The Relationship Between Human Papillomavirus and Epstein-Barr Virus Infections in Relation to Age of Patients With Cervical Adenocarcinoma

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SUMMARY

Objective: To examine the relationship between human papillomavirus (HPV) and Epstein-Barr virus (EBV) infections in relation to age of patients with cervical adenocarcinoma.

Materials and Methods: Thirty samples of human cervical adenocarcinoma tissue were collected from the surgical pathology archive at Taipei Veterans General Hospital from 1996 to 2008. All samples were examined for EBV, HPV-16 and HPV-18 E6 DNA by conventional and real-time quantitative polymerase chain reaction assays.

Results: HPV-16 DNA was detected in 10 cases (33.3%), HPV-18 DNA in 12 cases (40%), and EBV DNA in three cases (10%); there were negative findings in seven cases (23.3%). EBV combined with HPV-16 or HPV-18 was also detected in one case each. No link could be demonstrated between HPV and EBV in endocervical lesions. When 20 patients £ 45 years old were compared with 10 patients > 45 years old, HPV-18 E6 DNA was detected in 45% *vs*. 30% (9/20 *vs*. 3/10), HPV-16 E6 DNA in 40% *vs*. 20% (8/20 *vs*. 2/10), EBV DNA in 10% *vs*. 10% (2/20 *vs*. 1/10), and no virus DNA was detected in 10% *vs*. 50% (2/20 *vs.* 5/10). HPV and EBV were significantly more common in younger women ($p < 0.001$).

Conclusion: HPV-18 plays a major role in adenocarcinomas at any age. A high prevalence of HPV DNA is significantly associated with cervical adenocarcinoma, especially in younger women. The results do not support a role for EBV in cervical adenocarcinogenesis or any relationship between EBV and HPV infection in adenocarcinoma. [*Taiwan J Obstet Gynecol* 2009;48(4):370–374]

Key Words: cervical adenocarcinoma, Epstein-Barr virus, HPV-16, HPV-18

Introduction

The incidence of all invasive cervical cancers and squamous cell cancers has decreased over the last few decades. In this context, cervical adenocarcinoma stands out, because its incidence has been increasing in recent years, particularly in younger women, despite widespread screening programs and histology-specific cancer registration [1–4]. The epidemiologic factors underlying this differential trend are yet to be investigated. According the data of the Taiwan Cancer Registry, the proportion of adenocarcinomas relative to squamous cell carcinomas and to all cervical cancers was 1.6 between 1996 and 2005; the incidence of squamous cell carcinoma has declined but that of adenocarcinoma in the population at risk has steadily increased over this period.

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Human papillomaviruses (HPVs) are considered to be necessary and central etiologic agents for cervical carcinogenesis according to results of epidemiologic and molecular biologic studies [5–7]. To date, more than 100 HPV genotypes have been characterized and more than 20 different types have been isolated from patients with cervical carcinoma [8,9]. HPV-16, HPV-18, HPV-58, HPV-45, HPV-52 and HPV-31 have been frequently associated with cervical carcinoma in Taiwan. Squamous cell carcinoma of the cervix is linked to infection with oncogenic types of HPV, but the pathogenesis of adenocarcinoma is less well understood. The reported prevalence of HPV DNA in adenocarcinoma varies significantly from 32% to 100%, depending on the detection method used. A strong association between a sexually transmitted agent (HPV) and the risk of developing cervical squamous cell carcinoma has been clearly established; however, the relationship between HPV and cervical adenocarcinoma remains uncertain [4]. Some reports have shown that the epidemiologic cofactors for cervical adenocarcinoma include HPV positivity, no history of schooling, poor hygiene, sexual behavior-related variables, long-term use of hormonal contraception, high parity, and herpes simplex virus-2 seropositivity [4,10]. However, little information is available concerning the cofactors and geographic variation of HPV types in adenocarcinoma.

Epstein-Barr virus (EBV) receptors have been found in the cervical epithelium. It is a ubiquitous herpes virus associated with a number of human malignancies, and its DNA has been detected in other tumors, such as carcinomas from the tonsils, salivary glands and thymus, and in malignancies of the female genital tract [11]. van den Brule et al [12] suggested the existence of a previously undescribed association between EBV and squamous cell carcinoma of the cervix, but Payne et al [13] and other researchers [14,15] have failed to confirm this finding.

In this study, we used highly sensitive polymerase chain reaction (PCR)-based methods, including E6 type-specific primers and probes, to detect HPV and EBV DNA in cervical adenocarcinoma. The prevalence and possible etiologic link between HPV, EBV and age were examined in Taiwanese women.

Materials and Methods

Tissue samples

Thirty samples of human cervical adenocarcinoma tissue were collected from the surgical pathology archive at Taipei Veterans General Hospital from January 1996 to January 2008. The specimens were obtained through radical surgery, tumor removal, and debulking. Small biopsies unsuitable for further evaluation were excluded. The sampled cases had undergone complete clinicopathologic evaluations, including immunophenotypic and some molecular studies, to ascertain the diagnosis and rule out double tumors. Hematoxylin and eosin-stained slides of each primary case were reviewed and classified according to the 2003 World Health Organization classification of tumors. All studies were approved by the institutional review board of Taipei Veterans General Hospital.

DNA extraction

One adenocarcinoma tissue section (6 mm thick) was cut from each paraffin block using a disposable microtome blade and placed on a glass slide. The slides were then baked in an oven at 65°C for 30 minutes, and then deparaffinized by serial xylene and ethanol washes, according standard pathologic procedures. After rehydration, the slides were left to air dry at room temperature. Cellular proteins were digested in 10 mL of proteinase K (1 mg/mL) applied to the slide at room temperature. The cellular debris, including DNA, was aspirated by pipette and placed in a 100-mL microtube containing 15 mL of GeneReleaser (BioVentures Inc., Murfreesboro, TN, USA). DNA extraction was performed in a traditional 96-well PCR machine (Palm-Cycler; Corbett Research, Sydney, NSW, Australia) under the following conditions: 50°C for 180 minutes, 65°C for 30 seconds, 8°C for 30 seconds, 65°C for 90 seconds, 97°C for 180 seconds, 80°C for 60 seconds, 65°C for 180 seconds, 97°C for 60 seconds, 65°C for 60 seconds, and 80°C for 60 minutes, in accordance with the manufacturer's protocol. The suitability of samples for PCR amplification was ascertained by testing for the β-globin gene. Successful amplification of the β-globin gene fragments indicated that the DNA sample was adequate for PCR analysis and that no PCR inhibitors were present.

PCR amplification

Purified genomic DNA was amplified by PCR for the EBV, HPV-16 and HPV-18 E6 genes, as well as for the internal reference gene, β-globin. EBV nuclear antigen primers were used for EBV amplification: primer, 5′-AGTCATCATCATCCGGGTCTCC-3′; and probe, 5′-(FAM)-CGCAGGCCCCCTCCAGGTAGAA-(Black Hole Quencher)-3′. HPV primers were as follows: HPV-16 E6 primer, 5′-GAACCGGACAGAGCCCATTAC-3′, and probe, 5′-(CY5)-ACAACCGAAGCGTAGAGTCACACTTGC-(Black Hole Quencher)-3′; HPV-18 E6 primer, 5′-GAGGCCAGTGCCATTCGTG-3′, and probe, 5′-(ROX)-CAACCGAGCACGACAGGAACGACT-(Black Hole Quencher)-3′.

Multiplex real-time PCR

Each PCR reaction mixture contained 10 mL of 2X Qiagen Multiplex PCR Master mix (Qiagen Inc., Valencia, CA, USA), 1 mL of template DNA, and 0.5M (final concentration) of each primer pair and their corresponding probes. The final PCR mixture volume was made up to 20 mL with deionized water. The PCR reaction was programmed as: (1) Taq activation, 95°C for 14 minutes; and (2) amplification cycles, 94°C for 15 seconds, 58°C for 50 seconds, repeat for 60 cycles. The PCR reaction was run in duplicate in a Bio-Rad DNA Engine thermocycler coupled with a Chromo4 detector (Bio-Rad Gradient Real-Time PCR; MJ Research, Waltham, MA, USA). The PCR machine was controlled by a Windows-based laptop running Opticon Monitor 3.1 software (MJ Research).

Statistical analysis

The patients were divided into two groups: those ≤ 45 years old and those > 45 years old. Differences between the two groups were tested by univariate analysis using χ^2 tests and Fisher's exact tests, as appropriate. Overall survival was analyzed by log-rank tests. A p value of < 0.05 was considered statistically significant.

Results

Coexistence of cervical intraepithelial neoplasia (CIN) with HPV-16 DNA was detected in three of 30 cases of adenocarcinoma. The results of quantitative PCR analysis for HPV-18, HPV-16 and EBV DNA are shown in Figures 1, 2 and 3, respectively. HPV-16 DNA was detected in 10 cases (33.3%), HPV-18 DNA in 12 cases (40%), and EBV in three cases (10%); there were negative findings in seven cases (23.3%). EBV combined with HPV-16 or HPV-18 was also detected in one case each. Detection of virus DNA was significantly associated with younger age ($p < 0.001$).

When comparing the 20 patients aged ≤ 45 years with the 10 patients aged > 45 years, we found HPV-18 E6 DNA in 45% *vs*. 30% (9/20 *vs*. 3/10), HPV-16 E6 DNA in 40% *vs*. 20% (8/20 *vs*. 2/10), EBV in 10% *vs*. 10% (2/20 *vs*. 1/10), and negative findings of virus DNA in 10% *vs*. 50% (2/20 *vs*. 5/10) (Table). Overall survival of the two groups is shown in Figure 4. Patients aged ≤ 45 years had significantly shorter overall survival than those aged > 45 years ($p = 0.013$). No significant differences between the incidences of HPV-16 and HPV-18 DNA were noted in adenocarcinoma, but only HPV-16 was detected in cases with coexisting CIN. These results suggest that HPV-16 can occur in both

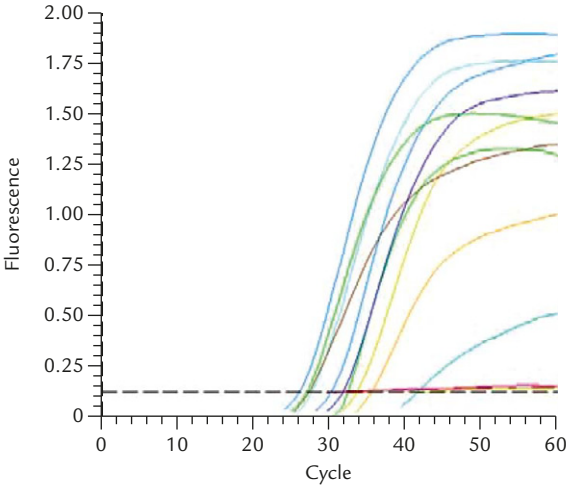

Figure 2. Quantitative polymerase chain reaction for human papillomavirus-16.

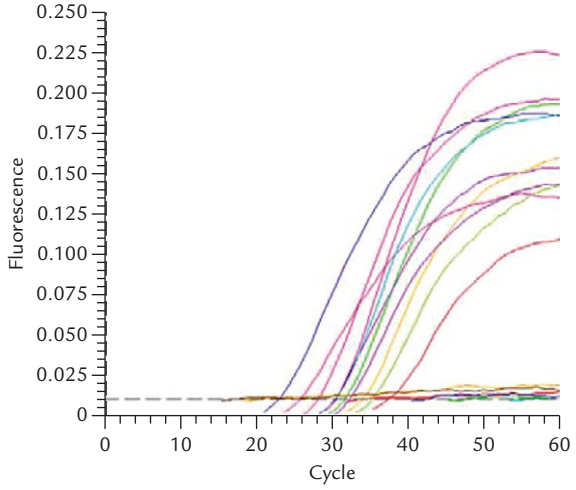

Figure 1. Quantitative polymerase chain reaction for human papillomavirus-18.

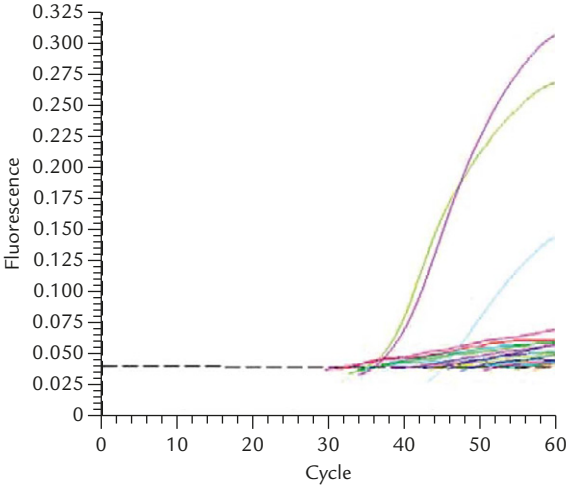

Figure 3. Quantitative polymerase chain reaction for Epstein-Barr virus.

Table. Detection of human papillomavirus (HPV)-16, HPV-18 and Epstein-Barr virus (EBV) in cervical adenocarcinoma in relation to age*

	Patients ≤ 45 yr (n = 20)	Patients > 45 yr (n = 10)	p
Age, median (range) (yr)	40.6 (32–44)	47 (47–69)	
BMI			0.89
< 18.5	2 (10)	1 (10)	
18.5–24.9	16 (80)	7 (70)	
≥ 25	2 (10)	2 (20)	
Smoking habit			0.66
Non-smoker	14 (70)	6 (60)	
Ex-smoker	4 (20)	2 (20)	
Smoker	2 (10)	2 (20)	
Virus DNA			< 0.001
Positive			
HPV-16	8 (40)	1 (10)	
HPV-18	8 (40)	3 (30)	
EBV	1 (5)	0	
HPV-16 + EBV	0	1 (10)	
HPV-18 + EBV	1 (5)	0	
Negative	2 (10)	5 (50)	

**Data are presented as n (%). BMI = body mass index.*

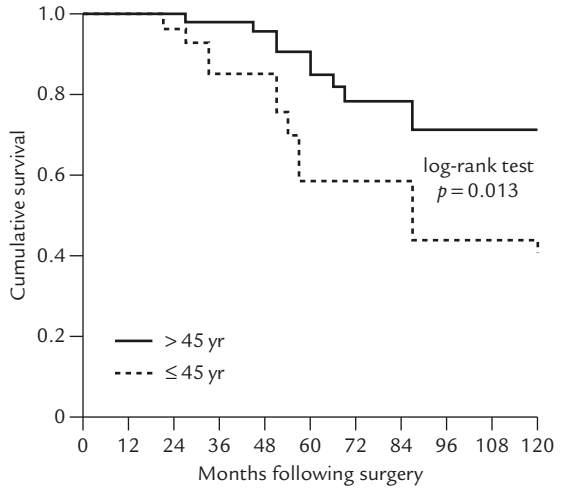

Figure 4. Overall survival.

CIN and adenocarcinoma, while HPV-18 occurs predominantly in adenocarcinoma and mainly in younger women. There was no significant link between the presence of EBV DNA and adenocarcinoma or HPV infection.

Discussion

The incidence of squamous cell carcinoma has decreased over the last few decades, while the incidence of cervical adenocarcinoma has increased in the United States since the early 1970s, especially in younger women [16,17]. This observation may be explained by changes in sexual behavior, HPV infection or an insufficient ability to detect a substantial proportion of adenocarcinoma precursor lesions in current screening practices [10].

HPV DNA, mainly HPV-16, has been detected in squamous cell carcinoma of the uterine cervix and its precursor lesion, CIN [16]. Coexistence of CIN with adenocarcinoma or the presence of HPV in established human cell lines derived from adenocarcinoma suggests that HPV could play the same etiologic role in adenocarcinoma as in squamous cell carcinoma.

HPV types 16 and 18 are those most often related to the development of squamous, adeno- and small cell carcinoma of the cervix and to advanced metastasis of cervical cancer [18]. Schiffman et al [5] reported that adenocarcinoma was more often associated with HPV type 18 than with squamous cell carcinoma. HPV-18 has also been shown to be associated with a poorer prognosis than other HPV types [6,7]. In this study, three of 10 cases with HPV-16 DNA had associated CIN, compared with none of the cases with HPV-18 DNA. These results suggest that HPV-18 is a dependent risk factor for the development of adenocarcinoma, whereas HPV is central to the carcinogenesis of both histologic types of cervical cancer. These distribution results suggest that HPV is central to the carcinogenesis

of adenocarcinoma and is associated with a poorer prognosis ($p = 0.013$) in younger women, compared with older women. Moreover, no viral DNA was detected in five of 10 patients (50%) > 45 years old. This may indicate that other epidemiologic cofactors are related to adenocarcinoma development in older women. However, further studies are needed to confirm the results of this small series.

Although little is known about the molecular genetic events involved in the pathogenesis of cervical adenocarcinoma after HPV infection, it is well established that expression of the high-risk HPV E6 and E7 oncoproteins in keratinocytes (squamous cells) disrupts the function of the cell cycle-regulating proteins p53 and pRB, respectively [19]. It is assumed that the same mechanism of HPV-related carcinogenesis occurs in cervical glandular epithelium.

Furthermore, the presence of EBV in tumor epithelium was detected in only three cases, and was combined with HPV-16 or HPV-18 in two cases. This finding suggests that this agent does not play a role in the pathogenesis of neoplasms of the cervix and that it has no significant relationship with HPV type. Although this study indicates that EBV is unlikely to be a major etiologic factor in the development of cervical adenocarcinoma in this population, it does not necessarily exclude its role elsewhere [20].

In conclusion, our findings confirm that HPV is a central cause of cervical adenocarcinoma and that the same HPV types known to be involved in squamous cell carcinoma are also involved in cervical adenocarcinogenesis. The introduction of HPV testing and Papanicolaou tests to primary screening programs for cervical cancer should improve their efficiency for detecting precancerous glandular lesions, especially in younger women.

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