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Association of EBV and HPV co-infection with the development of cervical cancer in ethnic Uyghur women

A. Abudoukadeer[1], M. Niyazi[2], A. Aikula[3], M. Kamilijiang[3], X. Sulaiman[3], A. Mutailipu[1], A. Abudula[3,4]

[1] Department of Gynecology, First Affiliated Hospital of Xinjiang Medical University, Urumqi
[2] Department of Gynecology, the General Hospital of Xinjiang Uyghur Autonomous Region, Urumqi
[3] Department of Biochemistry and Molecular Biology, Xinjiang Medical University, Urumqi
[4] Key Research Laboratory of Molecular Biology for Endemic Diseases, Xinjiang Medical University, Urumqi (China)

Summary

Objective: Study on the role of Epstein-Barr virus (EBV) and human papillomavirus (HPV) infection in the development of cervical cancer. *Materials and Methods:* We collected 178 cases of cervical tissue specimens of Uyghur women with cervicitis, cervical intraepithelial neoplasia (CIN I, CIN II-III), and cervical squamous cell carcinoma (CSCC). EBV- and HPV-DNA were detected by PCR of tissue DNA. EBV protein expression was checked by immunohistochemistry. *Results:* HPV-DNA was detectable in 2.5, 12.5, 68.0, and 96.4% of cases of cervicitis, CIN I, CIN II- III, and cervical cancer, respectively. For EBV-DNA, these numbers were 0, 3.1, 28.0, and 69.6%. There was a significant difference between the groups of cervicitis, CIN II-III, and cancer with respect to both HPV and EBV positivity rates ($p < 0.05$). Further analysis indicated that cervical lesion pathogenesis was not only accompanied by a gradually increasing rate of HPV or EBV-DNA alone, but also by an increasing rate of HPV-EBV dual infection ($r = 0.46$; $p < 0.01$). EBV protein expression was positive in 89.7% of EBV-DNA positive cases (34/39) and 6% of EBV-DNA negative cases (1/17). *Conclusion:* Cervical cancer development and progression may be closely associated with the dual-infection by HPV and EBV.

Key words: Cervical cancer; Human papillomavirus; Epstein-Barr virus.

Introduction

Cervical cancer continues as a leading female genital cancer, threatening the life of women living in developing countries, where about 80% of all cases occur [1-3]. In China, the highest incidence of cervical cancer has been documented for ethnic Uyghur women in Xinjiang (490~560/100,000), and the mortality is three to four times higher than the average in the country [4-5]. High-risk human papillomavirus (HPV) infection is a necessary but not a sufficient cause of cervical cancer [6-8]. In the present authors' previous study, they detected a high HPV infection rate in Uyghur women with cervical cancer, with HPV-16 as the most common infection type [9].

Nearly all women will become infected with HPV during their lifetime, but only a minority of these infections will progress to invasive cancer. The existence of long latent period of cancer development after HPV infection suggests the involvement of other etiologies in this malignancy process. Results of previous studies suggested [10-14] that Epstein-Barr virus (EBV) could be a co-factor in HPV associated carcinogenesis and that EBV itself may participate in cervical carcinogenesis. These findings are in contradiction to what has been reported by others [15-18]. The role of EBV in the cervix carcinoma thus remains a topic of great debate. The contribution of EBV to the development of malignancies could not yet been defined clearly. Despite the fact that the implication of HPV in carcinogenesis and prognosis of cervical cancer is well established, the impact of a co-infection with high risk HPV and EBV is still not fully understood. Therefore, the main aim of this study was to investigate the prevalence of EBV infection and HPV-EBV dual infections in cervical tissue samples from Uyghur patients with different cervical lesions such as cervicitis, cervical intraepithelial neoplasia (CIN), and cervical cancer, as well as to evaluate its association with the development of cervical cancer and precursor lesions at the high-risk population.

Materials and Methods

Cervical tissue specimen

A total of 178 formalin-fixed paraffin-embedded (FFPE) cervical tissue specimens from Uyghur patients who had been diagnosed or hospitalized at the Department of Gynecology of First Affiliated Hospital of Xinjiang Medical University between January 2003 and December 2007 were analyzed. Cervical tissue specimens derived from punch biopsies, loop electrosurgical excisions, cone biopsies, and hysterectomies. The pathology slides were reviewed and original histological diagnoses of samples were confirmed by experienced pathologists. The diagnoses were as follows: non-neoplastic cervix (cervicitis), n = 40; CIN, n = 82 (CIN I, n = 32; CIN II-III, n =

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50); and squamous cell carcinoma (SCC), n = 56. Patient's age ranged from 30 to 60 years, with a mean of 48 years. Cervical tissue samples were taken on the date of diagnosis and before initial treatment. All biopsy results were reviewed by two pathologists. Ethical approval for use of all specimens was obtained from the research ethics committee of the First Affiliated Hospital of Xinjiang Medical University.

DNA extraction

The paraffin-embedded tissue sections were deparaffinized with xylene, rehydrated by decreasing concentrations of ethanol and double distilled water (ddH2O), followed by digestion with 100 mg/ml proteinase K. The genomic DNA was extracted by the standard phenol-chloroform (1:1) extraction and ethanol precipitation. Purified DNA was then quantified using a spectrophotometer and stored at -20ºC until further use.

Screening HPV and EBV-DNA in tissue specimens

To screen HPV positive samples, the genomic DNA was analyzed with HPV specific general primer pairs MY09/11 (MY09: 5′-CGTCCMARRGGAWA CTGATC-3′; MY11: 5′- GCMCAGGGWCATAAYAATGG-3′, R=A+G, W=A+T, Y=C+T, product length 452 bp) by PCR amplification. Standard PCR was carried out in a total volume of 25 μL using a Taq DNA polymerase, with the following conditions: 94° C for three minutes, followed by 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 60 seconds, and a final extension at 72°C for ten minutes. Omission of the DNA template occurred in negative controls. To screen EBV-DNA positive samples, the genomic DNA was analyzed with specific EBV DNA PCR primer pairs (5'-CCAGACAGCAGCCAATGTC-3' and 5'-GGTAGAAGACCCCC TCTYAC-3') under the same PCR conditions to amplify a 129 bp fragment. Detection of amplification products occurred by electrophoresis on 2% agarose gel labeled with ethidium bromide and ultraviolet visualization.

Immunohistochemical detection of EBV expression

Immunohistochemical (IHC) staining was performed using a primary antibody recognizing the target protein and an IHC kit containing the biotin-labeled secondary antibody. Briefly, four to five μm-thick sections were cut from the paraffin-embedded tissue blocks. After being dewaxed in xylene and rehydrated in alcohol and distilled water, antigen was retrieved by heating in the microwave oven for 15 minutes at 95°C in EDTA buffer (pH 8.0). After cooling and rinsing in distilled water, endogenous peroxidase activity was blocked by incubating sections for 15 minutes in 3% H_2O_2, followed by rinsing in 0.01 M PBS (pH 7.4) for ten minutes. Samples were preincubated with a protein blocking solution for ten minutes and the sections were incubated at 4°C overnight in a humid chamber with the EBV coat protein LMPl specific antibody. Slides were washed three times in PBS and then incubated with a biotinylated secondary antibody for 15 minutes at room temperature. The reaction products were visualized with diaminobenzidine. PBS was used in place of the primary antibody as a negative control and slides were counterstained with hematoxylin, dehydrated, and evaluated under light microscope. The percentage and intensity of positively stained tumor cells in each lesion was investigated by two pathologists who had no knowledge of the patients' characteristics. A consensus number was reached for each tumor sample between the two investigators. Results were scored on a scale from 0 to 3 by the percentage and intensity of positive cells among tumor cells.

Statistical analysis

All statistical analyses were performed with the SPSS Version 17 software package. All p values were two-sided and the significance level was $p < 0.05$ or $p < 0.01$. Mann-Whitney test were used to test continuous variables for differences in scores of DNA- or protein-based EBV detection by PCR or immunohistochemistry among tumor and normal tissues. Fisher's exact test was used for evaluation of associations with clinical pathological parameters. Linear associations between two continuous variables were quantified by Pearson correlation coefficient.

Table 1. — *Analysis of cervical lesions HPV and EBV-DNA detection.*

Group	N	Virus DNA detection		
		HPV	EBV	HPV-EBV[α]
CV	40	1 (2.5)	0	0
CINI	32	4 (12.)	1 (3.1)	0
CINII-III	50	34 (68.0)	14 (28.0)	8 (16.0)
CSCC	56	54 (96.4)	39 (69.6)	39 (69.6)

[α] = cases of co-infection by HPV and EBV; CV = cervicitis; CIN = cervical intraepithelial neoplasia; CSCC = cervical squamous cell carcinoma. Analysis of the statistical significance among groups by chi-square (χ^2) test: HPV, $\chi^2 = 108.73$, $p < 0.01$; EBV, $\chi^2 = 69.70$, $p < 0.01$; HPV-EBV, $\chi^2 = 82.50$, $p < 0.01$.

Results

In this study, HPV infection was highly associated with cervical cancer development in Uyghur women. Among cervicitis, CIN (CINI, CINII-III) and cervical cancer patients, the overall HPV positive rate detected by MY09/11 was 2.5, 12.5, 68.0, and 96.4%, respectively. HPV infection rate increased along with the increasing severity of cervical histologic lesions, with a clear tendency of cervicitis < CINI < CINII-III < CSCC (Table 1). HPV infection was significantly higher in genomic DNA of CSCC than in either CIN or chronic cervicitis ($p < 0.01$, Table 1). Although HPV infection rate seemed to be higher in CINI than in cervicitis, the difference did not reach statistical significance ($\chi^2 = 2.71$, $p > 0.05$). HPV infection rate gradually increased with cervical disease pathogenesis ($r = 0.764$, $p < 0.01$).

EBV-DNA positivity rate in cervicitis, CIN (CINI, CINII-III) and CSCC patients was 0, 3.1, 28.0, and 69.6%, respectively. EBV infection rate gradually increased with cervical disease pathogenesis ($r = 0.606$, $p < 0.01$). The highest EBV positivity rate was observed in Uyghur patients with CSCC. The detection rate of EBV was higher in CSCC and CIN than in cervicitis. In addition, EBV was not found in any sample of cervicitis. The differences of EBV-DNA infection rates in different pathologic cervical lesions were statistically significant ($p < 0.01$). We found that EBV and HPV infection rate behaved similar: cervicitis < CINI < CINII-III < CSCC (Table 1)

Further analysis indicated that cervical lesion pathogenesis was not only accompanied by a gradually increasing rate of HPV or EBV DNA alone, but that it also correlated positively with the increase of HPV-EBV dual infection ($r = 0.46$; $p < 0.01$) (Table 1).

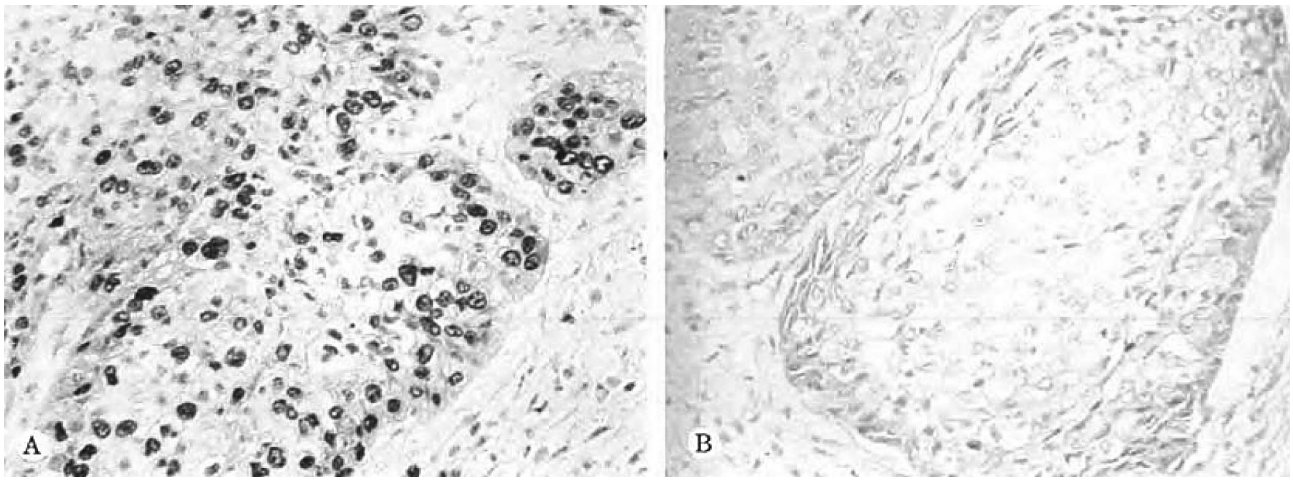

Figure 1. — Detection of viral envelope protein LMP1 coded by EBV in cervical cancer specimens by immunohistochemistry (IHC×400). A: EBV positive cervical cancer tissue. EBV is located in the nucleus of cells. B: EBV negative cervical cancer tissue.

Table 2. — *Comparative analysis of DNA- and protein-based EBV detection by PCR and immunohistochemistry.*

DNA-based EBV detection	All cases	Protein-based EBV detection positive	negative	*p*	κ
Positive	39	35 (89.7%)	4	0.375	0.799
Negative	17	1	16 (94%)		

We analyzed the EBV protein expression on 56 cases of cervical cancer specimens by IHC using an antibody specific to viral envelope protein LMP1, to assess the false positive and false negative rates of DNA-based EBV detection by PCR described above. It was evident that EBV protein expression was located in the nucleus of CSCC cells (Figure 1). EBV protein was detectable in 35 cases among 36 cases positive for EBV DNA (64.3%), whereas only one case was positive for EBV protein in EBV-DNA negative cases (89.7%) (Table 2). If the detection of protein expression by immunohistochemistry is assumed to be the golden standard, the results suggested that EBV-DNA detection by PCR has a certain false positive rate (4/39), and also misses some cases with EBV infection (1/17). Nevertheless, there is no statistically significant difference between the results of the two methods concerning detection of EBV infection ($p > 0.05$), and the coincidence degree of the two methods was very high (k = 0.80).

Discussion

Around the world, infection is one of the most important causes of cancer [19]. It was estimated conservatively that in the year 2002, 18% of all malignancies were attributable to infectious agents [20].

Molecular epidemiologic evidence originating from studies using polymerase chain reaction techniques has firmly established HPV as a causal factor in cervical cancer development [21-23]. Since high-risk HPV is detected in virtually all cases of cervical cancer, the attributable fraction for this cancer is admittedly 100%. HPV-16 and HPV-18 are the most virulent types and account for approximately 70% of cervical neoplasms [24]. Results of our previous studies have also shown that HPV-16 was the most frequent type in Uyghur patients [9].

In this and previous research, we found that HPV infection rate increased along with the increase in severity of cervical lesions, with a clear tendency of cervicitis < CINI < CINII-III < CSCC.

Although HPV infection is assumed to be required for the development of cervical cancer, some investigators reported that human herpes viruses (HHVs) could act as initiators of HPV carcinogenesis [25]. Human herpes virus 4 (HHV-4) or EBV belongs to the genus lymphocryptovirus of the human γ-herpesvirus family and infects more than 90% of the worldwide adult population [26].

Currently, it is known that EBV is associated with a human benign disease, infectious mononucleosis, and with multiple human malignancies, including nasopharyngeal carcinoma, gastric carcinoma, almost half of the cases of Hodgkin's lymphoma and B-cell lymphoma in immunocompromised patients [27, 28].

EBV was found in cervical samples and therefore many investigators have attempted to clarify its role in HPV-associated cervical carcinogenesis [29]. In the present study, EBV-DNA positivity rate in cervicitis, CIN (CINI, CINII-III) and CSCC were 0, 3.1, 28.0, and 69.6%, respectively. EBV infection rate gradually increased with cervical dis-

ease pathogenesis (r=0.606, $p < 0.01$). The highest EBV positivity rate was observed in Uyghur women with CSCC. The analysis showed a clear and statistically significant association between EBV and the development of cervical lesions.

The prevalence of EBV infection in cervical samples has been studied by several researchers: Silver *et al.* [30] described a prevalence of 20%; Szostek *et al.* [31] found a prevalence of 22% in HPV-16 positive women, and Voog [32] found a prevalence of 38% in HIV positive women. The overall prevalence of EBV in CIN and cervical cancer in this study was 30.3% (54/178), in cervical cancer only, the prevalence of EBV was 69.6%, which is significantly higher than that described in literature. The absence of EBV infection in the cervicitis group and the highest EBV infection rate in the cervical cancer group suggest that EBV infection occurs late in cervical oncogenesis. It may be that the EBV is acting as a cofactor of HPV, in induction of uterine cervix pathology; the suggestion was confirmed by studies of Szkaradkiewicz *et al.* [33].

Preliminary presented results of EBV and HR-HPV co-infection in Irish, North American, Thai, and Japanese SCC cases were recently reported [12, 34]. In the present research, EBV plus HPV in the same specimen were identified in 69.6% of cancer, 16.0% of CINII-III, and 0% of non-neoplastic cervix, the differences being significant. This is particularly noteworthy because of recent experimental evidence demonstrating that EBV and HPV can collaborate to increase proliferation of cultured cervical cells [35]. These findings also confirm that the uterine cervix is a habitat for multiple viral and other infections, some of which have oncogenic potential.

In addition the present results of immunohistochemical detection of EBV expression confirm the location of EBV in the nucleus of the malignant cells. EBV protein expression also was consistent with the presence of EBV-DNA in cervical cancer tissue. We showed that the immunohistochemical assay is a sensitive and simple method for detection of EBV infection in cervical cancer and provides similar results as EBV-DNA detection by PCR. Therefore, the joint application of the two methods for detection of EBV infection could reduce the false positive and false negative rate, and thus improve the accuracy of EBV detection.

In conclusion, Uyghur patients with cervical cancer were HPV-EBV co-infected. The highest rate of HPV and EBV co-infection was found in CSCC, a lesser degree of co-infection was observed in pre-cancerous lesions of the cervix. The cervical cancer development in Uyghur women may be associated with HPV/EBV dual infection, whereby EBV infection is incriminated in cervical cancer progression. However the role of dual infection in cervical oncogenesis needs further investigation.

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Address reprint requests to:
A. ABUDULA, M.D.
Key Research Laboratory of Molecular Biology
for Endemic Diseases
2011 Collaborative Innovation Center
Xinjiang Medical University
Xinyi Road 393
830011 Urumqi (China)
e-mail: abulizi_a@126.com