at stages ranging from Ib to IIa, based on the FIGO staging system. We also used ELISA to assess the antibodies against the HPV-18 E6, E7, and L1 proteins in the sera of these patients (patients) and in the sera of 20 healthy individuals (normal). Western blotting and immunohistochemical staining of these tissues confirmed the presence of the E6, E7, and L1 proteins. As compared to the normal controls, the patients' sera showed higher binding of the anti-E6, anti-E7, and anti-L1 antibodies. The anti-E6 and anti-E7 antibodies showed stronger binding than anti-L1 among all the 25 sera samples tested. The seropositive percentage of anti-E6 (88%) was higher than that of anti-E7 (32%) and anti-L1 (48%). These data suggest that elevated titer and seropositivity of the antibody to human papillomavirus E6 protein in the sera of cervical cancer patients. Interestingly, we also found that the 19 patients who indicated as anti-E6 seropositivity had a lower 5-year cumulate survival outcome.

Key Words: Human Papilloma Viruses (HPV), Cervical Cancer, anti-E6 antibody

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Running title: HPV E6 and cervical cancer

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Introduction

Cervical cancer ranks second among the most common cancers in women worldwide[1, 2]. An estimated 371,000 new cases of invasive cervical cancer are diagnosed every year, representing nearly 10% of all cancers in women worldwide[3, 4]. In addition, there is a strong epidemiological correlation between human papillomavirus (HPV) infections and cervical cancer[1, 2, 5, 6, 7].

More than 100 different HPV serotypes have been identified, and they are divided into cutaneous and mucosal types. Depending on the associated lesions, mucosal HPVs are further subdivided into "high-risk" and "low-risk" types. HPV-16, HPV-18, HPV-31, HPV-35, HPV-39, and HPV-45 are the high-risk (HR-HPV) types predominately associated with cervical, vulvar, vaginal, anal, perianal, skin, and penile cancers [1, 2, 8, 9], while HPV-6, HPV-11, HPV-42, HPV-43, and HPV-44 belong to the low-risk types that are commonly detected in genital warts and benign tumors [10, 11]. Furthermore, in the HR-HPV, HPV-16 and HPV-18 are commonly detected in high-grade cervical intraepithelial neoplasia (CIN) and invasive cervical cancer, while the low-risk HPV-6 and HPV-11 mainly occur in benign condyloma and mild dysplasia of the uterine cervix [8, 12].

The HPV genome consists two regions, the late (L) region that encodes major (L1) and minor (L2) capsid proteins, and the early (E) region that directs replication and transcriptional regulation [12]. The E6 and E7 proteins were shown to be associated with the development of cervical cancer [10, 13]. In addition, previous serological studies have reported the presence of serum antibodies against the HPV E2, E6, E7, and L1 proteins in patients with cervical cancer or HPV infection [3, 11, 14-19]. There is limited data on the comparison between the serum anti-E6, anti-E7, and anti-L1 antibodies in patients with cervical cancer or HPV infection.

In this study, the tissues were obtained from 25 HPV-18-positive patients diagnosed with cervical cancer at stages ranging from Ib to IIa, based on the FIGO staging system; in these cases, clinical diagnoses of cervical cancer had also been confirmed by molecular hybridization [20]. The expression of HPV-18 E6, E7, and L1 proteins in these tissues were detected by western blotting. The antibodies against the E6, E7, and L1 proteins in the sera of these patients (patients) and 20 healthy individuals (normal) were detected by ELISA.

Methods

Patient specimens

Sera and tissue samples were obtained from 25 cervical cancer patients from Dr. R.C. Hsu and Dr. P. H. Chou at the Department of Gynecology, Chunghua Christian Hospital, Chunghua City, Taiwan. Clinical diagnosis was established firstly by Papanicolaou (Pap) smears and subsequently by colposcopy. The tissue samples were obtained from the endocervix or exocervix. In addition, tumor sera and normal control sera were obtained from the patients and 20 healthy individuals, respectively.

Protein extraction

The 25 cervical tissues were minced in lysis buffer (100 mM NaCl; 10 mM Tris-HCl, pH 7.6; 1 mM EDTA, pH 8.0; 1 μg/ml aprotinin; and 100 μg/ml PMSF) and homogenized by sonication. The extracted proteins were quantified by a BCA assay kit (Pierce).

Construction of recombinant plasmids

The cDNA of the HPV-18 *E6*, *E7*, and *L1* genes were synthesized and amplified using primer sets as follows:
5′-CGGAATTCATGGCGCGCTTTGAG and 5′-CGGAATTCTTATACTTGTGTTTC for E6, 5′-CGGAATTCTTACTGCTGGGATGC and 5′-CGGAATTCATGCATGGACCTAAG for E7, 5′-CGGAATTCATGTGCCTGTATACA and
5′-CGGAATTCTTACTTCCTGGCACG for L1. Each primer set anchored at the *EcoRI* restriction site was used for construction of the recombinant expression plasmids. These gene fragments were cloned into the *EcoRI*-digested pGEX-5X-1 plasmid (Pharamacia). The sequences and orientation of the inserts were confirmed by auto-sequencing.

Purification of recombinant pGEX-5X-1 E6, E7, and L1 fusion proteins

The selected clones were transformed into DH5α cells and grown in ampicillin-containing LB broth at 37°C. The recombinant fusion proteins were induced by 0.1 mM IPTG for 6 h at 30°C and the cell pellets were collected. The cell lysate was prepared as according to manufacturer's instructions (Pharmacia) and the expressed GST E6, E7, and L1 fusion proteins were isolated using glutathione-Sepharose 4B beads (Pharmacia).

Western blotting

Protein extract obtained from purified recombinant fusion proteins (10 μg) and tissue extracts (20 μg) were subjected to Western blotting. Membranes were immunodetected by anti-human HPV-18 E6 antibody (Chemicon) or 1:1000 diluted anti-human HPV-18 E7 antibody (Biodesign) or 1:1000 diluted anti-human HPV-18 L1 antibody (Abcam).

Enzyme-linked immunosorbent assay (ELISA)

Sera from the cervical cancer patients were analyzed by ELISA. Direct ELISA was used to determine the extent of binding of the antibodies to purified GST, and the fusion proteins GST E6, E7, and L1. The fusion protein-coated plates were immobilized overnight at 37℃ with 100 ng of antigens. After blocking with 3% skimmed milk and extensive washing, the antigen-antibody complex was detected by a goat anti-mouse horseradish peroxidase-conjugated IgG and the oxidation of O-phenyldiamine (OPD) (Zymed).

Immunohistochemistry

The deparaffinized and rehydrated section was treated with 3% H_2O_2 for 10 min to eliminate endogenous peroxidase activity. Nonspecific binding sites were blocked with bovine serum albumin for 10 min. The section was incubated with antibodies raised for anti-human HPV-18 E6 antibody (Chemicon) or 1:1000 diluted anti-human HPV-18 E7 antibody (Biodesign) or 1:1000 diluted anti-human HPV-18 L1 antibody (Abcam) for 1 h at room temperature. The biotinylated secondary antibodies were added and final signal was expressed by avidin-biotin peroxidase technique in the presence of hydrogen peroxide. The sections were evaluated under light microscope (Olympus BX51, Japan) and the images were analyzed with Image- Pro Plus software (Media Cybernetics Co. Ltd. (USA) agent by Total-Integra Tech. Co. Ltd. in Taiwan).

Statistical test

Multiple comparisons for statistically significant differences in anti-E6, anti-E7, and anti-L1 antibody titers (measured by optical density, O.D.) between the sera of the cervical cancer patients and normal individuals were made by the *t* test. In addition, the significance of 5-year cumulate survival rate for the seropositivity of anti-E6, anti-E7, and /or anti-L1 antibodies were analyzed by the Chi-square test.

Results

Patient demographic characteristics were classified by cervical cancer stage

Patient demographic characteristics were classified based on the cervical cancer stage. Sera and tissue samples from 25 cervical cancer patients in the age range of 33 to 72 years were obtained from the Department of Gynecology, Chunghua Christian Hospital, Chunghua City, Taiwan. Clinical diagnosis was established first by Pap smears and subsequently by colposcopy. Furthermore, all the tissues from these patients were identified to be in stages ranging from Ib to IIa, based on the FIGO staging system; they were 20 (80%) and 5 (20%), respectively. In our previous study, the prevalence of HPV-18 infection in the 25 patients was 100%, and 7 out of these 25 cases (28%) were co-infected with HPV-16, as confirmed by molecular hybridization (20). The prevalent HPV infectious serotype was found to be HPV-18. However, these results differ from the worldwide prevalent type, which is HPV-16.

Western blotting of HPV-18 E6, E7 and L1 proteins in tissues of patients with cervical cancer

To measure the expression of HPV-18 antigens in cervical cancer patients, the crude extracts of the cervix were obtained as described. Total proteins were separated by 12% SDS-PAGE and then transferred to PVDF membranes. The HPV-18 antigens were detected by anti-HPV-18 E6, -E7, and -L1 antibodies. The HPV-18 E6, E7, and L1 proteins were all detected by western blotting in the cervical tissues of all 25 patients (Fig. 1). Furthermore, positive control included HPV-18-positive (HeLa) cervical cancer cell lines were all detected by anti-HPV-18 E6, -E7, and -L1 antibodies (Fig. 1). To elucidate and analyze the expression of the HPV-18 E6, E7, and L1 proteins detected in the cervical tissues of the patients (Fig. 1).

Immunohistochemical staining of HPV-E6, E7 and L1 in tissues of patients with cervical cancer

To elucidate the expression of HPV-18 E6, E7, and L1 proteins detected in all cervical tissues of the 25 cervical cancer patients, respectively (Fig. 1), we also used immunohistochemical staining as an approach to analyze the expression of HPV-18 E6, E7, and L1 proteins in cervical tissue of cervical cancer patient No. 4. The results showed that the HPV-18 E6, E7, and L1 proteins were detected in cervical tissue of cervical cancer patient No.4 (Fig. 2A, 2B, and 2C). In addition, the HPV-18 E6, E7, and L1 proteins were detected in all others cervical tissue of patients with cervical cancer (data not show) and results were similar with the results of western blotting.

Expression of the recombinant HPV-18 E6, E7 and L1 proteins

To study the presence of anti-HPV-18 E6, -E7, and -L1 antibodies and the magnitude of antibody reactivity to the expression of cervical tissues of patients with cervical cancer, we used GST-fusion protein expression systems as approaches to analyze. The GST-fusion protein expression system was used to produce recombinant HPV-18 E6, E7 and L1 proteins used as the coating antigen. The full-length coding region of HPV-18 E6, E7 an L1 were amplified by PCR and constructed into pGEX-5X-1 vector as the method described. The resulting pGEX-5X-1-E6, pGEX-5X-1-E7 and pGEX-5X-1-L1 expression constructs were confirmed by restriction enzyme digestion and sequencing. These fusion proteins were induced by IPTG and purified by glutathione sepharose 4B beads system and then analyzed by SDS-PAGE. The molecular sizes of fusion proteins of HPV-18 E6, E7 and L1 recombinant proteins were 43 kDa, 39 kDa and 89 kDa, respectively (Fig. 3, right column), and were used as antigens coated on an ELISA plate for screening the sera of patients with cervical cancer. In the western blotting results, the amount of recombinant proteins increased with induction time (Fig. 3, Left column).

Binding test in sera

The binding of the serum antibodies to HPV-18 E6, E7, and L1 was investigated by ELISA. Different amounts (300, 200, 100, 50, 25, 10, and 5 ng) of GST E6-, E7-, and L1-eluted fusion proteins (Fig. 2, right column) were coated on an ELISA plate and then reacted with different dilutions of the serum from patient No. 7, which had the highest titer against E6, E7, and L1. The optimal binding was with 100 ng of GST E6, E7, and L1 at a serum dilution of 1:100 (data not shown). Therefore, the binding of the serum antibodies to recombinant GST E6-, E7-, and L1-eluted proteins was measured under optimal dilution conditions and compared with the normal controls. ELISA revealed that the binding of anti-E6 and anti-L1 antibodies was significantly higher in the 25 patients than in the normal controls (Fig. 2, **: p value < 0.01; *: p value < 0.05). However, the binding of anti-E7 antibody was not significantly higher in the 25 patients than in the normal controls. Furthermore, the binding of anti-E6 and anti-E7 antibodies was higher than that of the anti-L1 antibody.

Comparing the seropositive percentage among antibodies against HPV-18 E6, E7, and L1 proteins in sera from patients with cervical cancer

The seropositive percentage among antibodies against HPV-18 E6, E7, and L1 proteins in the sera of cervical cancer patients was studied. Sera were considered antibody-positive (seropositivity) when their absorbance values were higher than each mean value of absorbance [15]. Based on the results, seropositivity was observed in 22 (88%), 8 (32%), and 12 (48%) patients presenting antibodies against the HPV-18 E6-, E7-, and L1-eluted proteins, respectively.

Comparing the seropositivity of anti-E6, anti-E7, and/or anti-L1 antibodies with the 5-year cumulate survival rate

The comparing of seropositivity of anti-E6, anti-E7, and/or anti-L1 with the 5-year cumulate survival rate was shown in Table 1. The results showed that the 19 patients who indicated as anti-E6 seropositivity had a lower 5-year cumulate survival outcome than those indicated as anti-E6 seronegativity (p value< 0.0001***). Furthermore, the patients who indicated as anti-E7 and anti-L1 seronegativity had a higher 5-year cumulate survival outcome (p value< 0.001**).

Discussion

Currently, cervical cytology screening and molecular detection are the universal diagnosis techniques of cervical carcinogenesis. The former is based on Pap staining of epithelial cells sampled from the cervix. However, the limitations of this technique are poor sensitivity, poor specificity, high cost, and manpower shortage [21]. On the other hand, HPV infection is always associated with the development of cervical cancer [1, 2,5-7]. At the molecular level, accurate HPV genotyping is essential for the classification of patients into low- and high-risk groups [8, 11]. The genome of HR-HPV may integrate with the chromosome of the host cell, whereas this integration is not found in low-risk HPV types. Results indicate that the ratio of HR-HPV-18 integration is higher than that of HR-HPV-16 in the lesions of invasive cervical cancer [7, 8]. Therefore, molecular detection of HPV genotyping is necessary for cervical carcinogenesis. However, no HPV-16 or HPV-18 DNA was detected in the blood of women with precursor cervical lesions, but it was detected in cervical cancer patients [22]. The advantages of serological studies are high sensitivity, high specificity, early tumor screening, and specific HPV genotyping without obtaining tissue samples [16, 23]. Consequently, serological diagnosis of HPV genotyping and early tumor screening are also necessary and potential indicators of cervical carcinogenesis.

Previous studies have reported that the antibody response to HPV-16 E6 appears to be dependent on the clinical stage of cervical cancer [3, 14, 17]. In addition, there is limited data on the comparison between the expression of E6, E7, and L1 in the sera of patients with cervical cancer or HPV infection [3, 14, 17]. Therefore, the tissues from 25 HPV-18-positive patients in whom cervical cancer had been clinically diagnosed and confirmed by molecular hybridization [20]. The prevalent HPV infectious serotype is HPV-18, which differs from the

worldwide prevalent type, HPV-16. The most dominant mutation among the 25 patients tested was a silent mutation, namely, C183G in the E6 coding region [20]. Furthermore, tissues from all 25 patients confirmed the stage of cervical cancer to range from stage Ib to IIa, based on the FIGO staging system. In the present study, western blotting showed that the HPV-18 E6, E7, and L1 proteins were present in all cervical tissues of the 25 cervical cancer patients (Fig. 1). Serological study revealed that the binding of the sera antibodies against the HPV-18 E6 antigen was significantly higher than that for the HPV-18 E7 and L1 antigens (Fig. 4). The elevated binding of anti-E6 was detected in 88% of the patients with cervical cancer at stages ranging from Ib to IIa. Rocha-Zavaleta et al. suggested that the antibody reactivity of L1 is induced in HPV-associated low-grade squamous intraepithelial lesions (LSIL) before invasion [19]. Nonetheless, Stanley suggested that the antibody reactivity of E6 and that of E7 are not induced before invasion, and it is not a useful early diagnostic marker of invasive cervical cancer [24]. However, a majority of the evidence indicated that the antibody reactivity to the HPV E6 and E7 oncoproteins had been good candidate markers for monitoring invasive cervical cancer [11, 15-18]. In addition, we also found that the patients with seropositivity of anti-E6 antibodies had a lower survival outcome than that the patients with seropositivity of anti-E7 or anti-L1 antibodies (Table 1). These results suggest that elevated titer and seropositivity of the antibody to human papillomavirus E6 protein in the sera of patients with cervical cancer. However, two pharmaceutical companies [Merck Sharp&Dohme (MSD) and GlaxoSmithKline (GSK)] have completed vaccine trails and HPV vaccine has been approved in most continents[25]. Ryding et al. reported that serological studies are the basis for design of a human papillomavirus vaccination program[26].

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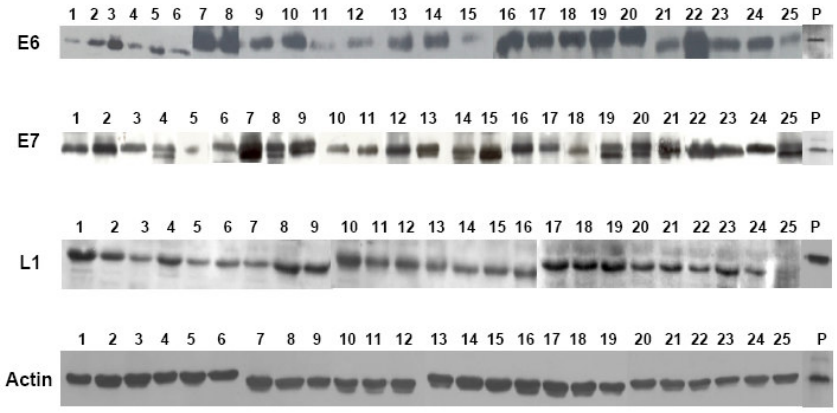

Figure 1. Western blotting of HPV-18 E6, E7, and L1 proteins in all tissues of cervical cancer patients. The HPV-18 E6, HPV-18 E7, and HPV-18 L1 proteins were detected in all the cell lysates of tissues from 25 cervical cancer patients by specific monoclonal antibodies. Actin was used as internal control. The crude extracts of the HPV-18- positive (HeLa) cervical cancer cells were used as positive controls.

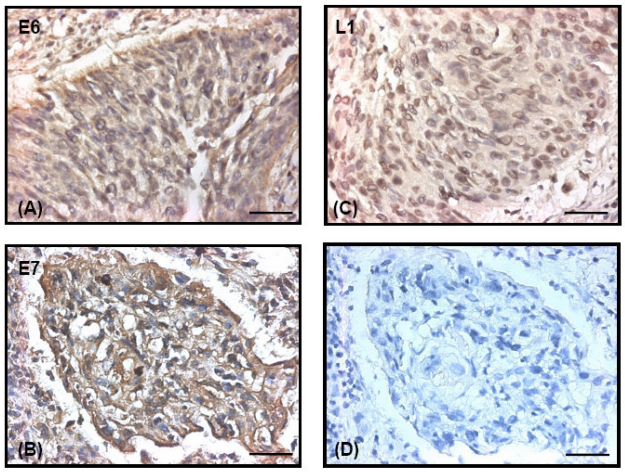

Figure 2. Immunohistochemical staining of HPV-E6, E7, and L1 in tissues of cervical cancer patients. The HPV-18 E6 (A), HPV-18 E7 (B), and HPV-18 L1 (C) proteins were detected in formalin-fixed paraffin sections (5 μm) of patient No. 4 with cervical cancer. The signals that confirm the specific reaction of HPV-18 E6 (A), HPV-18 E7 (B), and HPV-18 L1 (C) proteins to monoclonal antibodies (brown in color). The negative control was stained without the addition of the primary antibody (D). Bar: 50 μm size.

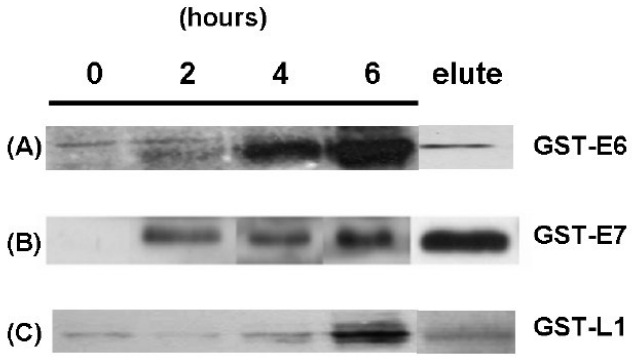

Figure 3. Western immunoblot analysis of the recombinant GST-E6 (A), GST-E7 (B), and GST-L1 proteins (C) at 0 h (lane 1), 2 h (lane 2), 4 h (lane 3), and 6 h (lane 4). Lanes 5 represents the elution of GST-E6 (A), GST-E7 (B), and GST-L1 (C) proteins, which were used as antigens coated on an ELISA plate for screening the sera of patients with cervical cancer.

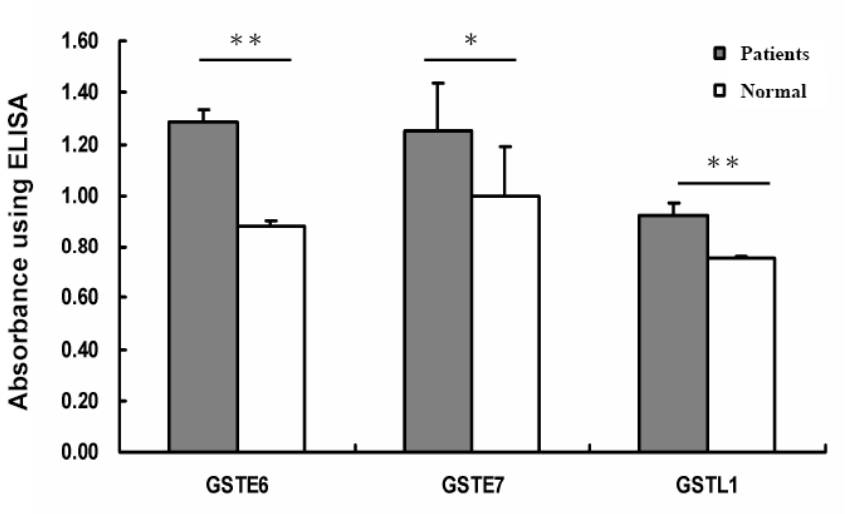

Figure 4. The ELISA results show the eluted GST-E6 (A), GST-E7 (B), and GST-L1 (C) proteins in the sera of patients with clinically confirmed cervical cancer (patients) and healthy individuals (normal). Equal quantities (100 ng/well) of the eluted GST-E6 (A), GST-E7 (B), and GST-L1 (C) proteins were coated on 96-well plates in triplicate. The asterisk indicates a statistically significant difference between the two groups (**: p value < 0.01; *: p value < 0.05).

TABLE 1. Comparing the seropositivity of anti-E6, anti-E7, and/or anti-L1 antibodies with the 5-year cumulate survival rate

Variable	5-year cumulate survival rate	*p*-value
Anti-E6 Ab		
(+)	**19/22**	**< 0.001*****
(-)	**3/3**	
Anti-E7 Ab		
(+)	**8/8**	**0.686**
(-)	**13/17**	
Anti-L1 Ab		
(+)	**10/12**	**0.875**
(-)	**11/13**	
Anti-E6 Ab + Anti-E7 Ab		
(+)	**4/4**	**0.788**
(-)	**3/3**	
Anti-E6 Ab + Anti-L1 Ab		
(+)	**10/12**	**0.055**
(-)	**3/3**	
Anti-E7 Ab + Anti-L1 Ab		
(+)	**0/0**	**0.009****
(-)	**7/9**	
Anti-E6 Ab + Anti-E7 Ab + Anti-L1 Ab		
(+)	**4/4**	**0.788**
(-)	**3/3**	

子宮頸癌病患血清中具有高效價與高血清陽性率的抗人類乳突瘤病毒 E6 抗體

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摘 要

目前已知子宮頸癌的形成與人類乳突瘤感染有密切的關係。然而，針對比較子宮頸癌病患血清中抗 E6、E7 與 L1 抗體表現量方面的資料略顯不足。在本實驗中，針對 25 位經臨床診斷(依照 FIGO 系統診斷爲 Ib 至 IIa 期)罹患子宮頸癌病患組織中，偵測病灶組織人類乳突瘤病毒血清型第 18 型非結構性蛋白質 E6、E7 與結構性蛋白質 L1 抗原的表現量。我們利用免疫酵素連結吸附檢測法測量 25 位病患和 20 位正常個體血清抗 E6、E7 與抗 L1 抗體的表現量。此外，我們也利用西方點墨法與免疫組織染色法偵測子宮頸癌病患組織中 E6、E7 與 L1 抗原的表現量。結果顯示，利用西方點墨法與免疫組織染色法已偵測到多數子宮頸癌病人腫瘤組織和相對正常組織含有血清型第 18 型 E6、E7 與 L1 抗原的表現。25 位病患抗 E6、E7 與 L1 抗體的表現量顯著高於 20 位正常個體。此外，病患血清中抗 E6、E7 抗體的結合能力高於抗 L1 抗體的結合能力。抗 E6 抗體的血清陽性率爲 88%，高於抗 E7 抗體的血清抗體陽性率(32%)與抗 L1 抗體的血清抗體陽性率(48%)。這些結果顯示，在子宮頸癌病患的血清中具有較高效價的抗人類乳突瘤病毒 E6 抗體與抗 E6 抗體的血清陽性率。有趣的是，我們發現了 19 位抗 E6 抗體血清陽性率的子宮頸癌病患，其五年累積存活機率較低。

關鍵字：人類乳突瘤病毒，子宮頸癌，抗 E6 抗體

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