

PATHOGENIC CONTRIBUTION OF HUMAN
PAPILLOMAVIRUSES AND EPSTEIN-BARR VIRUS
TO PROSTATE CANCER IN BULGARIAN PATIENTS

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Abstract

We investigate the prevalence and impact of Human Papillomaviruses (HPVs) and Epstein-Barr Virus (EBV) and other human herpesviruses (HHVs) in Formalin-fixed paraffin-embedded (FFPE) prostate cancer (PCa) samples ($n = 49$) and benign prostatic hyperplasia (BPH) controls ($n = 50$) from Bulgarian patients. DNA/RNA extraction, multiplex PCR, Real-time qPCR, ISH were applied.

EBV DNA was detected in 34.7% of PCa samples and 6% of BPH controls ($p = 0.0048$, Fisher's exact test – FET), suggesting a significant association with malignancy. Among EBV-positive PCa cases, 58% represented mono-infection, while 41.2% involved co-infections with other HHVs. The high prevalence of EBV supports its potential role in PCa development via inflammation and immune modulation. The immunological profile based on *IL1 β* , *IL10*, *IL18*, *TNF- α* , *TLR4*, *GATA3*, *CD68*-expression, revealed an inflammatory tumour microenvironment in EBV/HHVs positive PCa. Notably the majority of samples were classified as Gleason grades G3–G5, consistent with tumour aggressiveness and poor prognosis.

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HPV DNA was detected in 6.1% of PCa and 12% of BPH samples, showing no statistically significant difference ($p = 0.327$, FET). The lack of HPV association in PCa/BPH groups suggests a limited role in advanced disease. These results combined with the absence of hrHPV E6/E7 RNA supports the “hit and run” model, in which HPV contributes to early tumour initiation but is often absent in later stages.

Our findings suggest that long-term EBV/HHVs infection may contribute to immune dysregulation in the prostate and may serve as a predictor of aggressive PCa.

Key words: PCa, inflammation, oncogenic, virus

Introduction. Current evidence supports the hypothesis for a pivotal role of chronic viral intraprostatic inflammation provoking virus-mediated genetic alterations, immune dysregulation and the loss of apoptotic control in infected cells [1, 2]. Approximately 20% of human tumours in adults nowadays result from chronic inflammatory conditions, initiated by infectious agents or exposure to harmful environmental factors. Epidemiological and meta-data have confirmed a strong association between sexually transmitted infections including HPVs, polyomaviruses and HHV members with PCa onset and clinical behaviour [3, 4]. Current evidence indicates that nearly all immune cell populations in the human body may be affected by herpesvirus DNA, proteins, microRNAs, and other elements [5]. The described cascade in the case of primary active or secondary reactivated HHVs leads to multifactorial immunosuppression, especially in immunocompromised individuals, such as cancer patients [6]. The persistent latency of human HHVs is frequently associated with immunosenescence [7], a phenomenon that may also critically influence tumourigenesis. The cited high rate of EBV/HHVs infections into malignant prostate gland are explained by the presence of specific receptors for EBV/HHVs entry on its surface and core and by imbalanced androgen homeostasis facilitating the process [8].

Multiple studies report the increasing proportion – 41–63.5% – of hrHPV infected PCa tissues in contrast to significantly lower percentage of hrHPV in the group of BPH and adjacent normal prostate tissues and healthy controls [9]. It is essential to determine the physical state of the HPV genome – whether it is present in an “episomal-transient” state or integrated into the host genome, which is associated with transformation potential. In a study by KHATAMI et al. [10] integrated hrHPV was identified in 50% of PCa samples by hrHPV oncogene E6/E7 RNA testing. Their findings offer compelling insights into the potential role of HPV integration in PCa progression.

These findings led us to investigate the prevalence and potential role of 33 HPV genotypes (6, 11, 16, 18, 26, 31, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 66, 68, 70, 71, 72, 73, 81, 82, and 84), as well as Epstein–Barr virus (EBV) – and other members of the Herpesviridae family as EBV co-infection partners in the pathophysiology of PCa. By analyzing the expression of

IL-1 β , IL-10, IL-18, TNF- α , TLR4, GATA3, and CD68 markers in EBV/HHVs-infected and uninfected FFPE prostate tissues, we report additional evidence for an elevated inflammatory index in virus-positive PCa cases, potentially reflecting an immunocompromised tumour microenvironment.

Materials and methods. Patients and samples. A control group with BPH (50 fresh, unfixed samples) and a study group with histologically confirmed PCa FFPE (49 with tumour-rich areas marked) were selected. All patients signed informed consent for genetic testing.

DNA and RNA extraction. Total DNA from fresh “tru-cut” biopsies was extracted using the AmpliSens DNA isolation kit (Ecoli s.r.o, Slovak Republic). Total DNA from FFPE samples was extracted using the QIAamp DNA FFPE Tissue Kit and total RNA using the RNeasy DSP FFPE Kit. DNA/RNA concentration and purity were measured using a Nanodrop spectrophotometer.

Molecular analysis. The target HHVs viral DNA fragments were amplified using the AmpliSens real-time qPCR amplification kits (Ecoli s.r.o, Bratislava, Slovak Republic). Target HHV genomic regions were commercially designed on replication-essential genes: EBV-LMP (Ct $\leq$ 35), CMV-MIE exon 4 (Ct $\leq$ 36), and HHV6-DNA polymerase subunit (Ct $\leq$ 36). All clinical samples were investigated in triplicate.

RNA expression of IL1 β , IL10, IL18, TNF- α , TLR4, GATA3, and CD68 was analyzed using the SensiFAST cDNA Synthesis Kit (BIOLINE), followed by immunoprofiling and inflammatory index determination with the Immuno-Quantex C/V Real-Time PCR Kit (DNA Technology, Moscow). The inflammatory index was calculated using a proprietary scoring system provided by the assay kit, based on relative expression ratios: TLR4/GATA3, TNF- α /IL-18, IL-10/IL-18, and IL-1 β /CD68. According to the manufacturer’s instructions, an index value $> 50\%$ denotes an elevated inflammatory state. Index values were computed using normalized Δ Ct values and expressed as percentage scores.

FFPE prostate cancer samples were investigated for integrated hrHPV using the 14 High-Risk HPV E6/E7 mRNA Real-Time PCR Kit (Hybribio, Chaozhou, China), following the manufacturer’s instructions.

Alternative “in situ” hybridization method (ISH) by BOND Ready-to-Use ISH kit, (Leisa Biosystems, United Kingdom) on the EBV DNA positive PCa FFPE samples was applied. The EBV encoded small RNAs (EBER) were the target for qualitative identification of latent EBV using the automated BOND system (includes BOND-MAX system, BOND-III system and BOND-PRIME system). Nuclear brown staining within tumour cells is interpreted as positive for EBER expression, confirming EBV latency.

Results. Virological results. EBV detection in PCa FFPE and BPH samples. EBV DNA was detected by real-time PCR in 17 out of 49 (34.7%) FFPE PCa samples, compared to only 3 out of 50 (6%) BPH control samples (see Table 1). This difference was statistically significant, yielding a two-tailed

p -value of 0.0048 by Fisher's exact test, confirming a strong association between EBV infection and malignant prostate tissues. Chi-square analysis produced a χ^2 value of 9.432 with 1 degree of freedom (1 df, $p = 0.0021$), further supporting a significant relationship [11,12]. Approximately 59% of all EBV positive PCa FFPE samples have shown EBV mono-infection but in 41.2% a co-infection condition of EBV with alternative HHVs partners was observed, more precisely EBV/HHV6 (17.6%) and EBV/HHV7 (23.5%) EBER RNA transcripts were found in none of EBV or EBV/HHVs positive PCa FFPE samples by ISH, showing an absence of latent infection (see Fig. 1).

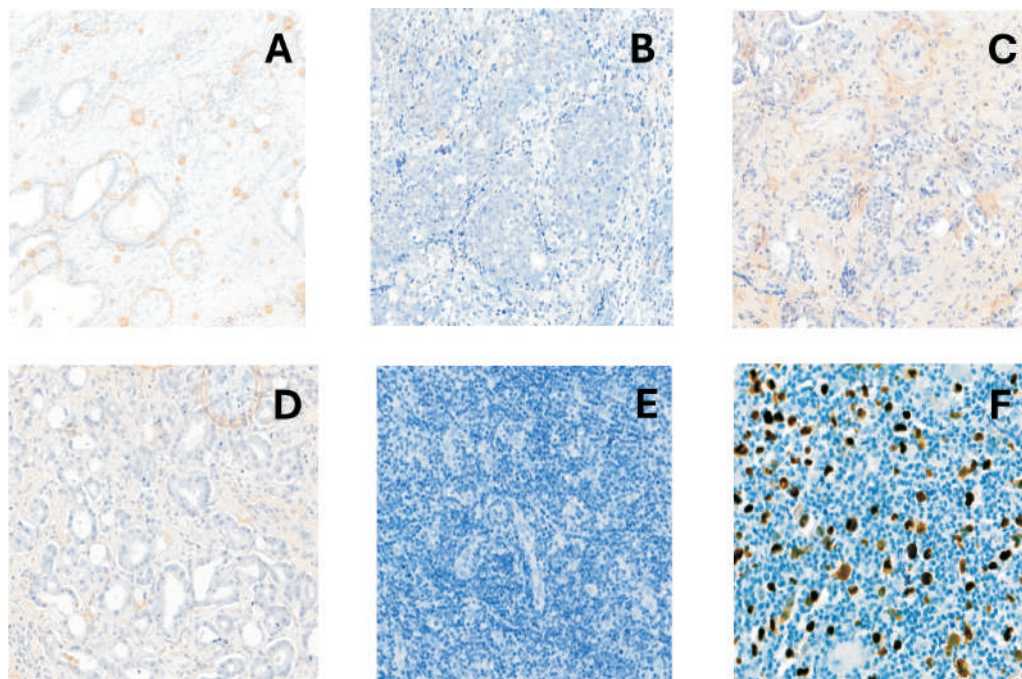


Fig. 1. ISH of EBER transcripts in EBV and EBV/HHVs DNA positive PCa FFPE samples. A) EBER negative moderately-differentiated glandular prostate carcinoma: Gleason score (3+4), G2; micro area of infiltration of the capsule; findings of perineural tumour infiltration; EBV DNA positive; B) EBER negative poorly differentiated acinar adenocarcinoma: Gleason Score (4+5=9), G5; perivascular and perineural infiltration, intratumoural lymphocyte infiltration; EBV/HHV7 DNA positive; C) EBER negative acinar adenocarcinoma in the apex (5% area) limited to the prostate-right lobe: Gleason Score (3 + 3 = 6), G1; BPH in the surrounding parenchyma; pronounced inflammatory reaction; EBV/HHV6 DNA positive; D) EBER negative acinar adenocarcinoma, covering 20% area limited to the prostate-left lobe: Gleason Score (3 + 4 = 7), G2; perineural invasion; BPH in the surrounding parenchyma; pronounced chronic inflammatory reaction; EBV/HHV6 DNA positive; E) EBER negative Hodgkin Lymphoma Mixed Cellularity: negative control; F) EBER positive Hodgkin Lymphoma Mixed Cellularity: positive control

HPV detection in PCa FFPE and BPH samples. HPV DNA was detected in 3 of 49 (6.1%) FFPE PCa samples (Table 1). None contained hr-, ir-,

T a b l e 1

Prevalence of HPV and EBV infections in patients with BPH and PCa

Group	Number	Sample type	HPV +/- (%)	EBV +/- (%)	Group information
Benign prostatic hyperplasia	50	Tru-cut biopsies	6/44 (12%/88%)	3/47 (6%/94%)	Randomly selected biopsies (from core samples)
Prostate cancer	49	FFPE tissue (Tumour-targeted)	3/46 (6.1%/93.9%)	17/32 (34.7%/65.3%)	Areas pre-identified as tumour target zones
Statistical significance	–	–	$p = 0.327$	$p^* = \underline{0.0048}$	–

(*) – Concerning EBV distribution rates in BPH (6%) and PCa (34.7) study groups, a statistically significant difference was found ($p = 0.0048$, Fisher’s exact test and χ^2 test).

or lr-HPV genotypes from the 33-type panel [10], though universal control amplification suggests non-targeted HPV types. In the BPH group, 6 of 50 (12%) were positive exclusively for low-risk HPV genotypes. Statistical analysis using Fisher’s exact test revealed no significant difference in HPV detection rates between PCa and BPH samples ($p = 0.327$). HrHPV onco E6/E7 RNA transcripts were detected in none of the studied PCa cases, regardless of HPV DNA status.

Histopathological results. EBV or EBV/HHVs-positive PCa cases have shown predominantly more unfavourable and aggressive tumour characteristics, including higher Gleason grading (ranging from $4 + 3 = 7$ to $4 + 5 = 9$) G3, 4, 5, etc. (Table 2). In contrast, uninfected FFPE PCa samples were overwhelmingly identified as well-differentiated, likely indolent prostate tumours, G1. In the BPH group some findings of inflammatory, atrophic, cystic alterations were described. Amyloid body formations, partial precancerous condition, and a cytopathic effect were registered but there is no difference between BPH EBV/HHVs/HPV infected and uninfected cases.

Immunological results. An inflammatory index was calculated based on the following cytokine/ILs expression ratios: TLR/GATA3; TNF- α /IL18; IL10/IL18; IL1B/CD68. As an elevated inflammatory index, a value > 50 is defined in the kit with a cut-off of 50%. Approximately 59% (58.8) of EBV positive FFPE exhibited an elevated inflammatory index > 50 . Furthermore, two thirds of these cases demonstrated a markedly high inflammatory index ($> 80\%$), showing robustly inflamed tumour microenvironment. Individual overexpression was observed for IL-1 β , IL-18, and TNF- α markers.

Discussion. EBV is a major oncogenic DNA virus, implicated in $\sim 38\%$ of virus-related cancers, similar to HPVs [13]. In our study, EBV DNA was detected

T a b l e 2

Virological and histopathological data in studied PCa

Study group PCa:	Histopathological characteristics:	Differences between viral infected/uninfected PCa sub-groups:
PCa study group EBV/HHVs infected (FFPE)	• intratumoural lymphocyte infiltration, fibrosis, necrosis • periductal chronic inflammatory reaction/ infiltration • granulomatous lesions with foci of abscesses and necrosis • chronic lymphadenitis • PIA, HGPIN • poorly or moderately differentiated prostatic tumours /G3, G4 and G5 • perineural; perivascular; lymphovascular invasion • seminal vesicle invasion • senile vesicle amyloidosis • lymph node metastasis	+ significant
PCa study group EBV/HHVs uninfected (FFPE)	• well-differentiated prostatic tumours without chronic inflammation and tumour invasion /G1/	

in 34.7% of FFPE PCa samples, a rate consistent with the reported detection range of 5–37% in prostate tumours. In contrast, we observed only 6% EBV positivity in BPH controls, lower than the 8–9% prevalence reported in earlier studies [11]. This difference was statistically significant (Fisher's exact test: $p = 0.0048$; Chi-square: 1 df, $p = 0.0021$; Table 1), supporting a strong association between EBV and malignant prostate tissue.

EBV's oncogenic activity has been previously documented in hyperplastic and dysplastic lesions, including PIN (prostatic intraepithelial neoplasia) and in situ breast carcinoma. Published data indicate that ~37% of PCa patients harbour EBV infection, confirmed via immunohistochemistry [13]. Our data aligns with prior reports showing EBV DNA and protein detection in prostate tumour epithelium [11–13]. Studies confirm EBV positivity across all Gleason grades (G1–G5), from well- to poorly-differentiated tumours, associated with aggressive clinical features. Notably, our study found EBV-positive PCa cases clustered in higher Gleason grades (G3–G5; Table 2), further suggesting a role in disease progression. Similar reports indicate elevated anti-EBV antibodies, viral load, and oncogene/oncomiR expression correlating with EMT and high-grade PCa [14].

To assess whether EBV DNA in PCa FFPE samples reflects direct epithelial infection or lymphocytic infiltration, ISH for EBV-encoded RNA (EBER) was performed. No EBER staining – indicative of latent EBV – was detected in PCR-confirmed EBV-positive PCa cases (Fig. 1). Given the strong RT-PCR signals (Ct 24–29; cutoff ≤ 35), this suggests active EBV infection with moderate to high viral load. This is consistent with literature reporting EBER negativity alongside PCR positivity in active EBV infections lacking latency markers [15]. To pinpoint EBV’s cellular localization, future studies will include immunohistochemical analysis.

Co-infections involving EBV and other herpes viruses have been reported to alter immune responses, enhance oncogenic potential, and complicate treatment strategies. HHV-6 is considered a potential co-factor in cancer development through interactions with EBV and others, although more evidence is needed to clarify its role. Data on HHV-7 remain scarce, and its oncogenic potential is still poorly understood [16]. Based on the observed co-infection rates of EBV/HHV-6 (17.6%) and EBV/HHV-7 (23.5%) in our study, we propose a mechanism involving complex virus-mediated tumourigenesis, where EBV may act synergistically with other herpes viruses to promote aberrant cellular proliferation.

Previous studies report a high frequency of HPV–EBV co-infection in prostate tumours ($\sim 55\%$) compared to healthy prostate tissues ($\sim 30\%$), with evidence suggesting that both viruses may cooperatively promote cell proliferation, particularly in cervical carcinoma models [4]. In contrast, we found no such co-infection in FFPE PCa samples. HPV DNA was absent in our PCa tissues aligning with the idea that HPV may contribute to early oncogenesis. The absence of HPV DNA in FFPE PCa samples suggests a limited role in advanced disease – consistent with prior reports showing low HPV prevalence in late-stage PCa [17]. Substantial evidence supports the hypothesis that HPV’s oncogenic activity is primarily an early event in prostate carcinogenesis [17]. The cited tumourigenic mechanism of HPV, called “hit-and-run” model initiates the oncogenic alterations in PCa, characterized by early integration of the viral genome into infected cells during the initial phases of malignant transformation. In advanced disease stages, HPV viral load often drops below detectable levels [18] – likely explaining the complete absence of the 33 HPV genotypes tested in our FFPE samples, despite detection of untyped HPV (6%). This is consistent with the advanced, high-grade histopathological profile observed in our PCa cohort.

These findings are in contrast with our earlier results from tumour-specific prostatic specimens (urine sediments post-DRE), which showed molecular alterations supporting HPV’s role as an inflammatory co-factor in PCa etiology [19]. This discrepancy likely stems from the non-invasive nature of urine-based PCa screening, which captures early or precancerous disease stages. In our previous study – using tru-cut biopsy samples primarily from early-stage or precancerous cases – we also detected HPV DNA in both BPH and PCa groups (data not

shown). Collectively, these observations support the hypothesis that HPV may initiate oncogenic transformation in prostate tissue, but its presence diminishes to undetectable levels as the disease advances.

Interestingly, no hrHPV E6/E7 RNA transcripts were detected in our PCa cohort, further emphasizing the limited role of active viral transcription in advanced disease stages. This underscores the unclear nature of hrHPV-driven tumourigenesis in prostate cancer and highlights the urgent need for functional studies. A key reason for discrepancies in global data may lie in differences in sample collection timing, potentially missing the critical window during which viral involvement is detectable.

In 59% of EBV-positive PCa samples, the inflammatory index was high (> 50 , often $> 80\%$), driven by elevated IL-1 β , IL-18, and TNF- α – cytokines linked to tumour progression [20]. TNF- α overexpression correlated with lymph metastasis, suggesting EBV may promote inflammation-driven progression in advanced PCa.

Conclusion. Clinically, EBV infection appears to be an adverse prognostic factor in prostate cancer, associated with cytopathic effects, inflammation, higher Gleason grades (G3–G5), and features of poor prognosis and metastatic potential. EBV persistence may contribute to more aggressive disease. In contrast, the role of HPV remains uncertain. Further studies are needed to explore HPV's oncogenic potential across all stages of prostate cancer.

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