Human Papillomavirus and Epstein Barr Virus in Prostate Cancer: Koilocytes Indicate Potential Oncogenic Influences of Human Papillomavirus in Prostate Cancer

Noel J. Whitaker,1 Wendy K. Glenn,1 Arisha Sahrudin,1 Matthew M. Orde,2 Warick Delprado,3 and James S. Lawson1*

1School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia
2Department of Forensic Medicine, Sydney South West Area Health Service, University of Sydney, Sydney, Australia
3Douglass, Hanly, Moir – Pathology, Sydney, Australia

INTRODUCTION. The purpose of this study is to determine if high risk human papillomaviruses (HPV) and Epstein Barr virus (EBV) are both present in the same prostate cancer specimens.

METHODS. We used a range of analytical techniques including in situ polymerase chain reaction (IS-PCR) and standard liquid PCR followed by sequencing of the product to seek to identify HPV and EBV in normal, benign, and malignant prostate tissues.

RESULTS. Both HPV type 18 and EBV gene sequences were identified in a high and approximately equal proportion of normal, benign, and prostate cancer specimens. These sequences were located in the nuclei of prostate epithelial cells. HPV associated koilocytes were identified in 24% of prostate cancer specimens.

CONCLUSIONS. The presence of both HPV and EBV gene sequences in most of the same normal, benign, and malignant prostate specimens is particularly noteworthy because of recent experimental evidence demonstrating that EBV and HPV can collaborate to increase proliferation of cultured cervical cells.

Because the presence of EBV and HPV in normal, benign, and malignant prostate tissues appears to be ubiquitous, it is possible that they are harmless. On the other hand HPV type 18 in particular, has high oncogenic potential and may be associated with some prostate cancers. The identification of HPV associated koilocytes in prostate cancer specimens is an indication of HPV infection and potential oncogenic influences of human papillomavirus in prostate cancer. Prostate © 2012 Wiley Periodicals, Inc.

KEY WORDS: prostate cancer; human papillomavirus; Epstein Barr virus; koilocytes

INTRODUCTION

The oncogenic potential of high risk human papillomaviruses (HPV) and Epstein Barr virus (EBV) is well established. In addition, there is recent experimental evidence which has demonstrated that HPV and EBV may enhance the oncogenicity of HPV positive cervical cancer cell cultures [1]. High risk HPVs have been repeatedly identified in both benign and malignant prostate tissues [2]. In contrast, while EBV is a ubiquitous virus with known oncogenic potential, a possible association with prostate cancer has seldom

Abbreviations: HPV, human papillomavirus; EBV, Epstein Barr virus; PCR, polymerase chain reaction.

Conflicts of interest: None.

*Correspondence to: James S. Lawson, School of Biotechnology and Biomolecular Sciences, University of New South Wales, NSW 2052, Australia. E-mail: james.lawson@unsw.edu.au
Received 21 March 2012; Accepted 18 June 2012
DOI 10.1002/pros.22562
Published online in Wiley Online Library (wileyonlinelibrary.com).

© 2012 Wiley Periodicals, Inc.
been investigated: We have identified only three reports in which EBV has been identified in prostate tissues. Grinstein et al. [3] identified EBV in 7 of 18 (37%) prostate adenocarcinomas in US males, Bergh et al. [4] identified EBV in 31 of 352 (8.8%) benign and malignant prostate tissues in Sweden, and Sfanos et al. [5] identified EBV in 16 of 200 (8%) of normal, benign, and malignant prostate tissues also in US males.

The purpose of this study is to determine if high risk HPVs and EBV are both present in the same prostate cancer specimens.

MATERIALS AND METHODS

There is no single method available which can conclusively identify the presence of viral sequences and potential oncogenic influences. For this reason we used several methods, including standard and in situ polymerase chain reaction (IS-PCR) plus the presence of HPV associated koilocytes characteristics in normal, benign, and malignant prostate tissues. When different techniques give consistent outcomes it is reasonable to assume the results are likely to be accurate.

Materials

The investigations were conducted on formalin fixed prostate specimens from the archives of a clinical pathology service and to gain a greater number of normal specimens, formalin fixed prostate specimens were obtained from deceased donors at a forensic pathology service. In addition DNA extracts were obtained from 10 fresh frozen prostate tumours. All the specimens were from Australian males. The identities of the donor patients were “de-identified” and clinical details were not available.

PCR

PCR techniques are extremely valuable for the detection of minute viral loads. However, because of amplification, standard liquid PCR is notoriously subject to contamination from personnel, the laboratory environment, and also positive control materials commonly used in laboratory. In addition, standard PCR cannot confirm that a positive outcome is based on the target cells rather than on infiltrating lymphocytes, leukocytes, and other cells. IS-PCR techniques largely, but not completely, overcome these problems. IS-PCR uses specific primers and dNTPs mix with DIG-labelled dUTP which is incorporated into the amplified DNA using the target viral gene as the template, within the nucleus of the cells. The DIG-dUTP labelled amplicons are detected with a colour reaction using AP-conjugated anti-DIG antibody. As the tissues are mounted on glass slides and kept intact, this allows the identification of specific cell types and the location of the viral sequences. Unfortunately IS-PCR is subject to both false positive and negative outcomes despite the careful use of controls [6]. The most common problem is false positive outcomes which are probably due to false priming by the nicked/denatured genomic DNA fragments. For this reason all specimens were screened by IS-PCR with the primers omitted. Specimens with false positive outcomes were then eliminated from further analyses. This procedure also eliminates true positives and therefore the outcomes do not give precise indications of prevalence of viral sequences in any series of specimens. The outcomes of IS-PCR can be confirmed by standard PCR followed by sequencing of the product.

Genomic DNA Preparation

Previously described protocols were used to extract genomic DNA from fresh frozen prostate cancer specimens [7]. The DNA quality was tested by the amplification of a 268-bp fragment of the β-globin gene using HotStarTaq DNA polymerase (Qiagen, Dusseldorf, Germany) with primers G073 (5'-GAAGAGCCAAGGACAGGTAC-3') and G074 (5'-CAACCTTCATCCACGTTACC-3'). The cycling conditions were 95°C for 9 min; followed by 35 cycles of 95°C for 30 sec; 55°C for 30 sec; 72°C for 1 min; and a final extension at 72°C for 10 min.

Screening for HPV Sequences

Standard PCR was carried out in a total volume of 50 µl using HotStarTaq DNA polymerase (Qiagen) with the following conditions: 95°C for 9 min; followed by 35 cycles of 95°C for 30 sec; 55°C for 40 sec; 72°C for 40 sec; and a final extension at 72°C for 10 min. The DNA from 10 fresh frozen prostate cancer specimens was screened using nested PCR with the MY/GP HPV consensus primers as described by Kroupis et al. [8], with omission of the DNA template for use as a negative control. PCR with the MY primers (MY11 5'-GCMAAGGGWCATAAYAATGG, MY09 5'-CGTCMMARRGGGWACTGAC) amplified a 452 bp (HPV-16) or 455 pb (HPV-18) fragment and the nested GP (GP5 5'-TTTGTTACTGTGACACGAC-3', GP6 5'-GAAAAATAAACTGTGGTGTAAC-3') primers amplified a 142 bp (HPV-16) or 155 bp (HPV-18) fragment of the L1 region of the HPV genome. The amplified products were sequenced to confirm the type of HPV. The PCR was independently repeated for each sample.

The Prostate
PCR Screening for EBV Sequences

The presence of EBV in DNA extracted from the fresh frozen prostate cancer specimens was determined using nested PCR primer sets described by Cinque et al. [9]. The first round of PCR with EB3-4 primers (EB3 5'-AAGGAGGGTGGTTTGAAAG, EB4 5'-AGCAATGGACTCCCTTAGC) amplified a 297 bp fragment and the nested EB1-2 primer pair (EB1 5'-ATCGTGGTCAAGGAGGTTCC, EB2 5'-ACT-CAATGGTGTAAGACGAC) amplified a 209 bp fragment of the EBNA-1 gene. The cycling conditions were 95 °C, 3 min; followed by 35 cycles of 95 °C, 30 sec; 55 °C, 30 sec; 72 °C, 30 sec; and a final extension at 72 °C, 5 min. Genomic DNA isolated from Raji (human lymphoma, EBV positive) was used as the positive control; negative controls were PCR with the omission of DNA template as well as genomic DNA from MDA-MB-453, an EBV negative breast cancer cell line. Nested PCR was independently repeated for each sample. The amplified products were visualized on 1.5% agarose gels. Randomly selected PCR products from the prostate tissue samples, were purified and sequenced with the identity of the sequences determined using the BLAST alignment system.

IS-PCR

In principle, the methods used for IS-PCR are the same as for standard PCR. The main difference is the use of fixed tissue sections which are placed on glass slides and the PCR is conducted with these tissues in situ. Four-micron thick sections of the prostate tissues were cut and placed onto silanized slides for IS-PCR. Positive controls (virus-positive tissue), and negative controls were PCR with the omission of DNA template as well as genomic DNA from MDA-MB-453, an EBV negative breast cancer cell line. Nested PCR was independently repeated for each sample. The amplified products were visualized on 1.5% agarose gels. Randomly selected PCR products from the prostate tissue samples, were purified and sequenced with the identity of the sequences determined using the BLAST alignment system.

Koilocytes

HPV associated koilocytes are associated with the activity of the high and low risk HPV E5 and E6 proteins and are recognised by their histological characteristics [10]. These characteristics are (i) large cells with cytoplasmatic vacuolisation—perinuclear halos surrounding cell nuclei and (ii) nuclear pyknosis—darkly stained, irregular sized, and shaped nuclei. In simple terms—large cells with clearing of the cytoplasm and associated nuclear atypia. The specimens were initially screened for the presence of koilocytes using light microscopy by co-authors J.L. and W.G. The representative example of koilocytes shown in Figure 4 was confirmed by an experienced pathologist co-author W.D. The presence of HPV sequences in these koilocytes was assessed by IS-PCR using the methods outlined above. The diagnosis of koilocytosis is subjective and, for this reason, prevalence estimates of koilocytosis are not reliable. The presence of HPV associated koilocytosis, however, is an indication of ongoing oncogenic processes [11].

Statistics

The chi-square test for categorical data was used to compare the proportions of normal, benign, and cancer prostate specimens according to whether EBV only, HPV only or both EBV and HPV were identified in the same specimen.

Ethics

This project has formal ethics approval by the University of New South Wales Human Research Ethics Committee—number HREC HC11421. De-identified archival specimens were used in this study.

RESULTS

There was little autolysis in the deceased donor specimens and the outcomes were similar to the clinical specimens. For this reason the data are presented in a single Table (Table I).

EBV only, was identified in 40% of cancer, 20% of benign, and 40% of normal fixed archival prostate tissue.

<table>
<thead>
<tr>
<th>Fixed prostate specimens</th>
<th>EBV only (n = 10)</th>
<th>HPV only (n = 10)</th>
<th>EBV plus HPV in same specimen (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>4/10 (40%)</td>
<td>7/10 (70%)</td>
<td>22/40 (55%)</td>
</tr>
<tr>
<td>Benign</td>
<td>2/10 (20%)</td>
<td>2/10 (20%)</td>
<td>6/40 (15%)</td>
</tr>
<tr>
<td>Normal</td>
<td>4/10 (40%)</td>
<td>1/10 (10%)</td>
<td>12/40 (30%)</td>
</tr>
</tbody>
</table>
specimens (these differences are not significant $P = 0.670$).

HPV only, was identified in 70% of cancer, 20% of benign, and 10% of normal fixed archival prostate specimens (these differences are significant $P = 0.045$).

EBV plus HPV in the same specimen was identified in 55% of cancer, 15% of benign, and 30% of normal fixed archival prostate specimens (these differences are significant $P = 0.007$).

EBV was identified in three (30%), HPV in three (30%) of the DNA extracts from the 10 fresh frozen prostate adenocarcinoma prostate specimens. The identification of EBV and HPV in the fresh specimens was confirmed by sequencing of the PCR product as shown in Figures 1 and 2 of selected samples of DNA extracts from the fresh frozen specimens. There were variations in the sequences (shown in red print in Figs. 1 and 2) which indicate there has probably not been contamination. Only HPV type 18 was identified.

**RAJI**

CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 021198
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 0333247
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 021488
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 021198
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 0333247
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 021488
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 041545
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 042096
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 0333247
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT
Specimen 041545
CTTCTGGCTAGGTGTCACAAATTTGGAGCTACCCGTAAGGCAATGGGGTCGCCGGTGCT

**Fig. 1.** HPV type 18 positive gene sequences identified in three selected fresh prostate cancers (compared to reference AY262282).

Both EBV and HPV sequences were co-located in the nuclei of most normal, benign, and malignant prostate tissues as shown in Figure 3.

Koilocytes were identified in 5 (19%) of 27 normal prostate specimens, 7 (30%) of 23 benign prostate specimens, and 12 (24%) of 50 prostate cancer specimens (example shown in Fig. 4). The presence of HPV sequences was confirmed by IS-PCR in 4 of 10 koilocyte containing specimens.

**DISCUSSION**

Both HPV and EBV gene sequences were identified in a very high proportion of normal, benign, and prostate cancer specimens. These sequences were located in the nuclei of prostate epithelial cells.

These outcomes are likely to be correct because of the use of a number of different analytical techniques on a range of specimens. The differences in prevalence of EBV and HPV sequences between the fixed and fresh specimens may reflect the greater sensitivity of IS-PCR technique used on the fixed specimens.

In addition, these observations confirm the frequent identification in many studies of high risk HPV in prostate cancer and the identification in three studies of EBV in prostate cancers [3–5]. The identification of HPV type 18 in these prostate specimens confirms a prior study of prostate cancer among Australian males in which only HPV type 18 was identified [12]. It is of interest that, among Australian HPV positive breast cancers, only HPV type 18 has been identified [13,14]. This is despite the identification of both HPV types 16 and 18 in Australian cervical cancer [15]. HPV type 18 is tropic to glandular as compared to squamous epithelial cells which may in part explain these findings [16]. An additional explanation may be that HPV type 18 is commonly transmitted among sexually active Australians.

The identification by IS-PCR of high risk HPV in the nuclei of koilocytes among normal, benign, and malignant prostate glandular epithelial cells, offers confirmation of HPV E5 and E6 activity and a potential oncogenic role for HPV in these prostate cancers. To the best of our knowledge, this is the first report of HPV associated koilocytes in prostate cancer. The appearance of koilocytes located among glandular epithelial prostate cancer cells, differs from the historical koilocyte patterns seen in squamous cell based cervical intraepithelial neoplasia (CIN) [10]. In CIN, the koilocyes are often present as sheets of cells which become increasingly abnormal toward the basal cell layers of the cervical epithelium [10]. In contrast to this common histological pattern, koilocytes in glandular cells are mostly single cells as shown in Figure 4. This single koilocyte histological pattern has
previously been observed in HPV associated breast cancer which is also based on glandular epithelial cells [17]. It is also known that with respect to lesions of the cervix, HPV associated koilocytosis is a specific and sensitive indicator of potential malignant progression [18].

The presence of both HPV and EBV gene sequences in the same specimens is particularly noteworthy because of recent experimental evidence demonstrating that EBV and HPV can collaborate to increase proliferation of cultured cervical cells [1]. However, these experimental studies were based on squamous cervical cells and any viral interactions may differ in glandular prostate cancer cells.

It is of interest that in this current study, there was an equally high proportion of HPV and EBV gene sequences identified in normal, benign, and prostate cancer specimens. It has long been recognised that EBV is ubiquitous and only rarely is this virus associated with oncogenesis. The reason for EBV to exert its oncogenic influences in a particular patient is unknown but is probably associated with co-factors. The HPV type 18 identified in these series of prostate cancer specimens is known to be very oncogenic in cervical glandular epithelial cells and there is no obvious reason why it should not be oncogenic in the prostate. Again it is possible that HPV exerts its oncogenic influences in concert with co-factors including a possible collaboration with EBV. This possibility is shown in this current study as EBV/HPV (that is both EBV and HPV were both present in the same specimen) positive prostate cancers, were a significantly higher proportion than EBV/HPV benign and normal prostate specimens (see Table I).

These observations confirm the assessment of Zambrano et al. [19] that the prostate is a habitat for multiple viral and other infections, some of which have oncogenic potential. They also confirm the co-

Fig. 3. Prostate intraepithelial neoplasia specimen containing EBV and HPV gene sequences in the nuclei of the same malignant epithelial cells. A: EBV positive, (B) HPV18 positive, (C) negative control.

Fig. 4. HPV associated koilocytes in invasive prostate cancer.
location of EBV and high risk HPV in the nuclei of the same normal, benign, and malignant prostate tissues.

HPV type 18 has high oncogenic potential and may cause some prostate cancers. This oncogenic potential may be enhanced by EBV. The identification of HPV associated koilocytes in prostate cancer specimens is an indication of HPV infection and potential oncogenic influences of human papillomavirus in prostate cancer.

AUTHORS' CONTRIBUTIONS

Noel J. Whitaker—carried out laboratory analyses, developed concepts, participated in writing the manuscript. Wendy K. Glenn—carried out laboratory analyses, developed concepts, participated in writing the manuscript. Arisha Sahrudin—carried out laboratory analyses, participated in writing the manuscript. Matthew M Orde—identified patients and collected specimens, conducted histological assessments, participated in writing the manuscript. James S. Lawson—conceived the study, participated in study design, identified patients and collected specimens, participated in writing the manuscript. James S. Lawson—conceived the study, participated in the study design, identified patients and collected specimens, participated in writing the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support from the Prostate Cancer Foundation of Australia. We are also grateful to Dr. Ronnie Cohen and Dr Bev Shannon for the donation of specimens.

REFERENCES


