

Increased Levels of Human Papillomavirus Type 16 DNA in a Subset of Prostate Cancers¹

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Abstract

Whether oncogenic human papilloma viruses (HPVs) are involved in the pathogenesis of prostate cancers has been a subject of great controversy. To clarify the contradictory results of investigations, with the aim of detecting viral nucleic acids in prostate cancers, we have carried out a comparative quantitation of the HPV16-E6 sequence in 84 prostate specimens. Using single-tube quantitative competitive PCR, we characterized 47 prostate cancers and 37 control tissues of benign prostatic hyperplasia. A subgroup of the prostate tumors (10 of 47; 21%) was detected as having significantly higher copy numbers of HPV16-E6 sequences when compared to the control tissue (1 of 37; 3%), using a cutoff value of 300 copies per 12,500 diploid cells (two-sided Fisher's exact test, $P = 0.02$). Our results indicate that the oncogenic HPV16 might contribute to the development of a subset of prostate tumors.

Introduction

It is assumed that viral infections are involved in the development of at least 15% of human malignancies (1). The detection of viral nucleic acids within tumor cells is one crucial prerequisite to demonstrating an involvement of viral infection in the development of human malignancies (2). Previously reported investigations aimed at the detection of HPVs³ in prostate cancer specimens revealed strikingly contradictory findings of low or no prevalence of the oncogenic HPVs on the one hand and significant or high prevalence on the other hand (for a comprehensive description, see Refs. 3 and 4). Moreover, former studies indicating the presence of viral DNA failed to demonstrate significant differences between malignant and benign prostatic tissue samples. Various reasons for these divergent findings have been discussed. For the pathogenesis of cervical carcinoma, the influence of geographical, ethnical, and individual factors on the detectability of the virus has already been established (5). Furthermore, when explaining these divergent findings, particular methodical problems due to (a) the presumably low copy number of HPVs in prostatic specimens (6, 7), (b) the different sensitivities of the various assays for detection of viral DNA (4, 5, 8), and (c) finally, the to some extent extraordinary histological and genetic heterogeneity of prostate tumors must also be taken into account. In this study, the highly sensitive and quantitative detection of HPV16 DNA is described. In contrast to a purely qualitative detection, the determination of viral sequence copy numbers, normalized to the number of cells analyzed, permits both comparison with quantitative results obtained during future studies and discrimination between the detection of "background" levels of viral DNA,

due to a possibly ubiquitous viral presence or urethral contamination, and probably higher levels of viral DNA, such as those assumed to be caused by an infection of prostatic cells. Here, we report the comparative quantitation of HPV16-E6 sequences in 47 prostate cancer specimens and 37 tissue samples from patients treated for benign prostatic hyperplasia, using the single-tube nested competitive PCR method, as described previously (9).

Materials and Methods

Tissue Samples. Specimens were intraoperatively obtained from 47 patients undergoing radical prostatectomy for the treatment of clinically localized prostate cancer (mean age, 67 years; range, 51–84 years) and from 37 patients surgically treated for BPH by transvesical prostatectomy (mean age, 70 years; range, 53–85 years). Tumor stages were classified according to the tumor-node-metastasis system as follows: T₁ in 5 cases (11%), T₂ in 22 cases (47%), T₃ in 18 cases (38%), and T₄ in 2 cases (4%). Histological grading was as follows: G1, 11 patients (23%); G2, 31 patients (66%) and G3, 5 patients (11%). Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C . For DNA extraction, tumor areas were dissected from the specimens according to the microscopical evaluation of corresponding H&E-stained tissue slides without further enrichment of tumor cells. To avoid contamination with possibly HPV-infected urothelial cells, we dissected the tissue from BPH specimens from intraglandular areas as far away from the urethra as possible. The presence of urothelial cells within the BPH samples was excluded by the microscopical evaluation of the corresponding H&E-stained fresh-frozen slides.

DNA Extraction, Quantitation, and Calculation of Cell Numbers. DNA was extracted using ~ 100 mg of the tissue specimens and a glass particle method (10). Subsequently, purified DNA was fluorimetrically quantitated with the PicoGreen reagent (Molecular Probes, Eugene, OR). For preparation of the standard curve of the fluorimetric assay, a medium scale-purified and spectrophotometrically quantitated DNA from a BPH specimen was used. Interference of RNA contamination with the fluorimetric DNA quantitation was excluded by RNase digestion experiments. Genomic DNA preparations were diluted to a standard concentration of 4.1 ng/ μl DNA and stored in aliquots of 20 μl , corresponding to 82 ng of total DNA-equivalent to $\sim 12,500$ diploid cells, at -80°C for single use only. Diploid cell equivalents were calculated using 3×10^9 bp per haploid genome (11) and M_r 660 per bp, which corresponds to a mass of 6.6 pg per diploid cell.

Quantitation of HPV16 DNA by Competitive Quantitative PCR. Quantitations of HPV16-E6 DNA sequences were carried out using the single-tube variant of competitive PCR, as described recently (9). Purified DNA, corresponding to 12,500 diploid cells (see above) was analyzed in duplicate using 1000 copies of the competitor sequence per reaction. The mean copy numbers were calculated and used for further data evaluation. Copy numbers that were below the limit of linear detection, i.e., ~ 50 copies, corresponding to 1.3 logs below the number of competitor sequences, were set to 0. The specificity of the PCR was additionally confirmed by direct PCR sequencing of selected samples containing high copy numbers of HPV16-E6 sequences (Fig. 1) using the Thermo Sequenase-radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech).

Statistical Analysis. For calculation of the statistical power, the two-sided Fisher's exact test was used.

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³ The abbreviations used are: HPV, human papilloma virus; BPH, benign prostatic hyperplasia.

Results and Discussion

Epidemiological data, in addition to cell culture experiments, indicate a possible role of HPVs for the pathogenesis of prostate tumors (12–14). However, this issue is controversial, in large part due to contradictory results regarding the detection of viral nucleic acids within prostate cancer. To our knowledge, this study is the first to describe both the highly sensitive and quantitative detection of the HPV16-E6 sequence in adenocarcinoma of the prostate (Fig. 2).

Our first finding, which became evident during the initial screening for the optimum competitor concentration (data not shown), was the low copy number detectable in most of the tumors and benign control samples. On average, only ~100–200 HPV16-E6 sequences per 12,500 diploid cells (Fig. 3, A and B) were detected, which means that, in most cases, only 1 or 2 copies of the HPV16-E6 sequence were present per 100 diploid cells. This result supports conclusions drawn from earlier investigations on the presence of HPV16 and HPV18 DNA in prostate specimens (6, 7).

One result of our quantitative study became evident upon examination of the frequency distribution shown in Fig. 3, A and B. The

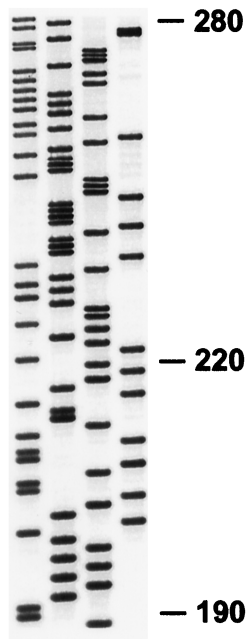


Fig. 1. Direct PCR sequence analysis of a HPV16-E6 amplicon of a selected prostate cancer specimen previously identified by competitive quantitative PCR analysis as containing increased copy numbers of HPV16. The numbers indicate the corresponding nucleotide positions of the complete circular DNA of HPV16 according to Ref. 17.

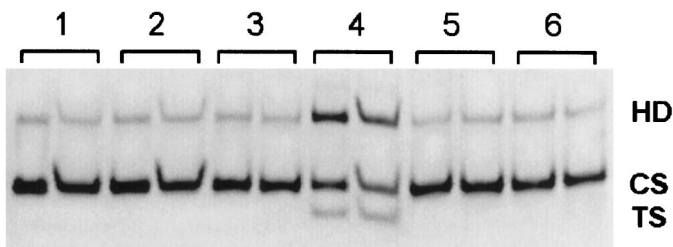


Fig. 2. Single-tube competitive PCR analysis of six prostate cancer samples (double measurements, inverse illustration of the ethidium bromide-stained gel). Sample 4 exhibited a higher copy number of HPV16-E6 sequences, indicated by the increased signals of the heteroduplex band (HD, 125 bp) and the target sequence band (TS, 95 bp) in relation to the signal of the competitor sequence (mutant HPV16-E6 sequence; CS, 98 bp). Note that the exact copy numbers were calculated from the three relative signals, as described previously (9).

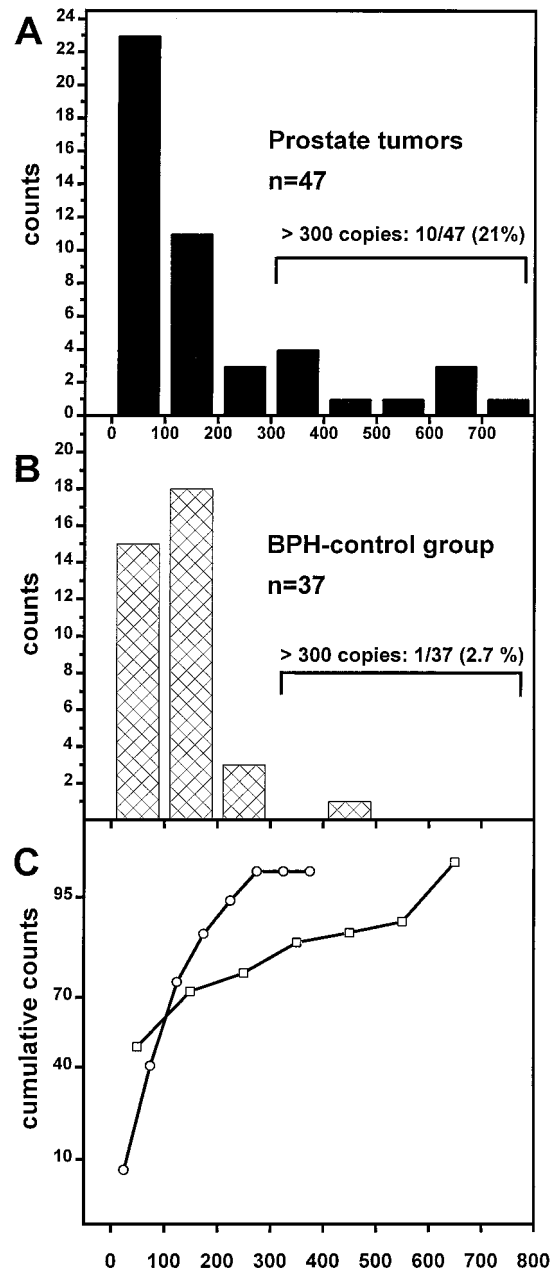


Fig. 3. A, frequency distribution of detected HPV16-E6 sequence copy numbers in prostate cancers (■). B, frequency distribution of detected HPV16-E6 sequence copy numbers in BPH control specimens (▨). C, probability plot of prostate cancer copy numbers (□) and BPH control copy numbers (○).

proportion of both HPV16-positive malignant and benign specimens, as would be detected by a purely qualitative assay, strongly depends upon the sensitivity of the respective assay. Decreasing the limit of detection would increase the number of positive samples detected and vice versa. Therefore, our data offer direct experimental evidence of the notion that different assay sensitivities of the various PCR variants are one reason for the contradictory results reported thus far (8).

The most interesting finding of our quantitative analysis is, however, the identification of a subset of the prostate cancers characterized by an increased copy number of HPV16-E6 DNA compared with benign control samples (see Figs. 3, A and B). Using a cutoff value of 300 copies, as suggested by Fig. 3, 10 of 47 (21%) prostatic carcinomas, in contrast to only 1 of 37 (3%), BPH controls were positive for HPV16 (Fig. 4). Although the number of samples analyzed is still

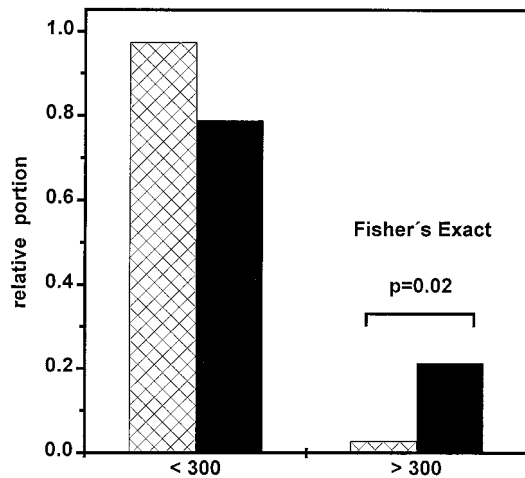


Fig. 4. Relative amounts of prostate cancer (■) and BPH samples (▨) above and below the cutoff value of 300 HPV16-E6 copies. The indicated statistical power was evaluated using the two-sided Fisher's exact test.

limited, this difference is statistically significant (two-sided Fisher's exact test, $P = 0.02$). As indicated by Fig. 3C, a moderate variation of the cutoff value used does not substantially alter the statistical significance of this result. However, the size of the corresponding subgroups would vary (data not shown). Therefore, our conclusion is that this statistical analysis is much more likely to demonstrate that a subgroup of prostate cancers exhibit increased copy numbers of HPV16 than to exactly define the absolute number of patients included in both groups. Consequently, the most important question is how to explain the elevated copy numbers detected in a subset of the prostate cancers as well as in one specimen of the control group. Additionally, the observation of intermediate copy numbers (200–300 copies), as found within both groups, remains to be explained. The detection of intermediate copy numbers, however, is most likely related both to the known histological and genetic heterogeneity (15) of each sample itself and the various tumor stages and histological differentiations of prostate cancers investigated. Moreover, small latent carcinomas, which occur in 30–50% of all prostates (16), might remain undetected within single BPH specimens during routinely performed histopathological examination. Therefore, the high HPV16 level that was observed in one of our BPH samples may result from unrecognized microscopic carcinoma.

In principal, the higher copy numbers of viral DNA detected in a subset of prostate cancer specimens compared with BPH samples might be due to either an intracellular or extracellular localization of the viral DNA. Consequently, two different explanations can be discussed. In the case of an intracellular localization of the HPV16-DNA, it can be concluded from the copy numbers detected in the HPV-positive tumor group and the assumption of a single viral DNA per diploid genome that the cells bearing the virus are either present within the sample with low frequency (estimated at ~3–8%) or that

only a fraction of the cells is carrying the viral DNA. Another explanation could be that focal infection of prostatic epithelial cells could likewise have been detected due to an intraglandular spread of the viruses themselves or of infected cells.

In conclusion, our data give additional evidence to explain some of the previously reported contradictory findings regarding the prevalence of viral DNA in prostate cancer and BPH specimens. However, to further analyze the potential role of oncogenic HPVs in a subset of prostate cancers, additional quantitative investigations, aiming at the detection of viral DNA on the cellular level, will be necessary.

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