

Epstein-Barr virus detection in invasive and pre-invasive lesions of the uterine cervix

NELLY BEATRIZ MODÓS SANTOS¹, FABIOLA ELIZABETH VILLANOVA²,
PRISCILA MARIA ANDRADE², JULISA RIBALTA¹, JOSÉ FOCCHI¹,
AUDREY YUMI OTSUKA² and ISMAEL DALE SILVA³

¹Colposcopy Section, Gynecology Department, Federal University of São Paulo,

²Urology Laboratory-LIM55, University of São Paulo, ³Molecular
Gynecology Laboratory, Federal University of São Paulo, Brazil

Received April 14, 2008; Accepted August 12, 2008

DOI: 10.3892/or_00000236

Abstract. In the present study, our aim was to investigate whether EBV DNA could be found in association with invasive and pre-invasive cervical cancer lesions. We hypothesize that EBV is not merely a commensal agent when present in malignant cervical lesions. DNA was extracted from cervical scrapings followed by nested PCR-based amplification. The patients were 66 women with high grade cervical intraepithelial neoplasia and 14 women with invasive cervical cancer. The control group consisted of 89 women with a normal Pap smear and colposcopy as well as a negative HPV DNA test. Analysis of our results, in conjunction with the work of other authors, leads us to propose that EBV is not merely a commensal agent when present in malignant cervical lesions. The presence of DNA from EBV is significantly associated with cervical cancer.

Introduction

Epstein-Barr virus (EBV) is a herpes virus that is one of the causative agents of mononucleosis (1-3). EBV is etiologically associated with two human tumors: Burkitt's lymphoma and nasopharyngeal carcinoma (4-6). More recently, there have been scattered reports linking EBV with conventional epithelial cancers of other primary sites including breast (7,8), lung (9,10), and gastric carcinomas (11,12).

With regards to cervical cancer, the role of EBV remains controversial. Sixbey *et al* (13) have demonstrated that uterine cervical cells can be infected by EBV, following the findings of Landers *et al* (14), who found EBV in 43% of

cervical cancer tissues, 8% of cervical intraepithelial neoplasias, and 0% of normal cervical tissues.

Se Thoe *et al* (15) were able to identify EBV in 63% of malignant cervical tissues, and more recently, Sasagawa *et al* (16) showed EBV in 55% of cervical cancers and in 26% of normal cervix tissues.

In contrast, some authors have been unable to identify EBV in biological samples from cervical cancer patients (17-21). Therefore, due to discrepancies in the literature, we undertook a systematic molecular study of a series of tissue samples from normal cervix, high-grade cervical intraepithelial neoplasias, and invasive cervical cancer using a nested polymerase chain reaction (PCR) approach in order to investigate the presence of EBV in cervical lesions.

Materials and methods

Specimens. Cervical-vaginal scrapings were collected using cervical brushes at the Colposcopy Section, Gynecology Department, Federal University of São Paulo-Escola Paulista de Medicina, São Paulo, Brazil, according to a protocol approved by the Human Investigations Committee. The patients were 66 women with high grade cervical intraepithelial neoplasias and 14 women with invasive cervical cancer. The control group consisted of 89 women with a normal Pap smear and colposcopy as well as a negative HPV DNA test [(PCR using MY9 and MY11 primers, Bernard *et al* (22))].

DNA extraction and nested PCR amplification: Cervical cells were collected and the cervical brushes used were placed in a tube containing TE buffer prior to DNA extraction and preserved at -80°C. DNA isolation was conducted using DNAzol (Life Technologies) according to the manufacturer's instructions. Nested PCR was performed using the kit, Master Mix for PCR (Eppendorf), under the following conditions: 5 min at 94°C as the initial denaturation step, followed by 30 sec at 94°C, 30 sec at 50°C, and 30 sec at 72°C for 30 cycles in a GeneAmp PCR System 9700 (Applied Biosystems) along with the following outer primers: 5'-GAT TCA GGC GTG GCT CTT GG-3' and 5'-GAG GAG GAA GAC GAC AAG AGT GG-3' (EBNA 3B GenBank M34440). A final

Correspondence to: Dr Priscila Maria Andrade, Urology Laboratory-LIM55, Urology Department, University of São Paulo, Av. Dr. Arnaldo 455, 2° andar-sala 2141, São Paulo, SP, Brazil
E-mail: pmborra@gmail.com

Key words: Epstein-Barr virus, cervical cancer, HPV

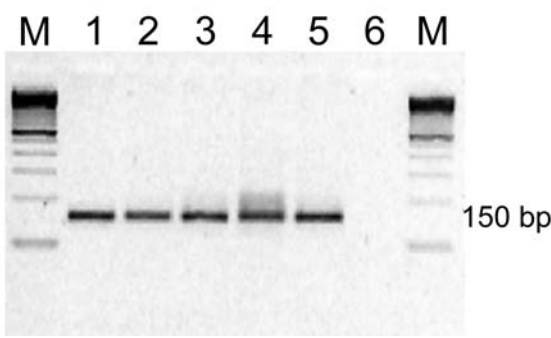


Figure 1. Agarose/ethidium bromide gel (2%) showing positivity for EBV DNA in cervical scrapings. Lanes 1-5, EBV positive cases (150 bp); lane 6, negative control and M, 100 bp DNA marker.

extension step of 72°C for 7 min was also included. Two microliters of the PCR product from the outer primer reaction were removed and transferred to a new 200 μ l thin-wall tube containing fresh reagents and 20 pmol of inner primers (5'-CAC TGC CGT ACA ATC CAA CA-3' and 5'-TGT TCT GGC TGC CTT CTT CT-3'; size 150 bp) (EBNA 3B GenBank M34440) and subjected to a further 30 cycles using the same conditions described above. The PCR products were separated by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR was also performed to amplify a β -globin gene as a control to monitor the ability to amplify a single gene copy (Fig. 1).

Cloning and DNA sequencing. In order to confirm the results of PCR amplification, we cloned the amplified fragment using the TA cloning kit (Invitrogen, San Diego, CA). The isolated fragment was sequenced using an automated DNA sequencer (Perkin Elmer ABI Prism 377) (Fig. 2).

Results

Among the 89 women from the control group, only eight patients (8.99%) tested positive for EBV DNA. Among patients with high-grade lesions (66 cases) and cervical cancer patients (14 cases), we identified EBV DNA in 21.21 and 64.29% of the cases, respectively (Fig. 3). The differences between the cancer group and the other two groups were statically significant; however, despite the apparent large difference between the normal and high-grade lesion groups,

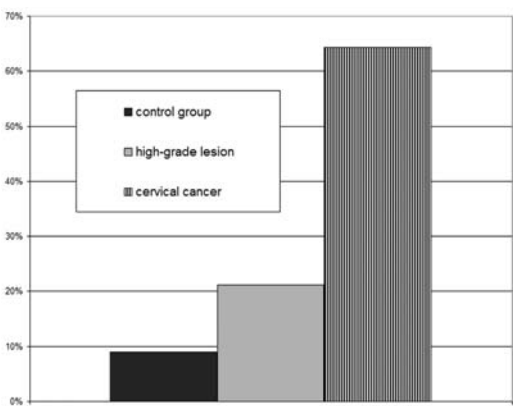


Figure 3. Percentage of patients with EBV DNA in control, high grade, and cervical cancer groups.

the difference between these groups was not statistically significant.

Discussion

EBV is an etiologic agent in infectious mononucleosis and is also implicated in some malignant tumors, such as lymphomas and lymphoepithelioma-like carcinomas. However, there have been some reports linking EBV with other types of cancer such as breast, stomach, and cervical cancers, based on controversial studies. According to some authors, there is no convincing evidence to suggest that other types of tumors are EBV-associated (23-25).

In the present study, we carried out a study using a nested PCR of fresh vaginal scrapings of patients included in a prospective study that incorporated 66 women with high grade cervical intraepithelial neoplasia, 14 with invasive cervical cancer, and 89 with a normal Pap smear, normal colposcopy, and a negative HPV DNA test (PCR using MY9 and MY11) as control group.

Our findings of EBV DNA in a high proportion of cervical carcinomas and also in premalignant epithelial proliferations suggest that the relationship between EBV and cervical carcinogenesis appears to be important, at least in a preliminary study. Our findings are in agreement with other authors such as Se Thoe *et al* (15), Landers *et al* (14), and more recently, Sasagawa *et al* (16), who showed similar results.

These data, combined with the fact that the carcinomas studied herein are exceedingly frequent, suggest that EBV,

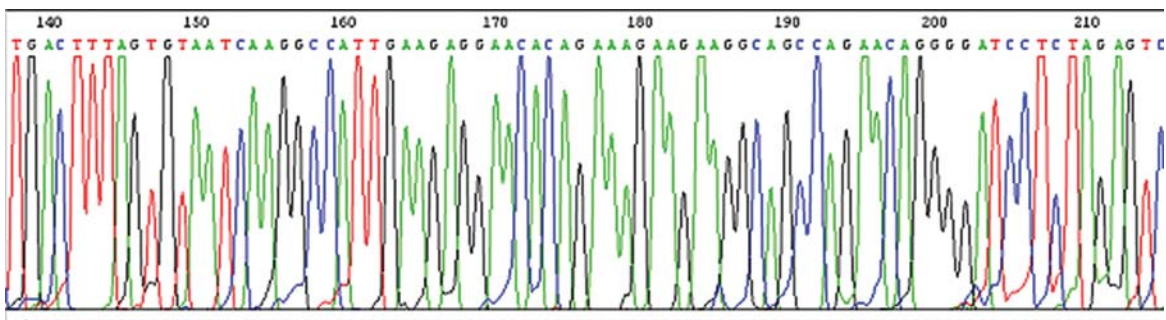



Figure 2. Electropherogram obtained after sequencing analysis of EBV PCR products.

 SPANDIDOS an being associated with restricted groups of malignancies, may play a wider role in human epithelial carcinogenesis.

We also would like to address the strengths and weaknesses of our study. First, in the present article we did not demonstrate the presence of virus inside malignant or premalignant cells by an *in situ* viral detection assay. Therefore, we cannot guarantee that our results represent the detection of viral particles in the nuclei of squamous cells.

However, Sasagawa *et al* (16) have shown by EBV RNA *in situ* hybridization analysis that EBER-1 was expressed in half (7/14) of their invasive squamous cell cervical cancer (ICC) cases and 35% (6/17) of cervical intraepithelial neoplasias (CINs). In addition, the same authors showed that *BamH*-W, which is a leader sequence of EBNA genes, was expressed in 86% (12/14) of ICCs and 71% (12/17) of CINs. LMP-1 and EBNA-2 proteins were also detected in ICC and CIN cells by immunofluorescence staining.

By analyzing our results together with other authors, we propose that the significance of EBV in cervical malignant proliferations it is not that of a simple commensal agent.

Acknowledgements

Supported by grant FAPESP/Ludwig 99/03366-6.

References

1. Macsween KF and Crawford DH: Epstein-Barr virus - recent advances. *Lancet Infect Dis* 3: 131-140, 2003.
2. Koffa M, Koumantakis E, Ergazaki M, Tsatsanis C and Spandidos DA: Association of herpesvirus infection with the development of genital cancer. *Int J Cancer* 63: 58-62, 1995.
3. Kurth J, Spieker T, Wustrow J, Strickler GJ, Hansmann LM, Rajewsky K and Küppers R: EBV-infected B cells in infectious mononucleosis: viral strategies for spreading in the B cell compartment and establishing latency. *Immunity* 13: 485-495, 2000.
4. Spano JP, Busson P, Atlan D, Bourhis J, Pignon JP, Esteban C and Armand JP: Nasopharyngeal carcinomas: an update. *Eur J Cancer* 39: 2121-2135, 2003.
5. Chan AT, Teo PM and Johnson PJ: Nasopharyngeal carcinoma. *Ann Oncol* 13: 1007-1015, 2002.
6. Niedobitek G, Meru N and Delecluse HJ: Epstein-Barr virus infection and human malignancies. *Int J Exp Pathol* 82: 149-170, 2001.
7. Bonnet M, Guinebretiere JM, Kremmer E, Grunewald V, Benhamou E, Contesso G and Joab I: Detection of Epstein-Barr virus in invasive breast cancers. *J Natl Cancer Inst* 91: 1376-1378, 1999.
8. Fina F, Romain S, Ouafik L, Palmari J, Ben Ayed F, Benharkat S, Bonnier P, *et al*: Frequency and genome load of Epstein-Barr virus in 509 breast cancers from different geographical areas. *Br J Cancer* 84: 783-790, 2001.
9. Grinstein S, Preciado MV, Gattuso P, Chabay PA, Warren WH, De Matteo E and Gould VE: Demonstration of Epstein-Barr virus in carcinomas of various sites. *Cancer Res* 62: 4876-4878, 2002.
10. Oda K, Koda K, Takiguchi N, Nunomura M, Seike K and Miyazaki M: Detection of Epstein-Barr virus in gastric carcinoma cells and surrounding lymphocytes. *Gastric Cancer* 6: 173-178, 2003.
11. Hsu JL and Glaser SL: Epstein-Barr virus-associated malignancies: epidemiologic patterns and etiologic implications. *Crit Rev Oncol Hematol* 34: 27-53, 2000.
12. Han AJ, Xiong M and Zong YS: Association of Epstein-Barr virus with lymphoepithelioma-like carcinoma of the lung in southern China. *Am J Clin Pathol* 114: 220-226, 2000.
13. Sixbey JW, Lemon SM and Pagano JS: A second site for Epstein-Barr virus shedding: the uterine cervix. *Lancet* 2: 1122-1124, 1986.
14. Landers RJ, O'Leary JJ, Crowley M, Healy I, Annis P, Burke L, O'Brien D, *et al*: Epstein-Barr virus in normal, pre-malignant, and malignant lesions of the uterine cervix. *J Clin Pathol* 46: 931-935, 1993.
15. Se Thoe SY, Wong KK, Pathmanathan R, Sam CK, Cheng HM and Prasad U: Elevated secretory IgA antibodies to Epstein-Barr virus (EBV) and presence of EBV DNA and EBV receptors in patients with cervical carcinoma. *Gynecol Oncol* 50: 168-172, 1999.
16. Sasagawa T, Shimakage M, Nakamura M, Sakaike J, Ishikawa H and Inoue M: Epstein-Barr virus (EBV) genes expression in cervical intraepithelial neoplasia and invasive cervical cancer: a comparative study with human papillomavirus (HPV) infection. *Hum Pathol* 31: 318-326, 2000.
17. Lanham S, Herbert A, Basarab A and Watt P: Detection of cervical infections in colposcopy clinic patients. *J Clin Microbiol* 39: 2946-2950, 2001.
18. O'Leary JJ, Landers RJ, Crowley M, Healy I, Kealy WF, Hogan J, Cullinane C, *et al*: Genotypic mapping of HPV and assessment of EBV prevalence in endocervical lesions. *J Clin Pathol* 50: 904-910, 1997.
19. Shoji Y, Saegusa M, Takano Y, Hashimura M and Okayasu I: Detection of the Epstein-Barr virus genome in cervical neoplasia is closely related to the degree of infiltrating lymphoid cells: a polymerase chain reaction and *in situ* hybridization approach. *Pathol Int* 47: 507-511, 1997.
20. Dillner J, Lenner P, Lehtinen M, Eklund C, Heino P, Wiklund F, Hallmans G, *et al*: A population-based seroepidemiological study of cervical cancer. *Cancer Res* 54: 134-141, 1994.
21. Hilton DA, Brown LJ, Pringle JH and Nandha H: Absence of Epstein-Barr virus in carcinoma of the cervix. *Cancer* 72: 1946-1948, 1993.
22. Bernard HU, Chan SY, Manos MM, Ong CK, Villa LL, Delius H, Peyton CL, *et al*: Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. *J Infect Dis* 170: 1077-1085, 1994.
23. Herrmann K and Niedobitek G: Epstein-Barr virus-associated carcinomas: facts and fiction. *J Pathol* 199: 140-145, 2003.
24. Speck P, Callen DF and Longnecker R: Absence of the Epstein-Barr virus genome in breast cancer-derived cell lines. *J Natl Cancer Inst* 95: 1253-1254, 2003.
25. Deshpande CG, Badve S, Kidwai N and Longnecker R: Lack of expression of the Epstein-Barr virus (EBV) gene products, EBERs, EBNA1, LMP1, and LMP2A, in breast cancer cells. *Lab Invest* 82: 1193-1199, 2002.