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ORIGINAL ARTICLE

# Low prevalence of HPV detection and genotyping in non-muscle invasive bladder cancer using single-step PCR followed by reverse line blot

**Renate Pichler[1] · Wegene Borena[2] · Georg Schäfer[3] · Claudia Manzl[3] · Zoran Culig[1] · Sebastian List[1] · Sabrina Neururer[4] · Dorothee Von Laer[2] · Isabel Heidegger[1] · Helmut Klocker[1] · Wolfgang Horninger[1] · Hannes Steiner[1] · Andrea Brunner[3]**

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**Abstract**
*Purpose* To clarify the role of human papillomavirus (HPV) in non-muscle invasive bladder cancer, HPV-DNA was scrutinized in formalin-fixed, paraffin-embedded (FFPE) bladder cancer tissue using single-step PCR (HPV L1) for HPV detection, followed by reverse line blot (RLB) for genotyping.
*Methods* A total of 186 patients who underwent transurethral resection of the bladder due to primary, non-muscle invasive bladder cancer from 2006 to 2009 were reviewed. A positive control group of 22 cervical tissues with cervical carcinoma was included.
*Results* Histology confirmed urothelial carcinoma in all patients: primary CIS, pTa, pT1 and pTa + pT1 in 14 (7.5 %), 134 (72 %), 36 (19.4 %) and two (1.1 %) patients, respectively. A total of 119 (63.9 %) of them were classified as low-risk, while 67 (36.1 %) were high-risk cancers. Tumor recurrence and progression (≥pT2) were seen in 79 and 11 patients (mean follow-up 45 months). The presence of HPV-DNA by single-step PCR was detected in four (2.2 %) patients. HPV 16 and HPV 6 were positive in two (1.1 %) and one (0.6 %) patient, respectively In one case, no HPV genotype listed on the RLB assay could be identified. In the control group, the HPV infection rate was 100 %: HPV 16 in 12 (54.6 %) patients, HPV 16/18 in four (18.3 %) patients, HPV 18 in two (9.1 %) patients, HPV 16/45 in one patient (4.5 %), HPV 18/33 in one (4.5 %) patient, HPV 16/33 in one (4.5 %) patient and HPV 33 in one (4.5 %) patient.
*Conclusions* Our study demonstrates low prevalence of HPV infection in FFPE bladder cancer tissue, arguing against the etiological role of HPV in non-muscle urothelial carcinogenesis.

**Keywords** Bladder cancer · Human papillomavirus · Infection · Carcinogenesis · PCR · Hybridization

## Introduction

Human papillomavirus (HPV) infections have been shown to play a significant role in the carcinogenesis of cervical, penile and anogenital tumor. Furthermore, high-risk HPV has been suggested to contribute to malignant progression in head and neck, lung, breast and also bladder cancer [1]. Various epidemiological studies evaluated the relationship between HPV infection and bladder cancer with controversial results regarding HPV prevalence between 0 and 60 % [2–4]. A recent meta-analysis included 2855 cases of bladder cancer confirming that the HPV incidence varied by region (highest rate in Asia), types of HPV-DNA specimen (higher in fresh samples than in fixed tissues) as well as PCR primers used [5]. The risk of HPV infection may be associated with the histological subtype of bladder cancer with an increased incidence in primary squamous differentiation (up to 17.1 %) [6], schistosomal cystitis and concomitant dysplasia or bladder cancer associated with schistosomiasis (up to 48.97 %) [7].

✉ Andrea Brunner
Andrea.Brunner@i-med.ac.at

1 Division of Experimental Urology, Department of Urology, Medical University of Innsbruck, Innsbruck, Austria

2 Section of Virology, Department of Hygiene, Microbiology and Social Medicine, Innsbruck, Austria

3 Division of General Pathology, Department of Pathology, Medical University Innsbruck, Innsbruck, Austria

4 Department of Medical Statistics, Informatics and Health Economics, Müllerstrasse 44A-6020, Innsbruck, Austria

Currently, more than 100 HPV assays are commercially available with different genotyping capacities [8]. Important for genotype analyses are those areas that identify the type-specific polymorphism. The late region gene L1 is the most polymorphic, highly conserved, and therefore a reliable region for genotyping based on PCR. Sequence polymorphism exists especially in the early region genes, E6/E7 being responsible for the oncogenic transformation potential of individual HPV types [9, 10]. Based on PCR amplification of virus DNA fragments, HPV genotyping can be performed by several molecular methods like reverse line blot (RLB) hybridization [11] or detecting amplified genotype-specific DNA by selective hybridization with oligonucleotides immobilized on nylon membranes. Using RLB assays, individual HPV genotypes can be classified with a very high sensitivity [9, 11]. The use of direct nucleic acid probe methods for HPV analysis on FFPE tissue is not recommended because detection of viral nucleic acids can be complicated by DNA/RNA degradation due to fixation or prolonged storage [12]. Therefore, we screened for HPV-DNA in the largest collection of bladder cancer specimens currently described in the literature using a reliable assay, which allowed us to amplify the L1 gene by real-time PCR, followed by RLB for genotyping.

## Materials and methods

The study has been approved by the local ethical committee (study number UN4974; 322/4.18). All patients underwent transurethral resection of the bladder (TURB) due to primary non-muscle invasive bladder cancer (NMIBC) at the Urology Department of Innsbruck. Demographic and histopathological characteristics of the study population such as patient age, gender, body mass index (BMI), preoperative C-reactive protein (CRP), tumor staging (according to TNM 2009), tumor grading (according to WHO 1973 and 2004), cancer recurrence and progression during a minimum follow-up of 3 years were recorded. A positive control group of 22 FFPE cervical tissues with invasive cervical carcinoma was included. A flowchart of the study design is shown in Fig. 1.

### Tissue selection and DNA extraction

After obtaining written consent, tumor samples were re-evaluated for tumor grade and stage by two experienced uropathologists who also selected and marked the specific areas of interest on H&E-stained slides of relevant paraffin

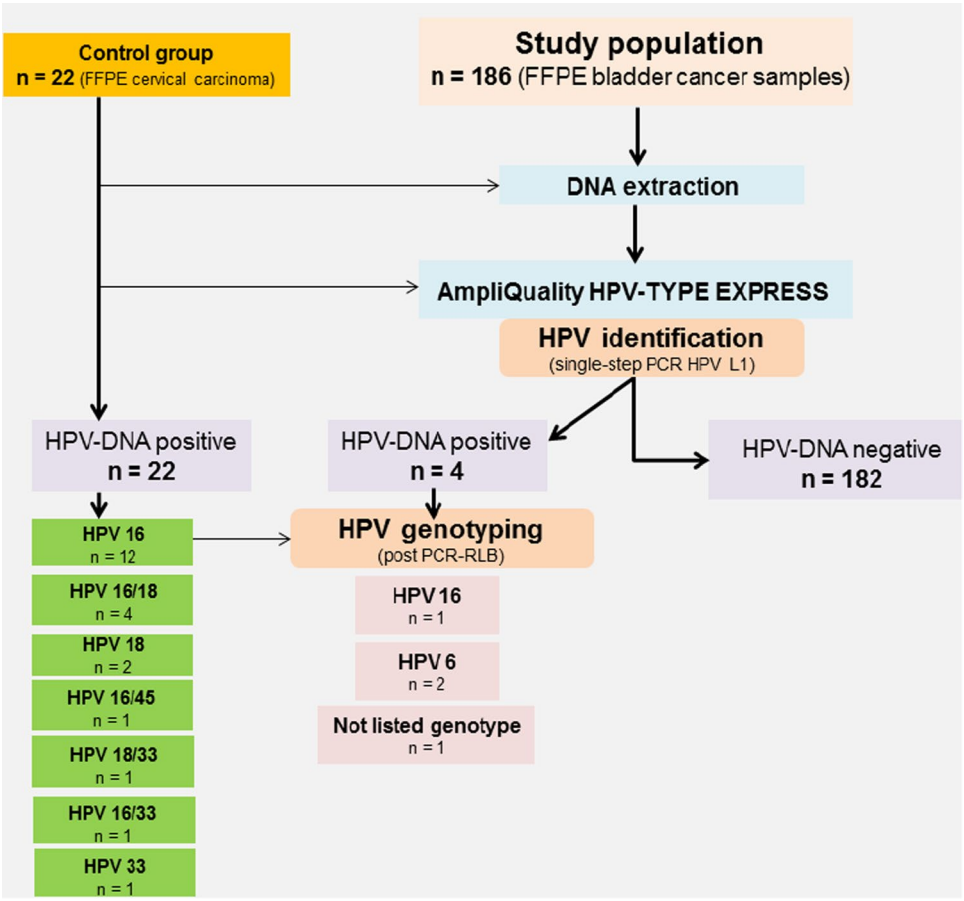


**Fig. 1** Flowchart of the study design. After DNA extraction, HPV detection and genotyping were performed by AmpliQuality HPV-Type Express assay in 186 FFPE urothelial cancer samples and a control group of 22 cervical carcinomas

blocks used for HPV testing. Genomic DNA was extracted from FFPE samples using EZ1 QIAGEN DNA easy kit (Qiagen, Düsseldorf, Germany) as recommended by the manufacturer's protocol.

Briefly, for DNA extraction, up to three tissue slices (5–10 μm) per specimen were cut using a new microtome blade for every specimen. The initial scroll of each specimen was discharged. Tissue samples were then transferred into a 1.5-ml screw-capped tube and 190 μl of lysis buffer G2 was added and incubated at 75 °C for 5 min. Thereafter, samples were cooled down to room temperature and 10 μl of proteinase K solution (600 mAU/ml) was added, mixed gently and incubated at 56 °C overnight. After a centrifugation step (2.000 rpm, 2 min), supernatant was transferred into a fresh 2-ml vial (Eppendorf, Germany) and subsequently processed on the EZ1 workstation using the EZ1 Paraffin Card. After approximately 20 min, genomic DNA isolation was finished and the concentration of DNA was evaluated by photometric analysis. The DNA was stored at 2–8 °C before HPV testing.

### Detection and genotyping of HPV

HPV testing and genotyping were conducted at the Virology Division of Medical University of Innsbruck. The kit AmpliQuality HPV-TYPE EXPRESS (AB Analytica, Padova, Italy) was used for both identification and genotyping of HPV by single-step PCR and RLB hybridization, respectively. This test kit is under use (not only for cervical samples) since the last couple of years. Quality of the test kit is guaranteed through regular external quality assessment procedures including evaluations from the virology section of the University of Köln, a national HPV reference center for Germany. Moreover, our test results show compatibility with those from WHO HPV LabNet proficiency study for international comparability of test.

Real-time PCR allows the detection of HPV by amplification of the gene region encoding the L1 protein based on SYBR Green detection and subsequent melting curve analysis. A dUTP/UNG system for the prevention of contamination due to carryover is included. To control for DNA quality, β-globin (BG) internal control fragment is amplified in parallel.

Subtype classification is based on direct amplicon genotyping on strips. Distinction of the different virus genotypes is based on reverse hybridization of the biotinylated viral L1 gene PCR product with specific probes immobilized on a nylon strip. Labeling of the PCR product is performed during the amplification. The assay allows the identification of 40 genotypes: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68a/b, 69, 70, 71, 72, 73, 81, 82, 83, 84, 87, 89, and 90. In addition, a specific probe for TST gene detection, used as an amplification control of the extracted DNA, is included. For other HPV genotypes, the hybridization will give a positive result to a universal HPV sequence on the strip. This genotyping test recognizes only the virus DNA sequences that are bound by the probes contained in this test. In case of ambiguous results, DNA sequencing may provide additional information.

All statistical analyses were performed using SPSS 21.0 (IBM SPSS Statistics for Windows). Figures were designed using GraphPad Prism (version 5.0 for Mac OS X, GraphPad Software, La Jolla, California, USA). The Kolmogorov–Smirnov test was used to test for normal distribution. The unpaired Student's $t$ test, Mann–Whitney $U$ test and Fisher's exact test were used to analyze intergroup differences. All statistical tests were done two-sided, and $p$ values $<0.05$ were considered statistically significant.

## Results

A total of 186 patients (149 men and 37 women) with a mean (range) age of 72 (24–93) years and a mean (range) BMI of 25.2 (14.1–40.4) kg/m$^2$ fulfilled the inclusion criteria and were included in the study population. All patients have been treated for primary bladder cancer with TURB between January 2006 and December 2009. Histological evaluation confirmed urothelial carcinoma in every patient: pTa in 134 (72 %) patients, pT1 in 36 (19.4 %) patients, primary carcinoma in situ (CIS) in 12 (7.5 %) and multifocal tumors (pTa in combination with pT1) in two (1.1 %) patients. Regarding tumor grading (according to WHO 1973), G1, G2, and G3 were noticed in 75 (40.3 %), 77 (41.4 %) and 34 (18.3 %) patients, respectively. A total of 119 (63.9 %) of them were reclassified as low-risk, and 67 (36.1 %) as high-risk cancers (according to WHO 2004). Recurrence and progression (defined as ≥pT2 tumor) were identified in 79 (42.4 %) and 11 (5.9 %) patients after a mean follow-up of 45 (35–83) months, respectively. Gender-related demographic and histopathological characteristics of the study population are summarized in Table 1. DNA was efficiently extracted from all FFPE tissue samples, and its integrity was tested by PCR amplification of human BG housekeeping gene. Four (2.2 %) of the 186 tested bladder cancer samples were positive for HPV-DNA. Low-risk HPV 6 was detected in 1.1 % of samples ($n = 2/186$). A high-risk HPV 16 genotype was identified in one sample (0.6 %). In one case, genotyping was not possible as the HPV type was not included in the RLB hybridization assay. HPV 6 was associated with low-risk tumor (pTa G1) in both positive patients, while HPV 16 was found in high-risk cancer (pTa G3). All four HPV-positive patients did not show tumor progression and recurrence after a mean (range) follow-up of 38 (36–47) months.

**Table 1** Gender-related baseline characteristics of study population

| Factors | Men | Women | *p* value |
|---|---|---|---|
| *n* | 149 | 37 | |
| Age (years) | 70.2 + 11.1 | 67.0 + 14.3 | 0.143 |
| BMI (kg/m)* | 24.3 ± 7.3 | 23.6 + 7.6 | 0.315 |
| CRP (mg/dl) | 0.95 + 1.4 | 0.53 + 0.9 | 0.010* |
| Staging [*n* (%)] | | | |
| pTa | 108 (72.5) | 26 (70.3) | |
| pT1 | 27 (18.1) | 9 (24.3) | 0.710 |
| Primary CIS | 12 (8.1) | 2 (5.4) | |
| pTa + pT1 | 2 (1.3) | – | |
| Grading (WHO 1973) [*n* (%)] | | | |
| G1 | 63 (42.3) | 12 (32.4) | |
| G2 | 58 (38.9) | 19 (51.4) | 0.382 |
| G3 | 28 (18.8) | 6 (16.2) | |
| Grading (WHO 2004) [*n* (%)] | | | |
| Low risk | 99 (66.4) | 20 (54.1) | 0.160 |
| High risk | 50 (33.6) | 17 (45.9) | |
| Recurrence [*n* (%)] | 65 (43.6) | 14 (37.8) | 0.524 |
| Progression [*n* (%)] | 9 (6.1) | 2 (5.4) | 0.884 |
| Follow-up (months) | 44.1 ± 27.0 | 45.1 + 29.0 | 0.844 |
| HPV prevalence [*n* (%)] | 2 (1.3) | 2 (5.4) | 0.127 |

Statistical analysis revealed a higher level of preoperative CRP in male patients compared to women (* $p = 0.010$). All other characteristics showed no statistically significant differences

In the cervical carcinoma control group, the HPV infection rate was 100 %: HPV 16 positivity in 12 (54.6 %), HPV 16/18 in four (18.3 %), HPV 18 in two (9.1 %), HPV 16/45 in one (4.5 %), HPV 18/33 in one (4.5 %), HPV 16/33 in one (4.5 %) and HPV 33 in one (4.5 %) patient.

## Discussion

High-risk HPV infection is the most important cause regarding cervical cancer being linked also to malignant progression in tumors of the anogenital and oropharyngeal entity. The HPV-DNA genome is expressing high levels of viral oncogenes like E6 and E7, which block cellular pRb and p53 proteins and thus induce cell cycle activation, cell growth and stimulate viability [13, 14]. As a result of pRB targeting by the viral E7 protein, cyclin D1 and cyclin inhibitors, like p16INK4a, are overexpressed in HPV-transformed cells [14, 15]. Therefore, immunohistochemistry for p16INK4a is a surrogate biomarker of HPV-E7 expression and HPV-transformed cells, which is used for histopathology and cytology in order to increase the diagnostic accuracy and sensitivity for detection of cervical precancer lesions. Interestingly, p16INK4a overexpression is common in different histological subtypes of bladder cancer including adenocarcinoma (67 %), urothelial carcinoma with squamous differentiation (45.7 %), high-grade urothelial cancers (85.9 %) and urothelial CIS (92.6 %). However, contrary to gynecological tumors, p16INK4a is not a surrogate marker for evidence of HPV infection in bladder cancer [6, 16–18].

In contrast to indirect HPV screening by IHC staining, detection of HPV-DNA is a direct, although more elaborate, method. HPV-DNA detection can be achieved by direct nucleic acid probe methods (Southern blotting or ISH), hybridization signal amplification methods (hybrid capture II kit or target nucleic acid amplification) and most notably PCR-based target amplification methods (consensus primers or genotype-specific primers) [9].

Although no exact consensus exists about the molecular technology that should be used for HPV detection in FFPE tissue, PCR with type-specific primers represents the gold standard with an overall HPV-DNA detection rate of 33.2 % in bladder cancer [5]. As post-PCR hybridization assays are a suitable alternative for the parallel detection of a larger number of different PCR products, we performed RLB, which captures and detects specific subtypes on a membrane support. RLB has the advantage that specific HPV genotypes and multiple infections can be detected, but has the disadvantage of being time consuming and laborious [19]. To the best of our knowledge, this is the first study using post-PCR RLB hybridization technique for HPV-DNA detection in FFPE bladder cancer tissue. HPV was detected most frequently in low-grade carcinoma [20] which can be explained by the different biologic pathways in the carcinogenic process of non-muscle invasive versus invasive bladder cancer: While low-grade cancer is associated with chromosome nine allelic loss and p16 overexpression, high-grade and invasive carcinoma is linked to lack of p16 expression and p53 mutation [20–22]. Therefore, we focused on NMIBC samples in this trial.

Various studies have addressed a possible relationship between the prevalence of HPV-DNA and bladder cancer recently, but convincing evidence for a potential role of HPV in bladder carcinogenesis is still missing. Results remain controversial depending mostly on the HPV-DNA detection method used. This fact may explain the discrepancies between several study results [2]. An overview of published studies regarding HPV prevalence among patients with bladder cancer is shown in Table 2. For example, 78 paraffin-embedded urothelial cancer samples were tested for the presence of HPV comparing both PCR (GP5 +/6 + primers) and IHC (polyclonal rabbit antibody against HPV capsid protein). Contrasting results regarding HPV positivity (IHC 60.2 % vs. PCR 0 %) might have been caused by false positives resulting from the IHC as a non-specific, polyclonal rather than a monoclonal antibody was used in this study [3]. In addition, a lower rate of positivity

**Table 2** Overview of published studies on HPV prevalence among patients with bladder cancer

| HPV detection method | Author | No. of cases | HPV-DNA specimen | HPV prevalence (%) |
|---|---|---|---|---|
| *PCR-based* | | | | |
| HPV-L1 | Yavuzer et al. [31] | 70 | FFPE | 0 |
| HPV-L1/E1 | Knowles et al. [33] | 109 | FFPE | 0 |
| HPV-L1 | Panagiotakis et al. [32] | 30 | FFPE | 0 |
| HPV-L1 + RLB | Current study | 186 | FFPE | 2.2 |
| *Direct nucleic acid probe* | | | | |
| ISH | De Gaetoni et al. [34] | 43 | FFPE | 39.5 |
| ISH (biotin-labeled DNA) | Lu et al. [35] | 31 | FFPE | 0 |
| *Immunohistochemistry* | | | | |
| IHC | Alexander et al. [18] | 36 | FFPE | 67 (p16)<br>58 (p53) |
| *Combined methods* | | | | |
| PCR (HPV-L1)<br>IHC (HPV-L1) (p16INK4a) | Shigehara et al. [20] | 117 | FFPE | 15<br>0<br>13.6 |
| ISH<br>IHC (p16INK4a) | Alexander et al. [4] | 69 | FFPE | 0<br>31 |
| RT-PCR (HPV-L1)<br>IHC | Youshya et al. [3] | 78 | FFPE | 0<br>60 |
| IHC (polyclonal antibody HPVc Ag)<br>ISH (HPV 6/11, 16/18, 31/33/35)<br>PCR | Lopez-Beltran et al. [23, 24] | 76 | FFPE | 32.8<br>15.7<br>9 |

Results are grouped according to the applied HPV detection method

for HPV 16-DNA (9.21 %) was confirmed by PCR compared to HPV capsid antigen (32 %) by IHC and ISH (15.7 %) using a biotinylated DNA probe that recognizes HPV 6/11, HPV 16/18 and HPV 31/33/35 in 76 bladder cancer samples [23, 24]. In a previous work by our study group, the prevalence of HPV in preputial FFPE samples of 250 asymptomatic boys and men was investigated by RT-PCR and in situ hybridization (ISH) with discrepant results, too. ISH verified a significantly higher prevalence of high-risk and low-risk HPV (49.8 and 41.9 %) than real-time PCR (4 and 5.8 %) [25, 26].

Such differences are most likely due to the diverse analysis methods and sample heterogeneity [23]. Type and time of fixation can influence the preservation of DNA. Some fixation methods damage DNA, affecting subsequent analysis. For example, formalin fixation has an inhibitory effect on real-time PCR, resulting in an underestimation of starting targeting DNA quantity [27]. The sensitivity of the PCR is strongly influenced by the size of the PCR product and of the input DNA [28]. In addition, consensus primer PCR assays frequently underestimate the HPV prevalence in FFPE tissue compared to type-specific primer amplification as the use of consensus primers results in amplification products of different sizes and thus different levels of detection sensitivity [29]. A high rate of false-negative results in FFPE material is the result of damaged and fragmented DNA [30]. Therefore, to avoid false-negative results, we used a HPV identification assay which included amplification of a BG gene fragment as an internal control, detected on the strip by a dedicated band, to ensure sufficient extracted DNA quantity and quality and all samples passed this test.

## Conclusions

In conclusion, HPV infection seems to have no influence on non-muscle invasive urothelial carcinogenesis. Therefore, routine screening for HPV infection seems not to increase the prognostic accuracy in urothelial cancer neither in women nor in men. Regarding post-PCR-based RLB hybridization technique, adequate DNA quantity and quality were provided using BG gene amplification; amplification step included the dUTP/UNG system to prevent carryover contamination; a simultaneous control group confirmed the validity of this assay. Taken together, the applied protocol and the included quality controls excluded false-negative results with high probability.

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**Conflict of interest** The authors had no conflicts of interest to declare.

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