Infectious Agents and Cancer

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Highly sensitive deep panel sequencing of 27 HPV genotypes in prostate cancer biopsies results in very low detection rates and indicates that HPV is not a major etiological driver of this malignancy

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Abstract

Background Human papillomavirus (HPV) has been proposed to contribute to the carcinogenesis of prostate cancer. However, previous studies have yielded conflicting results. This study aims to add useful information to the ongoing discussion concerning the association between HPV infection and prostate cancer.

Methods We used two high-throughput next-generation sequencing (NGS) approaches to detect HPV RNA in malignant and adjacent normal (AN) prostate tissue (cohorts 1 and 2) and HPV DNA from carcinogenic and probably/possibly carcinogenic-classified HPV types (cohort 3) in malignant prostate, AN prostate, and benign prostatic hyperplasia (BPH) tissues.

Results In total, 0% (cohort 1: 0/83, cohort 2: 0/16) of the malignant prostate tissue samples and 0% (cohort 1: 0/23, cohort 2: 0/8) of the AN prostate tissue samples were positive for HPV RNA. A total of 8.3% (1/12) of the BPH samples, 0% (0/28) of the AN samples, and 0.8% (1/132) of the malignant prostate samples were positive for HPV16 DNA. However, the normalized read count of the HPV16-positive malignant sample was close to the cut-off. In addition, no other carcinogenic-classified HPV types were detected in any of the BPH, AN, or malignant prostate tissue samples.

Conclusion Our study does not support HPV infection as a major contributor to the etiology of prostate cancer.

Keywords Prostate cancer, HPV, Human papillomavirus, NGS, Next-generation sequencing, RNA-seq

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Background

Prostate cancer (PC) is the second most common cancer among men worldwide, with a global incidence of 1,414,000 and a mortality of 375,304 men in 2020 [1]. Although some risk factors for PC development have been identified, the etiology remains largely unknown [2]. For the past three decades, researchers have suggested and explored the contribution of human papillomavirus (HPV) to prostate carcinogenesis [3–5].

HPV is the etiological cause of almost all cervical cancers [6]. In addition, HPV contributes to the development of other anogenital cancers, including penile, anal, vaginal, and vulvar cancer and a subset of head and neck cancers [7]. In total, HPV is present in up to 4.5% of all cancers worldwide [7], and over 200 different HPV types are known [8]. Only a subset of these viruses can infect mucosal epithelial cells and have been classified by the International Agency for Research on Cancer (IARC) as either carcinogenic to humans (high-risk), probably/possibly carcinogenic to humans, or low-risk [9]. HPV is a double-stranded DNA virus of approximately 8 kb, and the HPV genome comprises six early genes, E1, E2, E4, E5, E6, and E7; two late genes, L1 and L2; and a long control region, LCR. E6 and E7 are particularly important for the carcinogenic potential of HPV. The E6 and E7 oncogenes produce the E6 and E7 proteins, which target the p53 and pRb tumor suppressor proteins for degradation, enabling cellular instability and uncontrolled proliferation [10].

Since HPV is involved in the development of several cancer types, infection with HPV has also been proposed to contribute to the carcinogenesis of PC. In support of this hypothesis, several studies from the 1990s have shown the ability of HPV to immortalize human prostate cells in vitro [3, 11–13]. Furthermore, in recent decades, multiple studies have examined the association between HPV infection and PC. In 2020, Lawson and Glenn published a meta-analysis of 26 case-control studies in which HPV was present in 325 (22.6%) of 1437 PC tissue samples compared with 113 (8.6%) of 1313 normal or benign prostate tissue sample controls; thus concluding that a causal role of HPV in PC is highly likely [14]. However, considerable variation in HPV detection was observed in these 26 studies, ranging from 2 to 75% in PC tissue samples and from 0 to 82% in normal and benign tissue samples. Moreover, eight additional studies mentioned in the meta-analysis revealed no presence of HPV in PC tissue samples [14]. Polymerase chain reaction (PCR) was used in the vast majority of the studies included in the meta-analysis to detect HPV in either formalin-fixed paraffin-embedded (FFPE) or fresh-frozen prostate tissue samples, but few studies also performed in situ PCR and in situ hybridization [15, 16]. Moreover, several studies have examined the presence of HPV IgG antibodies in serum [5, 16–29]. This method is, however, not tissue-specific, and studies using this method were therefore excluded from the abovementioned meta-analysis [14]. In addition to the studies included in the meta-analysis, a few studies have used RNA sequencing to detect HPV expression in PC tissue samples. However, these studies have mainly provided negative results [30–33]. The evidence that HPV infections contribute to the carcinogenesis of PC is thus still not conclusive.

In this study, we used two different approaches for the detection of HPV RNA and HPV DNA in FFPE prostate tissue samples from men with localized PC. We included three independent cohorts of patients and employed a metatranscriptomic approach for the detection of HPV RNA and a custom targeted next-generation sequencing (NGS) panel [34] for the detection of HPV DNA. Notably, an RNA sequencing-based approach enables the identification of actively transcribing HPV and could indicate an active infection. In contrast, our DNA sequencing-based approach enables the identification of all tissue-resident HPV infections. The NGS panel targets eight separate regions within the L1, E2, E6, and E7 genes in each of the 25 HPV types classified by the IARC as carcinogenic, probably, or possibly carcinogenic to humans, as well as the two most common low-risk HPV types, namely, HPV6 and 11 [9], thereby allowing the detection of the 27 most relevant HPV types. Moreover, the panel is designed with primer pairs yielding short fragments of approximately 100 base pairs (bp). Together, these features limit the risk of false negative results, which is crucial, especially when working with partly degraded samples such as FFPE tissue samples. Thus, by using two orthogonal and high-throughput NGS approaches to detect both HPV DNA and RNA, we aim to add useful information to the ongoing discussion concerning the association between HPV infection and PC.

Methods

Study cohort

We used three independent cohorts of PC patients who underwent curatively intended radical prostatectomy (RP). An overview of the study design is provided in Fig. 1.

Cohort 1 (total RNAseq) included 106 fresh-frozen prostate tissue samples (83 malignant and 23 adjacent normal (AN)) from an in-house cohort of 94 patients treated by RP (2004–2017) with histologically verified, clinically localized PC at the Department of Urology, Aarhus University Hospital (AUH), Denmark, previously published by Salachan et al. [35]. Before RNA extraction, each tissue sample was marked as either benign or malignant by an experienced pathologist at AUH. The metatranscriptome was profiled via total RNA sequencing,

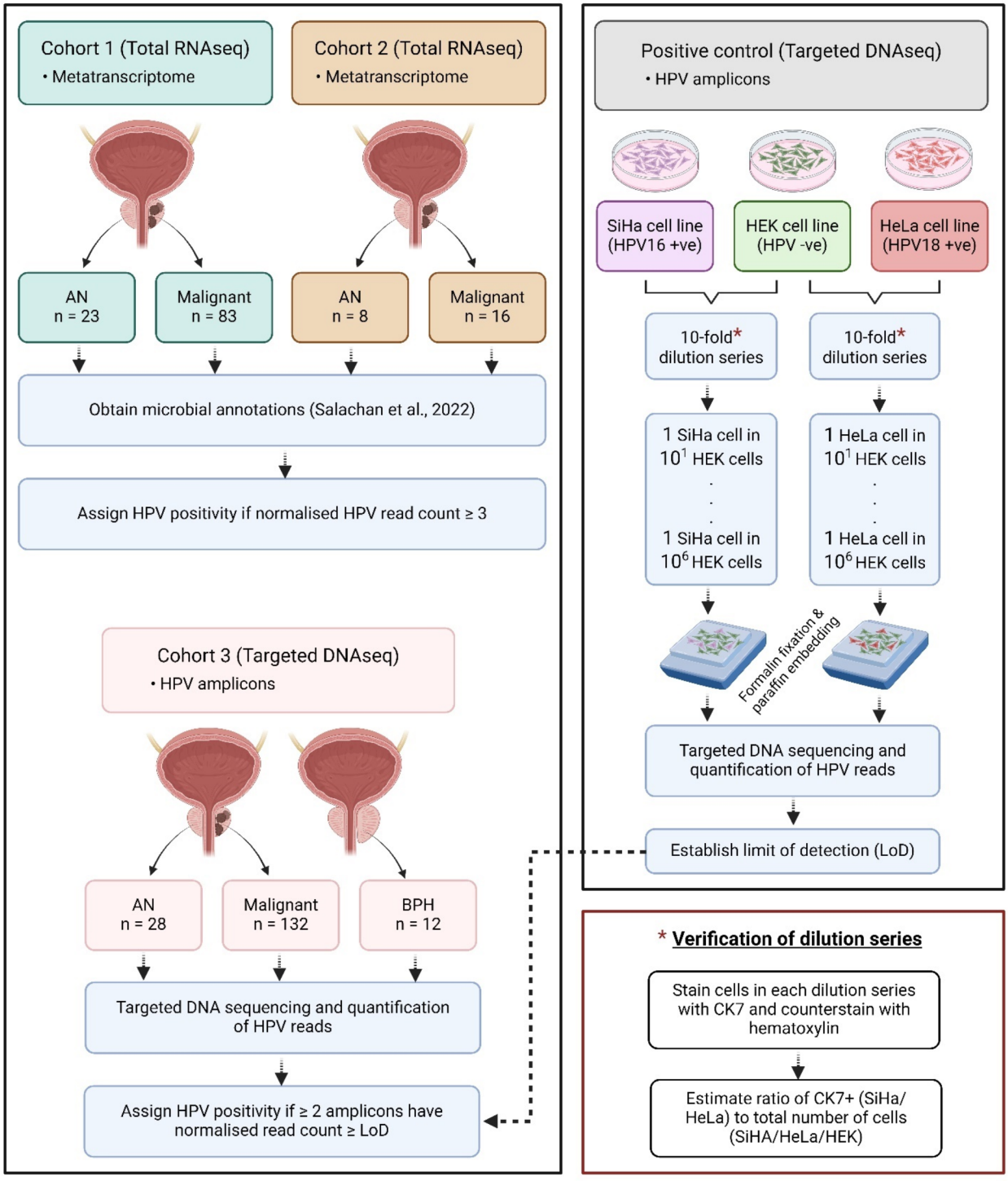

Fig. 1 Overview of the study design for the detection of HPV. AN, adjacent normal. BPH, benign prostatic hyperplasia. +ve, positive. -ve, negative. CK7, cytokeratin 7

as described previously in detail [35]. Only the microbial annotations were analyzed in this study.

Cohort 2 (total RNAseq) included 24 prostate tissue samples (16 malignant and 8 AN) from an external cohort of patients treated with RP for localized PC in France, which was previously described by Pinskaya et al. [36]. Formalin-fixed paraffin-embedded prostate tissue samples were processed for total RNA extraction and

subjected to total RNA sequencing, as described previously [36]. The metatranscriptome was profiled prior to this study [35]. Only the microbial annotations were analyzed in this study.

Cohort 3 (targeted DNAseq) included a total of 173 FFPE prostate tissue samples (132 malignant and 29 AN tissue samples from men with cancer and 12 benign tissue samples from men without cancer) obtained from an in-house cohort of 158 patients who underwent RP (1999–2016) for clinically localized PC and 12 cancer-free patients with benign prostatic hyperplasia (BPH) who underwent transurethral resection of the prostate (TURP; 2001–2007) at the Department of Urology, Aarhus University Hospital (AUH), Denmark. One sample was excluded from targeted DNA sequencing due to failed library preparation. Thus, the final cohort 3 consisted of 172 samples (132 malignant and 28 AN tissue samples from men with PC and 12 benign tissue samples from men with BPH) from 156 PC patients and 12 BPH patients. The clinicopathological characteristics of the cohorts are provided in Table 1.

Sample preparation

Prostate tissue samples for targeted DNA sequencing

For cohort 3, punch biopsies were obtained from FFPE blocks from areas with >80% cancer cells, as described previously [37]. Before DNA extraction, each tissue sample was marked as either AN or malignant by an experienced pathologist at AUH. The samples from the BPH patients were similarly verified to ensure that no carcinoma was present and were labeled as BPH. DNA was extracted from FFPE tissue samples using the QIAamp DNA FFPE Tissue Kit (Qiagen). The DNA concentration was measured using a Qubit dsDNA broad-range assay kit (Thermo Fisher Scientific), and the DNA was diluted to a concentration of 10 ng/µl for the next-generation sequencing (NGS) assay.

Table 1 Clinicopathological characteristics of patients with PC in cohorts 1, 2, and 3

Characteristic	Cohort 1	Cohort 2	Cohort 3
Malignant samples, *N*	83	16	132
Age (years) at RP, median (range)	65 (45–76)	N/A	64 (50–76)
Preoperative PSA (ng/ml)			
≤ 10, *N* (%)	39 (47.0%)	N/A	42 (31.8%)
> 10, *N* (%)	39 (47.0%)	N/A	90 (68.2%)
Unknown, *N* (%)	5 (6.0%)	N/A	0 (0.0%)
ISUP Grade Group			
≤ 3, *N* (%)	63 (75.9%)	9 (56.2%)	110 (83.3%)
> 3, *N* (%)	20 (24.1%)	7 (43.7%)	22 (16.7%)
Pathological T-stage			
T2, *N* (%)	51 (61.4%)	4 (25.0%)	85 (64.4%)
T3, *N* (%)	31 (37.4)	10 (62.5%)	47 (35.6%)
Unknown/T4, *N* (%)	1 (1.2%)	2 (12.5%)	0 (0.0%)
Biochemical recurrence (BCR) status			
BCR, *N* (%)	28 (33.7%)	6 (37.5%)	70 (53.0%)
BCR-free, *N* (%)	31 (63.9%)	10 (62.5%)	62 (47.0%)
Unknown, *N* (%)	2 (2.4%)	0 (0.0%)	0 (0.0%)
Total follow-up in months, median (range)	52.4 (17.6-178.9)	N/A	147.7 (6.0-267.6)

Positive control samples for targeted DNA sequencing

As a positive control and to assess the analytical sensitivity of our custom targeted NGS panel [34], we used FFPE cell blocks made from dilutions of the HPV-positive SiHa and HeLa cell lines in the background of the HPV-negative cell line HEK. HeLa cells contain approximately 10–50 copies of integrated HPV18 DNA [38, 39] per cell, whereas SiHa cells contain 1–2 copies of integrated HPV16 DNA [40] per cell. The cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. All reagents were obtained from Gibco (Thermo Fisher Scientific). The cells were incubated in a humidified incubator (5% CO2/95% air atmosphere at 37 °C) and harvested via trypsin treatment (0.25% Trypsin, 0.02% EDTA solution, Sigma-Aldrich ApS), followed by washing with 1× PBS. The titration was performed at 10-fold intervals, ranging from 1 SiHa or HeLa cell per 10^1 HEK cells to 1 SiHa or HeLa cell per 10^6 HEK cells, on the basis of an initial cell count using a Bürker-Türk cell counting chamber (Sigma-Aldrich ApS). The FFPE cell blocks were made by centrifuging the cell dilutions and discarding the supernatants. The cell pellets were subsequently washed with 1x PBS, the supernatant was removed, and the cell pellets were gently resuspended in 60 µL of human serum before 40 µL of thrombin was added to each cell dilution to form a cell clot. After coagulation, the cell clots were fixed in formalin for 24 h and then embedded in paraffin to mimic clinical FFPE biopsies.

Two sections of 4 µm thickness were cut from each FFPE cell dilution block containing dilutions ranging from 1 SiHa or HeLa cell per 10^1 HEK cells to 1 SiHa or HeLa cell per 10^4 HEK cells. Each of these two sections was mounted on glass slides and stained with hematoxylin (3 min of incubation) and cytokeratin (CK) 7 to confirm the correct dilution of the cells in the FFPE cell dilution blocks. Hematoxylin was used to stain the cell nuclei of both the HPV-positive and the HPV-negative cells, whereas CK7 was used to stain only the HPV-positive cells since the SiHa and HeLa cervical squamous cell carcinoma cell lines are CK7 positive, whereas the HEK cells are not [41]. The slides were stained for CK7 using the cytokeratin 7 (OV-TL 12/30) mouse monoclonal antibody (Agilent Technologies) with the EnVision FLEX, high pH visualization system (Agilent Technologies) and

the DAB+Substrate Chromogen System (Agilent Technologies) on Dako Omnis (Agilent Technologies). The IHC, Nuclei Detection, AI app in Visiopharm was used to calculate the ratio of SiHa or HeLa cells to HEK cells. To avoid the more cell-dense areas of the slides, the app was employed on three specific regions of each slide located away from these cell-dense areas. The dilution of HPV-positive cells in each of the slides (two slides per FFPE cell dilution block) was calculated as the number of CK7-positive cells (SiHa or HeLa) divided by the total number of cells. The average dilution was subsequently calculated from the two stained slides for each dilution. For this analysis, dilutions of $1{:}10^5$ and $1{:}10^6$ were not included, since the number of CK7-positive cells in these sections was expected to be too low to obtain a reliable dilution estimation.

In addition to the sections cut for immunohistochemistry, five sections of 10 µm thickness were cut from each FFPE cell dilution block containing dilutions ranging from 1 SiHa or HeLa cell per 10^1 HEK cells to 1 SiHa or HeLa cell per 10^6 HEK cells. From these sections, DNA was extracted using the QIAsymphony DSP DNA Mini Kit (Qiagen) on the QIAsymphony Instrument (Qiagen). The DNA concentration was measured via the Qubit dsDNA HS Assay Kit (Invitrogen) on a Qubit 2.0 Fluorometer (Invitrogen), and targeted DNA NGS was subsequently performed as described below.

Targeted DNA sequencing

An in-house custom Ion Torrent NGS panel (Thermo Fisher Scientific) was used for the detection of HPV. The panel was designed via AmpliSeq Designer (Thermo Fisher Scientific) as previously described [34]. In brief, the panel enables the detection of 27 HPV types, including all carcinogenic types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), all probably/possibly carcinogenic types (HPV68, 26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, and 97), and the two most common low-risk types (HPV6 and 11) [9]. For each of the included HPV types, eight amplicons were designed, enabling the amplification of two areas in each of the E6 and E7 oncogenes, three areas in the E2 gene, and one area in the L1 gene. One amplicon was designed for each of the five human reference genes (BTF3, PABPN1, PPIE, RAB1B, and SRSF3) to confirm adequate amounts of DNA in each analyzed sample. The amplicons were designed using the cell-free DNA pipeline of the AmpliSeq Designer (Thermo Fisher Scientific), resulting in amplicons of approximately 100 bp. Thermo Fisher Scientific's Ion AmpliSeq Library Kit 2.0 was used to prepare the samples for sequencing. The preparation was performed according to the manufacturer's protocol, except that only half of the stated reaction volume was used. The amount of input DNA was 60 ng per sample, and the samples were labeled with Ion Xpress Barcode Adaptors (Thermo Fisher Scientific). After library preparation, the libraries were quantified using the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific) and diluted to 40 pM. Template preparation was performed using the Ion 510 & Ion 520 & Ion 530 Kit-Chef (Thermo Fisher Scientific) on the Ion Chef System (Thermo Fisher Scientific). Sequencing was performed using the Ion GeneStudio S5 System (Thermo Fisher Scientific), with the coverage analysis plugin set to a minimum aligned length of 50 bp.

Targeted DNA sequencing data analysis

The initial data analysis was performed as described by Andersen et al. [34]. The sequencing data were aligned to a reference genome comprising the human reference genome (hg19) and the genomes of 313 HPV types. A coverage analysis file including the total number of reads for all included amplicons in each included sample was generated by the S5 Torrent Server (Thermo Fisher Scientific). The five human reference genes were assessed to determine the validity of each sample. For a sample to be valid, all five human reference genes had to be amplified and sequenced. The HPV amplicons of each sample were normalized to the average number of reads from the reference genes of the corresponding sample. Normalization was performed by dividing the number of reads from an HPV amplicon by the average number of human reference reads for that sample and then multiplying by 10,000. Thus, the data were analyzed as the number of reads per 10,000 reference reads.

A cut-off for each HPV type was calculated using samples known to be negative for that specific HPV type. Thus, the six HPV16-positive samples (10-fold dilutions of SiHa cells in HEK cells ranging from 1 SiHa cell per 10^1 HEK cells to 1 SiHa cell per 10^6 HEK cells) were used in the calculation of cut-offs for all HPV types except for HPV16. Similarly, the six HPV18-positive samples (10-fold dilutions of HeLa cells in HEK cells ranging from 1 HeLa cell per 10^1 HEK cells to 1 HeLa cell per 10^6 HEK cells) were used in the calculation of cut-offs for all HPV types except for HPV18. The cut-offs were based on the limit of detection (LoD), which was calculated as $\text{LoD} = \text{mean}_{\text{blank}} + (2^{*}\text{SD}_{\text{blank}})$. For each of the control samples known to be HPV negative, the *blank* was determined as the amplicon presenting the highest normalized read count. The mean of the *blank* for each control sample known to be HPV negative was then used to calculate the LoD. See Table S1 (Additional file 1) for the specified cut-offs for each HPV type. For a sample to be rendered HPV positive, at least two of the eight HPV amplicons for an HPV type should contain a normalized read count above the calculated LoD. This was selected since, in our experience, reads present in only one amplicon often correspond to background.

Results

HPV detection using metatranscriptomics data

Given the discrepancy in the results from previous studies [4, 5, 17–20, 42, 43], we sought to investigate the association between HPV infection and prostate cancer via two different high-throughput NGS-based methods and three independent cohorts of early-stage (localized) PC patients recruited in Denmark (cohorts 1 and 3) and France (cohort 2). See Fig. 1 for an overview of the study design.

First, we used a previously published [35], metatranscriptomics dataset (cohort 1), comprising 23 benign and 83 malignant prostate tissue samples, which were subjected to total RNA sequencing. On average, approximately 1 million unmapped read pairs (i.e., read pairs that did not map to the human reference genome) per sample were available (Fig. 2A) for mapping to the microbial (bacterial and viral) reference sequences. This sequencing depth is sufficient for the detection of HPV even in samples that are PCR-negative for HPV, as shown for cervical cancer [44]. Using the microbial annotations reported by the SAMSA2 pipeline [45] from our previous study [35], we detected annotations for 'Human papillomavirus' in two of the malignant samples (2/83, 2.4%; Fig. 2B and Table S2, Additional file 1) and none of the benign samples (0/23, 0%; Fig. 2B). However, in each of the two potential HPV-positive samples, SAMSA2 detected only one read count (normalized counts of 1 and 1.3; Table S2, Additional file 1), which was below even a conservative threshold of a minimum of 3 reads, which we used pragmatically for assigning 'true' HPV positivity for the samples (Fig. 2B). Notably, such a low HPV read count occurred despite both samples having an unmapped read count greater than or equal to 1 million (Table S2, Additional file 1). Thus, using this dataset, we did not observe a clear indication of active HPV infection in any of the prostate tissue samples.

To explore whether the lack of an HPV signal was due to an inadequate sequencing depth, we used a previously published [35] metatranscriptomics dataset (cohort 2), comprising 8 benign and 16 malignant prostate tissue samples originally included in a French study [36]. The samples in this dataset were subjected to deep total RNA sequencing, and we obtained approximately 2.7 million unmapped read pairs per sample (Fig. 2C) for mapping to the microbial (bacterial and viral) reference sequences using the SAMSA2 pipeline [45]. Using this dataset with a much higher sequencing depth, we did not detect HPV reads in any of the prostate tissue samples (0/24, 0%; Fig. 2D). These results suggest that active HPV infection may not be clearly associated with the development or progression of PC.

Targeted DNA sequencing

Positive control samples for sensitivity assessment

To ensure that the lack of HPV detection in the metatranscriptomic data is not simply caused by a lack of active viral transcription, we decided to use deep panel DNA sequencing, which targets 27 HPV genotypes, to detect HPV in prostate tissue samples. To determine assay sensitivity, we made serial 10-fold dilutions of HPV16-positive SiHa cells and HPV18-positive HeLa cells in HPV-negative HEK cells (range: 1 SiHa or HeLa cell per 10^1 HEK cells to 1 SiHa or HeLa cell per 10^6 HEK cells). The resulting 12 dilutions were formalin-fixed and embedded in paraffin and served as positive controls ($n=12$) in the NGS analysis. Prior to NGS, slides made from the first four serial dilutions (i.e., $1{:}10^1$ to $1{:}10^4$) were stained with an antibody targeting CK7, which is expressed only by the HPV-positive SiHa or HeLa cells. This allowed us to cytologically verify that the correct serial dilutions of the cells were made. As expected, we observed CK7-positive reactions in all the dilutions (Fig. 3), indicating the presence of HPV-positive cells in each of the dilutions. Furthermore, the enumeration of the CK7-positive cells using the Visiopharm software revealed the expected cell counts in each of the dilutions (Figure S1, Table S3; Additional file 1), indicating correct serial dilutions.

Using the DNA sequencing panel, all five human reference genes were amplified in 100% (12/12) of the positive control samples (see Additional file 2). The mean number of reads for the reference amplicons was 57,389 (range: 10,183−78,432) for the HPV16-positive control samples and 55,837 (range: 27,345−88,180) for the HPV18-positive control samples (Fig. 4A). A proportional decrease in normalized reads was observed through the 10-fold dilutions of the HPV16- and HPV18-positive control samples (Fig. 4B and C), as also indicated by the slope of the trendline approaching −1 for each amplicon (Table S4, Additional file 1). For both the HPV16- and the HPV18-positive controls, no reads were observed within parts of the E2 gene. This could indicate integration of the viral genome into the host genome, which often results in partial or complete loss of the E2 gene [46–48]. This is consistent with previous reports of HPV integration in SiHa and HeLa cells [38–40].

A cut-off was applied to the normalized data on the basis of the calculated LoD for each HPV type of the targeted DNA sequencing panel (Methods). Thus, for a sample to be rendered HPV positive, at least two HPV amplicons should contain a normalized read count above the calculated LoD (LoD=0.50 for HPV16 and LoD=18.7 for HPV18). On the basis of this cut-off, HPV16 was detected at a dilution of one HPV16-positive SiHa cell per 10^5 HPV16-negative HEK cells. However, only three amplicons presented reads in this dilution, in contrast to

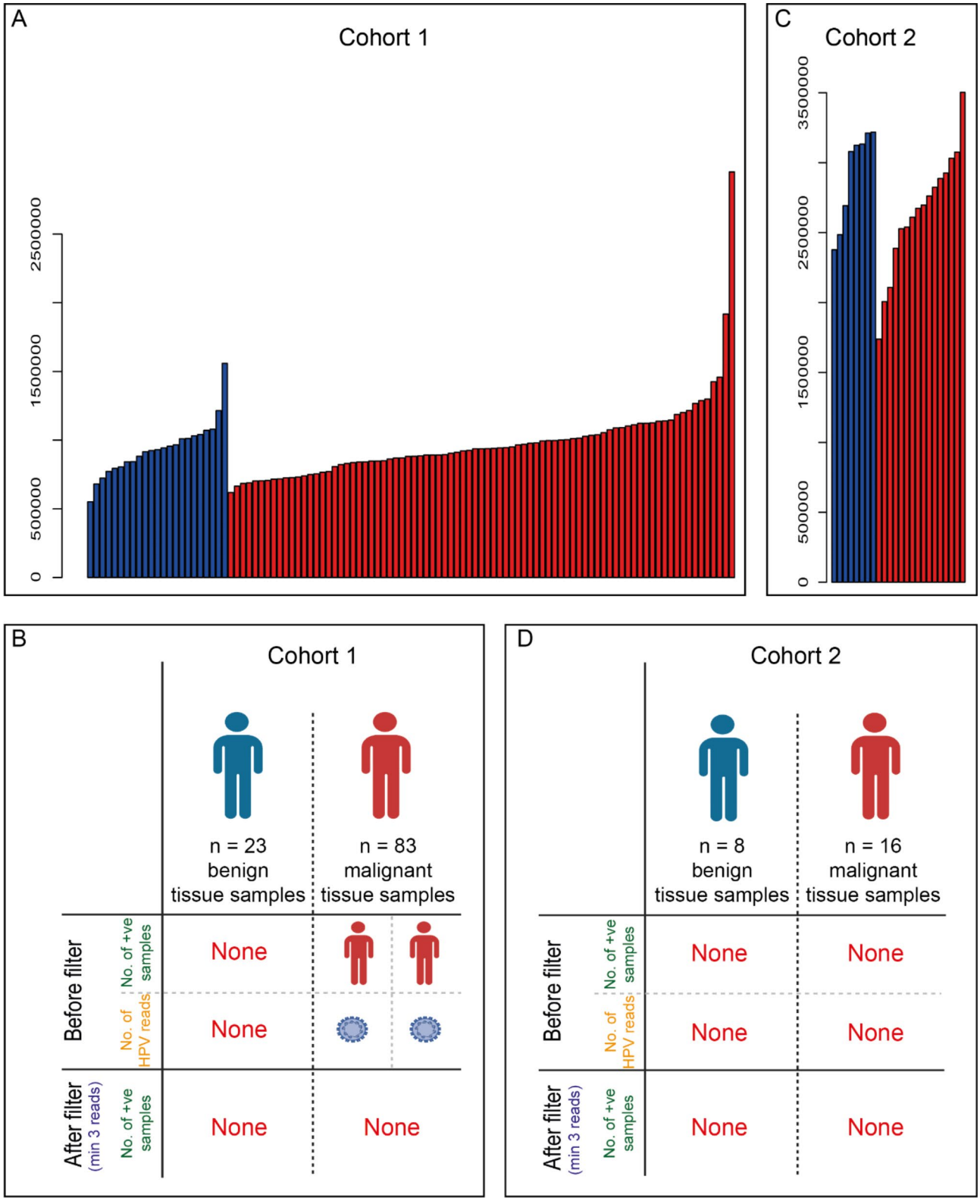

Fig. 2 **A**) Distribution of unmapped reads across all samples in cohort 1. The blue bars correspond to benign tissue samples ($n = 23$), and the red bars correspond to malignant tissue samples ($n = 83$). **B**) Overview of HPV detection in the metatranscriptomics data from cohort 1. **C**) Distribution of unmapped reads across all samples in cohort 2. The blue bars correspond to benign tissue samples ($n = 8$), and the red bars correspond to malignant tissue samples ($n = 16$). **D**) Overview of HPV detection in the metatranscriptomics data from cohort 2

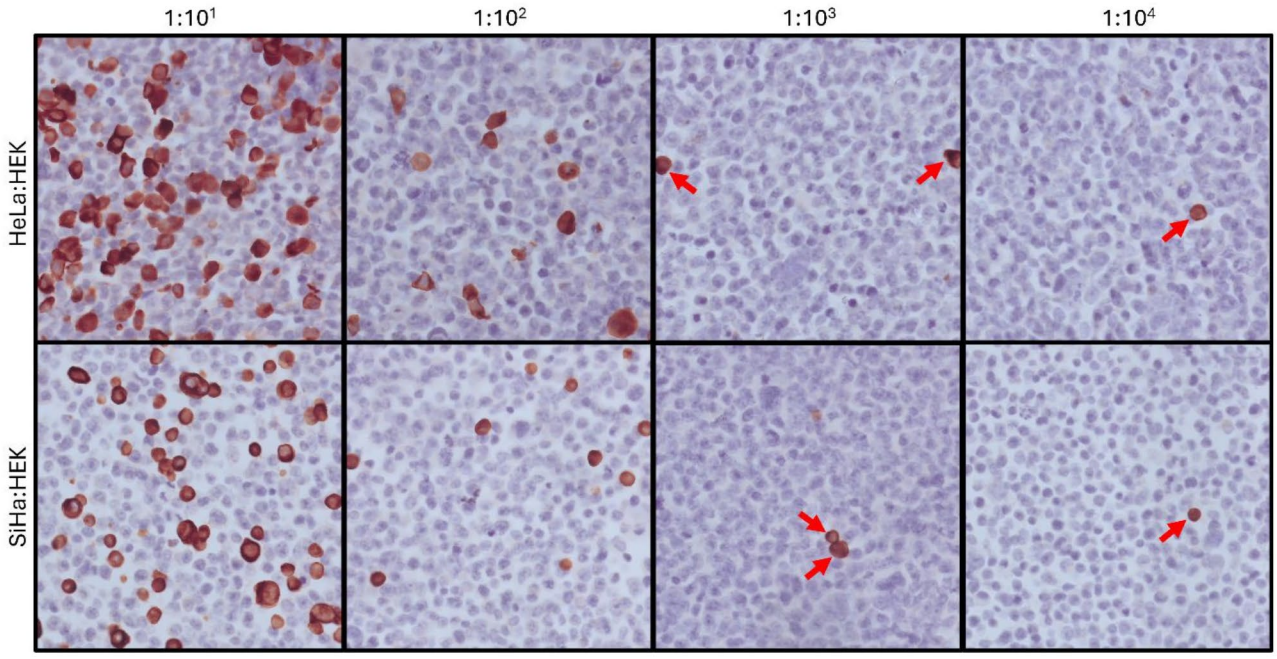

Fig. 3 Representative images of CK7-stained slides containing serial 10-fold dilutions of SiHa or HeLa cells in HEK cells. The slides were stained with hematoxylin and for cytokeratin (CK) 7 using 3,3'-diaminobenzidine (DAB) chromogen (brown cell nuclei: SiHa and HeLa cells (CK7 positive cells); purple/blue cell nuclei: HEK cells (CK7 negative cells, counterstained with hematoxylin)). 20X magnification

the detection of six amplicons in the less diluted samples. It was therefore concluded that HPV16 could be reliably detected at a dilution of one HPV16-positive SiHa cell per 10^4 HPV16-negative HEK cells, with all amplicons showing reads except for the E2 amplicons. This corresponds to a detection limit of approximately 1–2 HPV16 copies, based on the literature stating that the SiHa cell line contains 1–2 HPV16 copies per cell [40]. For the HeLa cell line, HPV18 was reliably detected in a dilution of one HPV18-positive HeLa cell per 10^4 HPV18-negative HEK cells when the cutoff was applied, corresponding to a detection limit of approximately 9–46 HPV18 copies based on the literature stating that the HeLa cell line contains 10–50 HPV18 copies per cell (Fig. 4B and C; Table S4 and calculations in Additional file 1).

Prostate tissue samples

One hundred and seventy-three prostate tissue samples were sequenced using our in-house targeted DNA HPV sequencing assay. These included 29 AN tissue samples, 12 BPH tissue samples, and 132 malignant prostate tissue samples. One of the AN tissue samples was subsequently excluded since the library formation yielded a DNA concentration too low for NGS analysis, leaving 172 prostate tissue samples for analysis. All five human reference genes were amplified in 100% (172/172) of the samples. The coverage, defined as the mean number of reads for the reference amplicons, was <1,000 reads in 14.3% (4/28) of the AN samples, none (0/12) of the BPH samples, and 6.1% (8/132) of the malignant prostate samples. One (0.8%, 1/132) of the malignant prostate samples yielded a coverage of 402,515 reads, corresponding to almost 10X the coverage of the sample with the second highest coverage, making this sample an obvious outlier in terms of coverage (Fig. 5A). When the outlier sample was removed, the mean coverage was 19,745 reads (range 66−54,441).

Using the cut-off criteria for HPV16 (≥2 amplicons with ≥0.50 normalized reads), 8.3% (1/12) of the BPH samples were infected with HPV16, with a normalized read count above the cut-off for 7/8 of the HPV16 amplicons (Fig. 5B). Moreover, 0.8% (1/132) of the malignant prostate samples were positive for infection with HPV16. However, for the malignant prostate sample, only 2/8 of the HPV16 amplicons presented with a normalized read count above the cut-off, and both amplicons presented a normalized read count very close to the cut-off. Thus, the HPV16 positivity of this sample may be questioned. None (0/28) of the AN samples were infected with HPV16.

Among the malignant prostate samples, 2.3% (3/132) were infected with HPV70 based on the cut-off of ≥2 amplicons with ≥2.39 normalized reads. Moreover, 6.8% (9/132) of the malignant prostate samples were infected with HPV73 based on the cut-off of ≥2 amplicons with ≥0.09 normalized reads (Fig. 5B). However, when taking a closer look at the samples yielding reads for HPV70 and HPV73, they all presented with reads only for the same two-three amplicons and were not distinguishable from

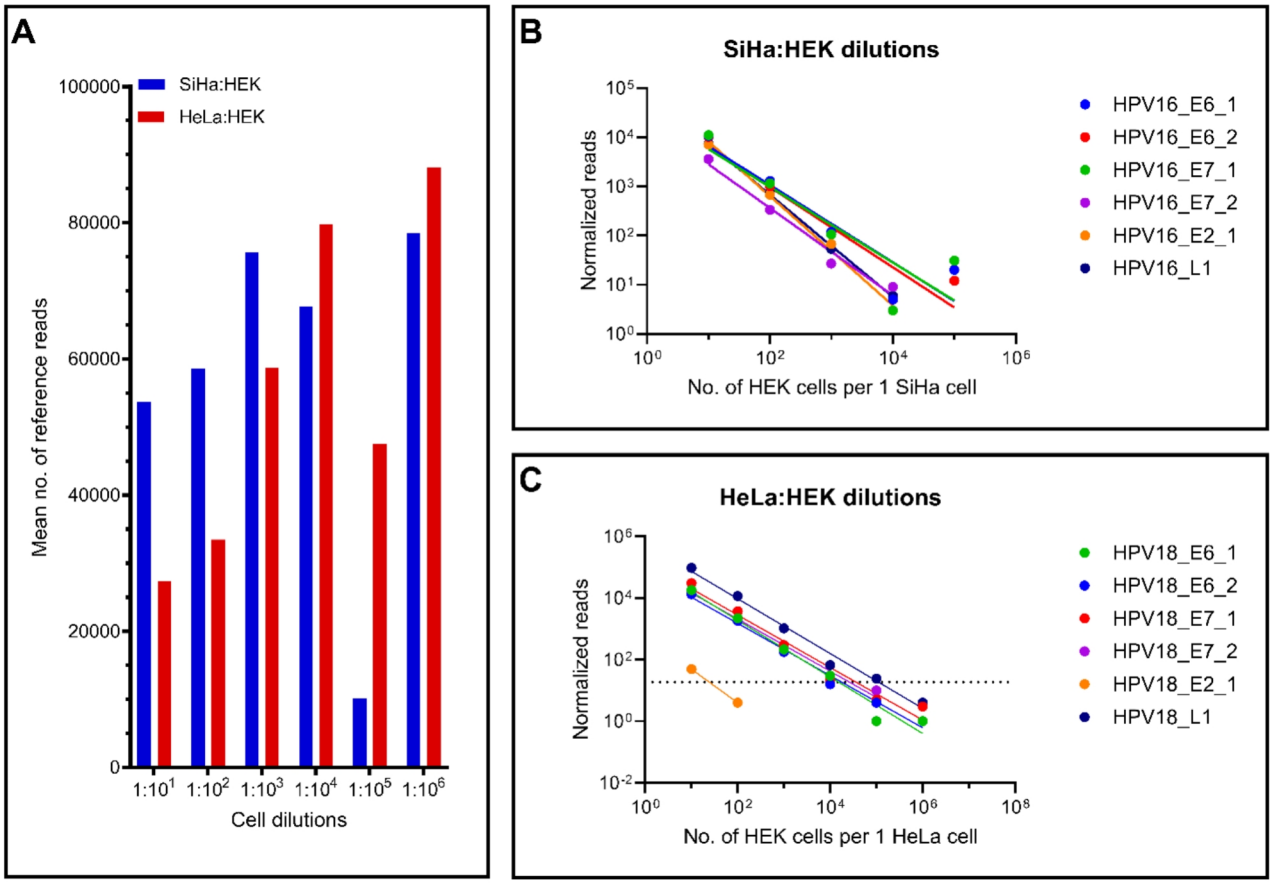

Fig. 4 **A**) The mean number of reference reads for serial 10-fold dilutions of SiHa and HeLa cells in HEK cells. **B**) The number of normalized reads (HPV amplicon reads per 10,000 reference reads) generated for each of the HPV16 amplicons in the analysis of the HPV16-positive SiHa: HEK dilutions. **C**) The number of normalized reads (HPV amplicon reads per 10,000 reference reads) generated for each of the HPV18 amplicons in the analysis of the HPV18-positive HeLa: HEK dilutions. Dotted horizontal line: normalized read cut-off

the HPV70- and HPV73-negative samples whereof some also presented with few normalized reads for these specific HPV70 and HPV73 amplicons (Fig. 5B). The eight amplicons for each of these HPV types have formerly been amplified [34], thereby eliminating the risk of faulty primers and making it more likely that these normalized reads for specific HPV70 and HPV73 amplicons correspond to unspecific binding or background and not to real HPV-positive samples. Moreover, HPV70 and HPV73 have, to our knowledge, not formerly been associated with prostate cancer, and these HPV types are not classified as carcinogenic to humans by the IARC [9].

None (0/28) of the AN samples, (0/12) BPH samples, or (0/132) malignant prostate samples were positive for any other HPV types. In conclusion, only 8.3% (1/12) of the BPH samples and 0.8% (1/132) of the malignant prostate samples were positive for HPV16. However, the HPV16-positive malignant prostate sample presented with a normalized read count very close to the cut-off, making the HPV positivity of this sample questionable. No other carcinogenic-classified HPV types were observed in any of the AN, BPH, or malignant prostate tissue samples.

Discussion

This study used two different high-throughput NGS-based methods to investigate the associations between HPV infection and prostate cancer. In the first method, we leveraged two independent cohorts of PC patients for whom we had total RNA sequencing data available and for whom we had previously performed unbiased profiling of the prostate tissue microbiome (i.e., metatranscriptome). Both bacterial and viral species were included in the annotations reported in our previous study, which allowed us to detect HPV annotations in the dataset. Metatranscriptomics, as an approach to detect HPV in tissue samples, has previously been shown to be highly successful in multiple cancer types [49–52]. Thus, the lack of an HPV signal in our metatranscriptomic cohorts could be considered a true lack of active HPV infection within the prostate of these patients. Consequently, we do not find support for an association between HPV

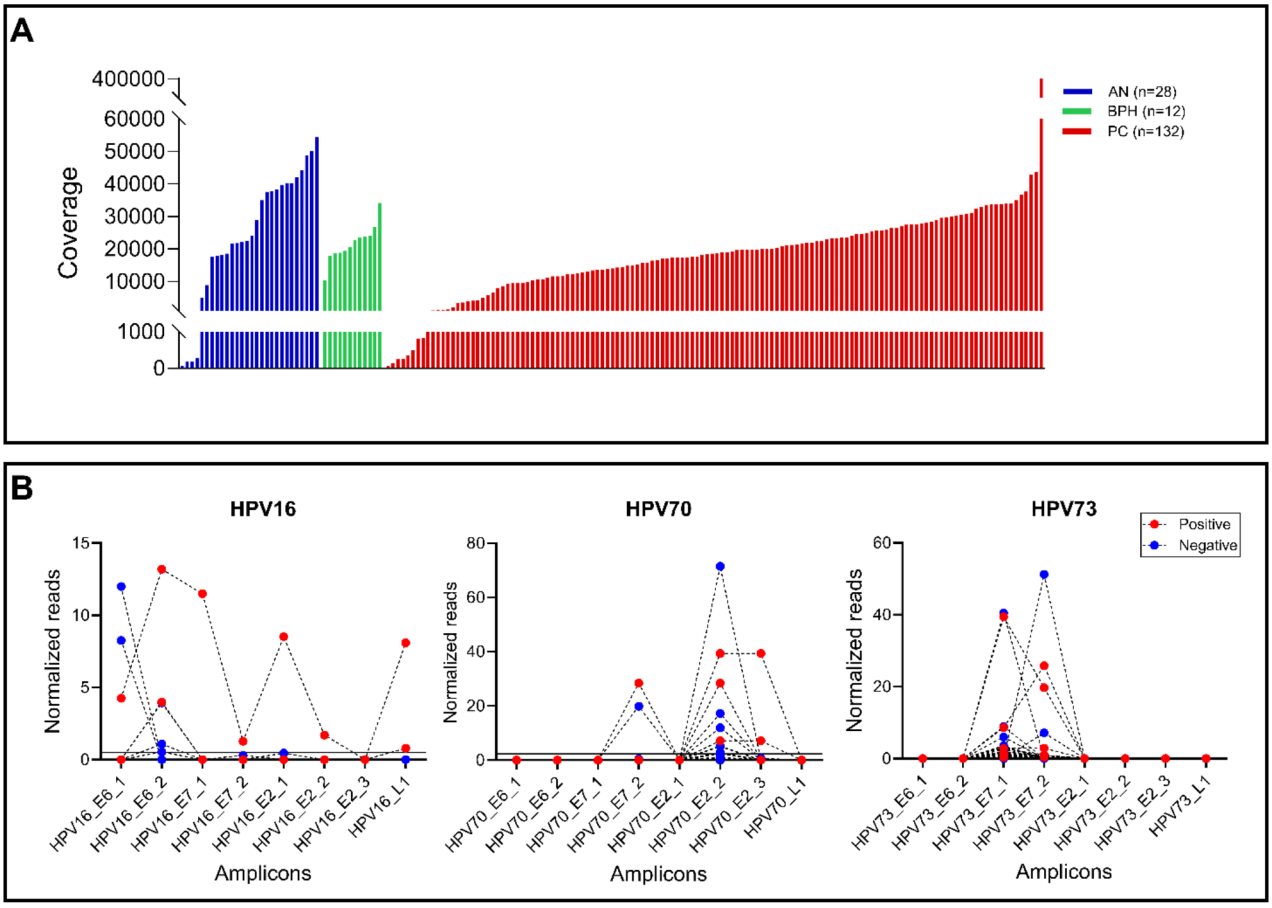

Fig. 5 **A**) Coverage, i.e., the average number of reference reads across all prostate tissue samples included in cohort 3. AN, Adjacent normal. BPH, benign prostatic hyperplasia. PC, prostate cancer. **B**) Normalized reads, i.e., the number of reads per 10,000 reference reads, of the HPV16, HPV70, and HPV73 amplicons for all the samples divided into HPV-positive (red) and HPV-negative (blue) groups. The dotted lines connect amplicons of individual samples. Horizontal line: cut-off for HPV positivity (two amplicons need to be above this line for a sample to be rendered HPV positive)

infection and PC, as opposed to the suggestion of such an association in the previous study [14]. It is of course possible that the prevalence of HPV is much lower in PC; thus, we might have missed these cases because of the relatively small cohort sizes in the two metatranscriptomic cohorts. However, if that was the case, then the prevalence of HPV in the prostate, based on our combined data (i.e., cohort 1+cohort 2), should be lower than 0.77%. In fact, a metatranscriptomic analysis of the PRAD cohort within TCGA detected HPV in only two of the 498 PC tissue samples [49], equating to a prevalence of approximately 0.4%, which is much lower than that reported in the previous study [14].

Nevertheless, to ensure that the metatranscriptomic approach did not detect HPV (1) because of a lack of assay sensitivity or (2) because of a lack of active viral transcription, we used deep panel DNA sequencing targeting 27 HPV genotypes as a novel method for the detection of HPV in PC tissue samples. This targeted NGS assay could detect HPV in FFPE samples containing as few as one HPV-positive SiHa or HeLa cell per 10,000 HPV-negative HEK cells in samples with a mean coverage of 56,513 reference reads. Nevertheless, only one out of 132 (0.8%) malignant prostate samples and one out of 12 (8.3%) BPH samples were positive for a carcinogenic-classified HPV type in our study. However, with a normalized HPV16 read count of the malignant sample very close to the cut-off, the HPV status within the prostate tissue from this patient could be questionable. The remaining 170 prostate tissue samples were rendered negative for carcinogenic-classified HPV types and should thus be considered either true high-risk HPV-negative samples or the frequency of HPV-infected cells in the samples should be extremely low. Notably, the limit of detection for the NGS assay also depends on the viral load of a sample, i.e., the number of HPV copies per cell, the number of HPV-positive cells present in the sample, and the sequencing depth of a sample. However, if HPV plays a role in the carcinogenesis of prostate cancer, HPV

is expected to be present in most of the cancer cells in malignant prostate tissue samples.

Since the sensitivity of our targeted NGS assay is strongly influenced by sequencing depth, a limitation of this study was the difference in sequencing depth between positive control samples, which were used for assay sensitivity assessment, and prostate tissue samples. Moreover, a difference in sequencing depth between prostate tissue samples was generally observed. This difference could be caused by variations in the DNA quality of the prostate tissue samples, resulting in a lower detection limit of HPV in samples with a lower sequencing depth.

For 160 (93.0%) of the prostate tissue samples, the coverage was >1,000 reads. For a sample with a coverage of 1,000 reads, the detection limit of HPV would, in theory, if an inversely proportional relationship with the coverage was assumed, be reduced by approximately 57X compared with the positive control samples with an average coverage of 56,513 reads. This would result in a detection limit conservatively calculated to be between 51 and 2,622 HPV copies (see Additional file 1 for calculations). This is, however, still superior to or equal to the detection limit of most PCR assays usually used for HPV detection [53–55]. Most PCR studies use the MY09/MY11 primers for HPV detection, which amplify a fragment of 450 bp of the L1 gene [4, 21, 56–58]. However, the length of primers is crucial, especially when working with partially degraded material such as DNA extracted from FFPE tissue samples, since longer fragments are then more difficult to amplify. Likewise, the genomic position of primers is important since L1 might be deleted following viral integration [4, 59]. Therefore, a strength of our study was the use of primers that amplify short DNA fragments of approximately 100 bp. We included primers to amplify eight separate genomic regions of each HPV type instead of just one region, limiting the risk of false negative results caused by, e.g., deletions in the viral genome, poor performance of specific primers, or partly degraded DNA material.

Differences in HPV detection methods are thus likely one of the reasons for the discrepancies between studies concerning HPV and prostate cancer. As mentioned, HPV has usually been detected using serum for the detection of HPV IgG antibodies or using prostate tissue for PCR-based HPV detection. The use of serum in association studies of HPV and prostate cancer has, however, been questioned since this method is not tissue-specific and therefore cannot be used to determine from where the HPV originates in positive samples [14]. Likewise, the earliest conducted PCR-based studies are thought to be very susceptible to contamination and thus false positive results, compromising the value of these results as well [14, 59]. Additionally, the analytical sensitivity of the assays used for HPV detection is essential since previous research has suggested a viral load of less than 0.1 HPV copies per cell in prostate cancer and non-neoplastic hyperplasia tissue [60]. This is contrary to the viral load in cervical cancers, where HPV is known to be the etiological cause of the cancer. Here, the viral load is reported to range from <1 to >1,000 copies per cell [61–63]. This indeed questions the role of HPV in the development of prostate cancer or at least suggests a different role of HPV in the carcinogenicity of prostate cancer than in that of cervical cancer. In 2017, Glenn et al. [30] detected significantly higher expression of the HPV E7 oncoprotein in benign prostate samples than in subsequent prostate cancer samples obtained from the same patients. Although not significant, they also detected a higher prevalence of the E7 gene in benign prostate samples (23/28, 82%) than in subsequent prostate cancer samples (19/28, 68%). They thus proposed the possibility of HPV being involved in the early stage of prostate carcinogenesis, explaining the very low or undetectable HPV in prostate cancers and supporting the "hit and run" model of oncogenic viruses proposed by several researchers as initiators of tumorigenesis [20, 64]. Our study found a higher frequency of HPV positivity in BPH samples (1/12, 8.3%) compared to malignant PC samples (1/132, 0.8%). However, this comparison is constrained by the limited number of BPH samples included. Nonetheless, the low frequency of HPV-positive samples in both BPH and malignant PC groups raises questions about the role of HPV in initiating prostate tumorigenesis. If HPV is an initiator of tumorigenesis in PC, we could hypothesize to observe HPV-positivity in early cancers. Our study material is collected from patients undergoing radical prostatectomy, a procedure typically performed for tumors identified at an early stage and, consequently, often of a low grade. Our cohort 3 does consist primarily of low-grade (≤3) malignant PC samples (110, 83.3%), and the only malignant sample which might be HPV-positive was from a patient with a grade 1 tumor. Hence, our study does not support the "hit and run" model of HPV as an initiator in PC tumorigenesis but more studies are needed to evaluate this hypothesis.

Conclusions

In conclusion, our study does not support HPV infection as a major contributor to the etiology of prostate cancer.

Abbreviations

HPV	Human papillomavirus
PC	Prostate cancer
AN	Adjacent normal
BPH	Benign prostatic hyperplasia
IARC	International Agency for Research on Cancer
PCR	Polymerase chain reaction
FFPE	Formalin-fixed paraffin-embedded
NGS	Next-generation sequencing

bp	Base pairs
RP	Radical prostatectomy
CK	Cytokeratin
LoD	Limit of detection
DAB	3,3′-diaminobenzidine

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13027-024-00619-x.

Supplementary Material 1: Additional file 1.Pdf: supplementary material, includes supplementary tables S1-S5, supplementary figure S1, and calculations for the theoretical change in HPV detection limit based on coverage

Supplementary Material 2: Additional file 2.Xlsx: data file, includes coverage matrix with normalized read counts for prostate tissue samples and positive control samples (cell lines)

Acknowledgements
We would like to thank the histology laboratory team at MOMA and the medical laboratory technologists at the Department of Pathology for excellent technical support. We thank the Danish Cancer Biobank for providing samples and information on storage and handling and GenomeDK for computational resources. We acknowledge BioRender.com, Adobe Illustrator, GraphPad Prism, and R for figure illustrations and Visiopharm for their software, which enabled digital analysis of the stained slides.

Author contributions
KA: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original draft, Visualization, Project administration, Funding acquisition; PVS: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original draft, Visualization; MB: Resources, Writing – Review and editing; BU: Formal analysis, Writing – Review and editing; MS: Writing – Review and editing, Funding acquisition; KDS: Conceptualization, Writing – Review and editing, Funding acquisition; TS: Conceptualization, Writing – Review and editing, Supervision.

Funding
This work was supported by the Danish Cancer Society, the Novo Nordisk Foundation, Independent Research Fund Denmark, Aase og Ejnar Danielsens Fond, and Torben og Alice Frimodts Fond. The funding sources were not involved in the conceptualization, design, data collection, analysis, decision to publish, or preparation of the manuscript.

Data availability
The metatranscriptomics datasets supporting the conclusions of this study are available from https://doi.org/10.1186/s13073-022-01011-3. The targeted DNAseq datasets supporting the conclusions of this article are included within the article and its additional files.

Declarations

Ethical approval and consent to participate
All research that formed the basis of this study was conducted in accordance with relevant rules, regulations, and guidelines. For samples included in cohorts 1 and 3, written informed consent was obtained from all participants who consented to donate tissue samples for a research biobank (approved by The Central Denmark Region Committees on Health Research Ethics [jr. nr. 2000 – 0299] and The Danish Data Protection Agency [jr. nr. 2013-41-2041 and jr. nr. 2007-58-0010]). The present study was approved by the Danish National Committee on Health Research Ethics [jr. nr. 1302791 and jr. nr. 1603542], who waived the requirement for informed consent for the specific sequencing analyses performed in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 15 August 2024 / Accepted: 28 October 2024
Published online: 14 November 2024

References

1. Wang L, Lu B, He M, Wang Y, Wang Z, Du L. Prostate Cancer incidence and mortality: global status and temporal trends in 89 countries from 2000 to 2019. Front Public Health. 2022;10:811044.
2. Bergengren O, Pekala KR, Matsoukas K, Fainberg J, Mungovan SF, Bratt O et al. 2022 update on prostate Cancer epidemiology and risk Factors-A systematic review. Eur Urol. 2023.
3. Rhim JS, Webber MM, Bello D, Lee MS, Arnstein P, Chen LS et al. Stepwise immortalization and transformation of adult human prostate epithelial cells by a combination of HPV-18 and v-Ki-ras. Proceedings of the National Academy of Sciences. 1994;91(25):11874-8.
4. Carozzi F, Lombardi FC, Zendron P, Confortini M, Sani C, Bisanzi S, et al. Association of human papillomavirus with prostate cancer: analysis of a consecutive series of prostate biopsies. Int J Biol Markers. 2004;19(4):257–61.
5. Zhao X, Zhou Z, Chen Y, Chen W, Ma H, Pu J. Role of antibodies to human papillomavirus 16 in prostate cancer: a seroscreening by peptide microarray. Tumour Biol. 2017;39(6):1010428317698371.
6. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol. 1999;189(1):12–9.
7. de Martel C, Plummer M, Vignat J, Franceschi S. Worldwide burden of cancer attributable to HPV by site, country and HPV type. Int J Cancer. 2017;141(4):664–70.
8. Karolinska Institutet. International Human Papillomavirus (HPV) Reference Center 2023 [updated 13-09-2023. https://www.hpvcenter.se/human_reference_clones/
9. The International Agency for Research on Cancer (IARC). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 90 Human Papillomaviruses2007.
10. Chan CK, Aimagambetova G, Ukybassova T, Kongrtay K, Azizan A. Human papillomavirus infection and cervical Cancer: Epidemiology, Screening, and vaccination-review of current perspectives. J Oncol. 2019;2019:3257939.
11. Naghashfar Z, DiPaolo JA, Woodworth CD, Passaniti A. Immortalization of human adult prostatic adenocarcinoma cells by human papilloma virus HPV16 and – 18 DNA. Cancer Lett. 1996;100(1–2):47–54.
12. Choo CK, Ling MT, Chan KW, Tsao SW, Zheng Z, Zhang D, et al. Immortalization of human prostate epithelial cells by HPV 16 E6/E7 open reading frames. Prostate. 1999;40(3):150–8.
13. Weijerman PC, König JJ, Wong ST, Niesters HG, Peehl DM. Lipofection-mediated immortalization of human prostatic epithelial cells of normal and malignant origin using human papillomavirus type 18 DNA. Cancer Res. 1994;54(21):5579–83.
14. Lawson JS, Glenn WK. Evidence for a causal role by human papillomaviruses in prostate cancer – a systematic review. Infect Agents Cancer. 2020;15(1).
15. Whitaker NJ, Glenn WK, Sahrudin A, Orde MM, Delprado W, Lawson JS. Human papillomavirus and Epstein Barr virus in prostate cancer: Koilocytes indicate potential oncogenic influences of human papillomavirus in prostate cancer. Prostate. 2013;73(3):236–41.
16. Chen ACH, Waterboer T, Keleher A, Morrison B, Jindal S, McMillan D, et al. Human papillomavirus in Benign Prostatic Hyperplasia and Prostatic Adenocarcinoma Patients. Pathol Oncol Res. 2011;17(3):613–7.
17. Dillner J, Knekt P, Boman J, Lehtinen M, Af Geijersstam V, Sapp M, et al. Seroepidemiological association between human-papillomavirus infection and risk of prostate cancer. Int J Cancer. 1998;75(4):564–7.
18. Korodi Z, Dillner J, Jellum E, Lumme S, Hallmans G, Thoresen S, et al. Human papillomavirus 16, 18, and 33 infections and risk of prostate cancer: a nordic nested case-control study. Cancer Epidemiol Biomarkers Prev. 2005;14(12):2952–5.
19. Sutcliffe S, Giovannucci E, Gaydos CA, Viscidi RP, Jenkins FJ, Zenilman JM, et al. Plasma antibodies against Chlamydia trachomatis, human papillomavirus, and human herpesvirus type 8 in relation to prostate cancer: a prospective study. Cancer Epidemiol Biomarkers Prev. 2007;16(8):1573–80.
20. Sutcliffe S, Viscidi RP, Till C, Goodman PJ, Hoque AM, Hsing AW, et al. Human papillomavirus types 16, 18, and 31 serostatus and prostate cancer risk in

the prostate Cancer Prevention Trial. Cancer Epidemiol Biomarkers Prev. 2010;19(2):614–8.
21. Tachezy R, Hrbacek J, Heracek J, Salakova M, Smahelova J, Ludvikova V, et al. HPV persistence and its oncogenic role in prostate tumors. J Med Virol. 2012;84(10):1636–45.
22. Hisada M, Rabkin CS, Strickler HD, Wright WE, Christianson RE, van den Berg BJ. Human papillomavirus antibody and risk of prostate cancer. JAMA. 2000;283(3):340–1.
23. Hayes RB, Pottern LM, Strickler H, Rabkin C, Pope V, Swanson GM, et al. Sexual behaviour, STDs and risks for prostate cancer. Br J Cancer. 2000;82(3):718–25.
24. Rosenblatt KA, Carter JJ, Iwasaki LM, Galloway DA, Stanford JL. Serologic evidence of human papillomavirus 16 and 18 infections and risk of prostate cancer. Cancer Epidemiol Biomarkers Prev. 2003;12(8):763–8.
25. Adami HO, Kuper H, Andersson SO, Bergström R, Dillner J. Prostate cancer risk and serologic evidence of human papilloma virus infection: a population-based case-control study. Cancer Epidemiol Biomarkers Prev. 2003;12(9):872–5.
26. Sitas F, Urban M, Stein L, Beral V, Ruff P, Hale M, et al. The relationship between anti-HPV-16 IgG seropositivity and cancer of the cervix, anogenital organs, oral cavity and pharynx, oesophagus and prostate in a black South African population. Infect Agents Cancer. 2007;2(1):6.
27. Huang W-Y, Hayes R, Pfeiffer R, Viscidi RP, Lee FK, Wang YF, et al. Sexually transmissible infections and prostate Cancer risk. Cancer Epidemiol Biomarkers Prev. 2008;17(9):2374–81.
28. Dennis LK, Coughlin JA, McKinnon BC, Wells TS, Gaydos CA, Hamsikova E, et al. Sexually transmitted infections and prostate cancer among men in the U.S. military. Cancer Epidemiol Biomarkers Prev. 2009;18(10):2665–71.
29. Hrbacek J, Urban M, Hamsikova E, Tachezy R, Eis V, Brabec M, et al. Serum antibodies against genitourinary infectious agents in prostate cancer and benign prostate hyperplasia patients: a case-control study. BMC Cancer. 2011;11(1):53.
30. Glenn WK, Ngan CC, Amos TG, Edwards RJ, Swift J, Lutze-Mann L et al. High risk human papilloma viruses (HPVs) are present in benign prostate tissues before development of HPV associated prostate cancer. Infect Agents Cancer. 2017;12(1).
31. Chen Y, Wei J. Identification of Pathogen signatures in prostate Cancer using RNA-seq. PLoS ONE. 2015;10(6):e0128955.
32. Tang K-W, Alaei-Mahabadi B, Samuelsson T, Lindh M, Larsson E. The landscape of viral expression and host gene fusion and adaptation in human cancer. Nat Commun. 2013;4(1).
33. Khoury JD, Tannir NM, Williams MD, Chen Y, Yao H, Zhang J, et al. Landscape of DNA Virus associations across Human malignant cancers: analysis of 3,775 cases using RNA-Seq. J Virol. 2013;87(16):8916–26.
34. Andersen K, Holm K, Tranberg M, Pedersen CL, Bønløkke S, Steiniche T et al. Targeted next generation sequencing for human papillomavirus genotyping in cervical liquid-based cytology samples. Cancers (Basel). 2022;14(3).
35. Salachan PV, Rasmussen M, Fredsøe J, Ulhøi B, Borre M, Sørensen KD. Microbiota of the prostate tumor environment investigated by whole-transcriptome profiling. Genome Med. 2022;14(1).
36. Pinskaya M, Saci Z, Gallopin M, Gabriel M, Nguyen HT, Firlej V et al. Reference-free transcriptome exploration reveals novel RNAs for prostate cancer diagnosis. Life Sci Alliance. 2019;2(6).
37. Strand SH, Switnicki M, Moller M, Haldrup C, Storebjerg TM, Hedegaard J, et al. RHCG and TCAF1 promoter hypermethylation predicts biochemical recurrence in prostate cancer patients treated by radical prostatectomy. Oncotarget. 2017;8(4):5774–8.
38. Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, et al. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature. 1985;314(6006):111–4.
39. Adey A, Burton JN, Kitzman JO, Hiatt JB, Lewis AP, Martin BK, et al. The haplotype-resolved genome and epigenome of the aneuploid HeLa cancer cell line. Nature. 2013;500(7461):207–11.
40. Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J Virol. 1987;61(4):962–71.
41. Chu P, Wu E, Weiss LM. Cytokeratin 7 and Cytokeratin 20 expression in epithelial neoplasms: a Survey of 435 cases. Mod Pathol. 2000;13(9):962–72.
42. Bergh J, Marklund I, Gustavsson C, Wiklund F, Grönberg H, Allard A, et al. No link between viral findings in the prostate and subsequent cancer development. Br J Cancer. 2007;96(1):137–9.
43. Balis V, Sourvinos G, Soulitzis N, Giannikaki E, Sofras F, Spandidos DA. Prevalence of BK virus and human papillomavirus in human prostate cancer. Int J Biol Markers. 2007;22(4):245–51.
44. Arroyo Mühr LS, Lagheden C, Lei J, Eklund C, Nordqvist Kleppe S, Sparén P, et al. Deep sequencing detects human papillomavirus (HPV) in cervical cancers negative for HPV by PCR. Br J Cancer. 2020;123(12):1790–5.
45. Westreich ST, Treiber ML, Mills DA, Korf I, Lemay DG. SAMSA2: a standalone metatranscriptome analysis pipeline. BMC Bioinformatics. 2018;19(1):175.
46. Nkili-Meyong AA, Moussavou-Boundzanga P, Labouba I, Koumakpayi IH, Jeannot E, Descorps-Declère S, et al. Genome-wide profiling of human papillomavirus DNA integration in liquid-based cytology specimens from a Gabonese female population using HPV capture technology. Sci Rep. 2019;9(1):1504.
47. Kulmala SM, Syrjänen SM, Gyllensten UB, Shabalova IP, Petrovichev N, Tosi P, et al. Early integration of high copy HPV16 detectable in women with normal and low grade cervical cytology and histology. J Clin Pathol. 2006;59(5):513–7.
48. Arias-Pulido H, Peyton CL, Joste NE, Vargas H, Wheeler CM. Human papillomavirus type 16 integration in cervical carcinoma in situ and in invasive cervical cancer. J Clin Microbiol. 2006;44(5):1755–62.
49. Ure A, Mukhedkar D, Arroyo Mühr LS. Using HPV-meta for human papillomavirus RNA quality detection. Sci Rep. 2022;12(1).
50. Chang Y-S, Hsu M-H, Tu S-J, Yen J-C, Lee Y-T, Fang H-Y, et al. Metatranscriptomic Analysis of Human Lung Metagenomes from patients with Lung Cancer. Genes. 2021;12(9):1458.
51. Ure AE, Lagheden C, Arroyo Mühr LS. Metatranscriptome analysis in human papillomavirus negative cervical cancers. Sci Rep. 2022;12(1).
52. Arroyo Mühr LS, Hultin E, Dillner J. Transcription of human papillomaviruses in nonmelanoma skin cancers of the immunosuppressed. Int J Cancer. 2021;149(6):1341–7.
53. Liewchalermwong S, Oranratanaphan S, Termrungruanglert W, Triratanachat S, Tantbirojn P, Kitkumthorn N, et al. High-risk human papillomavirus detection via Cobas® 4800 and REBA HPV-ID® assays. Viruses. 2022;14(12):2713.
54. Williams J, Kostiuk M, Biron VL. Molecular detection methods in HPV-Related cancers. Front Oncol. 2022;12:864820.
55. Hesselink AT, Berkhof J, Van Der Salm ML, Van Splunter AP, Geelen TH, Van Kemenade FJ, et al. Clinical validation of the HPV-Risk assay, a Novel Real-Time PCR assay for detection of high-risk human papillomavirus DNA by targeting the E7 region. J Clin Microbiol. 2014;52(3):890–6.
56. Medel-Flores O, Valenzuela-Rodríguez VA, Ocadiz-Delgado R, Castro-Muñoz LJ, Hernández-Leyva S, Lara-Hernández G, et al. Association between HPV infection and prostate cancer in a Mexican population. Genet Mol Biology. 2018;41(4):781–9.
57. Aghakhani A, Hamkar R, Parvin M, Ghavami N, Nadri M, Pakfetrat A, et al. The role of human papillomavirus infection in prostate carcinoma. Scand J Infect Dis. 2011;43(1):64–9.
58. Silvestre RVD, Leal MF, Demachki S, Nahum MCDS, Bernardes JGB, Rabenhorst SHB, et al. Low frequency of human papillomavirus detection in prostate tissue from individuals from Northern Brazil. Memórias do Instituto Oswaldo Cruz. 2009;104(4):665–7.
59. Terris MK, Peehl DM. Human papillomavirus detection by polymerase chain reaction in benign and malignant prostate tissue is dependent on the primer set utilized. Urology. 1997;50(1):150–6.
60. Rotola A, Monini P, Di Luca D, Savioli A, Simone R, Secchiero P, et al. Presence and physical state of HPV DNA in prostate and urinary-tract tissues. Int J Cancer. 1992;52(3):359–65.
61. Malagón T, Louvanto K, Ramanakumar AV, Koushik A, Coutlée F, Franco EL. Viral load of human papillomavirus types 16/18/31/33/45 as a predictor of cervical intraepithelial neoplasia and cancer by age. Gynecol Oncol. 2019;155(2):245–53.
62. Hong D, Liu J, Hu Y, Lu X, Li B, Li Y et al. Viral E6 is overexpressed via high viral load in invasive cervical cancer with episomal HPV16. BMC Cancer. 2017;17(1).

63. Trevisan A, Schlecht NF, Ramanakumar AV, Villa LL, Franco EL. Human papillomavirus type 16 viral load measurement as a predictor of infection clearance. J Gen Virol. 2013;94(8):1850–7.
64. Niller HH, Wolf H, Minarovits J. Viral hit and run-oncogenesis: genetic and epigenetic scenarios. Cancer Lett. 2011;305(2):200–17.

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