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, 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℃. 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℃ as the initial denaturation step, followed by 30 sec at 94℃, 30 sec at 50℃, and 30 sec at 72℃ 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 AAG AGT GG-3' (EBNA 3B GenBank M34440). A final
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 μl 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, 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,
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References