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# Human papillomavirus detection in fine needle aspiration cytology of lymph node metastasis of head and neck squamous cell cancer

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## ARTICLE INFO

## ABSTRACT

*Background:* Currently, testing on HPV in oropharyngeal squamous cell carcinoma (OPSCC) is performed on histological material. However, in a certain percentage of the cases who present with lymph node metastases no primary tumor can be identified and only fine needle aspiration cytology (FNAC) is available for analysis.
*Objectives:* Purpose of this study was to assess HPV status on FNAC and to validate it using histological material of the same patients.
*Study design:* Patients with cervical metastasis from OPSCC or cancer of an unknown primary tumor (CUP), diagnosed between 2007 and 2012 were included. In 6 of the 47 patients, no primary tumor could be identified. HPV detection and genotyping was performed in both FNAC slides scrapings and formalin fixed paraffin embedded (FFPE) histological material from the same patients, using the HPV $SPF_{10}$-$LiPA_{25}$ assay. HPV PCR analysis on FFPE material was considered the reference standard for HPV status of each case.
*Results:* Compared with HPV negative cases (n = 22), significantly more HPV positive cases (n = 25) presented initially with cervical metastasis (27% vs 56% respectively; p = 0.047). The HPV PCR assay on FNAC material showed a high sensitivity (96%; 95% CI 86.6–97.4) and specificity (100%; 95% CI 85.1–96.7) using the reference standard of HPV PCR analysis on FFPE material of the same patients.
*Conclusion:* In this study, testing on HPV in FNAC of cervical lymph node metastases of SCC is validated. It provides a valuable alternative for testing of HPV on histological material from patients with oropharyngeal squamous cell carcinoma or cancer of an unknown primary tumor.

## 1. Background

In the last decade it became evident that viral integration of high-risk human papillomavirus (hrHPV) types, especially HPV type 16, is an important factor in the oncogenesis of oropharyngeal squamous cell carcinoma (OPSCC) [1,2]. Through overexpression of the HPV oncoproteins E6 and E7, as a result of disruption of E2 after HPV-DNA integration into the host DNA, the host tumor-suppressor proteins p53 and retinoblastoma (Rb) are degraded. This subsequently leads to p53 dysfunction and p16$^{INK4A}$ upregulation [3,4]. This mechanism is responsible for the distinct molecular, clinical and pathological entity of HPV-related OPSCC as compared with HPV-unrelated tumors, which are associated with alcohol and smoking habits [1,5]. The prevalence of HPV-related OPSCC is rising and reported with a reported wide range between 20% and 90% [6,7].

It has become evident that HPV-positive OPSCCs patients have a better survival outcome with a significant reduction in mortality in comparison with patients with HPV negative tumors. This is thought to be partly due to a favorable response to ionizing

radiation and chemotherapy regimes, attributed to an intact p53 apoptosis pathway [8,9]. Therefore, HPV status has become a major prognostic factor in patients with OPSCC.

Despite extensive diagnostic workup, including a thorough clinical and endoscopical examination with the taking of biopsies and the use of several imaging techniques, two to five percent of cases who present with neck nodes containing metastatic SCC remain with an unidentified primary site of origin [10,11]. This is thought to be due to a possible involution of the primary tumor or a slow growth rate of the primary tumor as compared to their metastases, resulting in a very small and undetectable primary tumor with a more advanced N-stage [5]. In particular in case of a primary location in the tonsillar crypts and the base of the tongue such a small primary tumor may remain undetected. So it may be hypothesized that at least a part of the patients with cancer of an unknown primary tumor (CUP) could in fact suffer from small undetected OPSCCs. Therefore, important diagnostic and prognostic information can be expected to be provided by the HPV status of the cancer cells in CUP, as it may point towards the most likely site of origin of the primary tumor in case of HPV-positivity, namely the oropharynx. Testing on HPV in this category of patients with CUP to localize the primary tumor site in the oropharynx is an accepted concept now.

Currently, the HPV status of OPSCC is commonly determined through the accepted algorithm of HPV detection by PCR combined with p16 immunohistochemical (IHC) staining on histological tumor material [12,13]. However, in case of CUP, no histological material of a primary tumor is available for HPV analysis. If HPV status can be reliably established by testing on material obtained by fine needle aspiration cytology (FNAC) of metastatic lymph nodes, it can be used to locate the likely origin of the primary HNSCC in CUP without the necessity to obtain material from a neck dissection specimen.

To validate if testing on cytological material matches with testing on histological material, we evaluated HPV-PCR analysis in FNAC from cervical lymph node metastasis and compared it to the golden standard of HPV-PCR on histological material from the same patients. Although HPV analysis in FNAC material has been reported previously, the validation using histological material of the same patients has not been done before as far as we are aware of.

## 2. Study design

### 2.1. Case selection

FNAC samples obtained from metastatic lymph nodes of patients diagnosed with OPSCC or CUP of the head and neck diagnosed between 2007 and 2012 were identified and retrieved from the files of the Department of Pathology of the Radboud University Medical Center in Nijmegen, The Netherlands. Formalin fixed paraffin embedded (FFPE) histological material taken from the primary tumor or metastatic lymph node of the corresponding cases was retrieved as well.

Cases were included if the FFPE material was both HPV and p16 IHC negative or both HPV and p16 IHC positive. p16 IHC was considered positive in cases that showed both diffuse and intense nuclear and cytoplasmic staining in almost all tumor cells. Exclusion criteria were: a second primary tumor in the head and neck region to avoid uncertainty about the relationship between primary tumor and metastasis, insufficient cytological material for processing, and previous exposure of the neck to radiotherapy.

Patient medical records were reviewed to document the primary tumor site, metastatic lymph node location and general histopathological characteristics of the samples.

### 2.2. Tissue preparation and DNA purification

The detection and genotyping of HPV was performed on scraped FNAC material from archival slides. The FNAC slides were reviewed for determining tumor cell density and representativeness. After soaking in xylene to remove the coverglass, the cytological material on the slides was completely scraped off and transferred into 200 μL phosphate buffered saline (PBS) suspension. Isolation and purification of the DNA was performed with MagNa pure 96 (Roche Molecular Diagnostics). The purified DNA was diluted in 50 μL in elution buffer (Roche Molecular Diagnostics) and stored in −20 °C until further processing by PCR. In the process, internal extraction and amplification controls were included to ensure a valid en reliable procedure.

DNA was isolated from FFPE tissue sections (4 μM) with the EZ1 robot (Qiagen, Germany, with the DNA tissue kit of Qiagen) according to standard procedures [14] and used for PCR analysis. A negative water control was included with each batch of 10 samples.

#### 2.2.1. HPV-DNA detection and typing

Broad-spectrum HPV-DNA amplification was performed using a short-PCR-fragment assay (HPV SPF$_{10}$-LiPA$_{25}$, version 1; Labo Biomedical Products B.V, Rijswijk, Netherlands). This assay amplifies a 65-bp fragment of the L1 open reading frame of HPV genotypes, as described by Melchers et al. [14] HPV genotyping was performed using a cocktail of 9 conservative probes in a micro titer hybridization assay, the DNA enzyme immunoassay (DEIA). The samples positive for HPV by DEIA were then analyzed with the line probe assay (LiPA25) by reverse hybridization with type-specific probes for HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74. The LiPA strips were visually inspected and interpreted following the standardized reference guide. Phocine Herpesvirus (PhHV) was used as an internal control for amplification.

#### 2.2.2. Statistical analysis

Pathological and clinical characteristics were assessed using a contingency table $\chi^2$ tests. By applying cross tabulation sensitivity, specificity, positive predictive value and negative predictive value were calculated (and 95% confidence intervals (CI)) for final HPV 16 classifications compared with the 'golden standard'. Cohen κ was used to analyze the measure of agreement between histological and cytological HPV analysis by means of SPF-10 assay. Statistical analysis was conducted using the statistical software package SPSS 20.0 (IBM corporation, 2011).

## 3. Results

### 3.1. Clinical and pathological characteristics

In our institute approximately 100 OPSCCs and 10 CUPs are diagnosed and treated annually. Based on the availability of histological material already tested on HPV (in routine practice) and the availability of complementary FNAC material of these cases a selection was made. The inclusion criteria were met by 47 cases. In total, 25 HPV 16 positive cases and 22 HPV negative cases were included for analysis. Compared with HPV negative cases (n = 22), significantly more HPV positive cases (n = 25) presented initially with a metastasis to a regional lymph node (27.3% vs 56% respectively; p = 0.047).

The characteristics of both groups are presented in Table 1. The mean ages of both cohorts was similar (mean 57.7 years vs. 58.4 years; p = 0.4) as well as the distribution of gender in the two groups (p = 0.36). No clear difference in primary location between the two groups was observed (p = 0.48). Twenty cases presented initially with SCC in the neck only. In six out of the total of 47 cases (13%) no

**Table 1**
Patient group and histological characteristics.

| Variables | HPV positive (%)[a] N = 25 | HPV negative (%)[b] N = 22 | Total (%) N = 47 | p-value |
|---|---|---|---|---|
| Gender | | | | |
| – Male | 6 (24%) | 14 (64%) | 33 (70%) | 0.355 |
| – Female | 19 (76%) | 8 (36%) | 14 (30%) | |
| Age | | | | |
| – Mean | 57.7 yr | 58.4 yr | 58.0 yr | 0.432 |
| – Range | 34.6–77.3 yr | 28.9–71.3 yr | 28.9–77.2 yr | |
| Tumor stage[c] | | | | |
| – T0 | 4 (16%) | 2 (9%) | 6 (13%) | 0.129 |
| – T1 | 5 (20%) | 2 (9%) | 7 (15%) | |
| – T2 | 11 (44%) | 5 (23%) | 16 (34%) | |
| – T3 | 4 (16%) | 7 (32%) | 11 (23%) | |
| – T4 | 1 (4%) | 6 (27%) | 7 (15%) | |
| Nodal stage[c] | | | | |
| – N0 | 1 (4%) | 1 (5%) | 2 (4%) | 0.718 |
| – N1 | 3 (12%) | 4 (18%) | 7 (15%) | |
| – N2 | 21 (84%) | 17 (77%) | 38 (81%) | |
| – N2a | 1 (4%) | 3 (14%) | 4 (9%) | |
| – N2b | 11 (44%) | 7 (32%) | 18 (38%) | |
| – N2c | 9 (36%) | 7 (32%) | 16 (34%) | |
| – N3 | 0 (0%) | 0 (0%) | 0 (0%) | |
| Anatomical primary side tumor | | | | |
| – Tonsils | 11 (44%) | 8 (36%) | 19 (40%) | 0.484 |
| – Base of the tongue | 8 (32%) | 4 (18%) | 12 (26%) | |
| – Cancer of Unknown Origin | 4 (16%) | 2 (9%) | 6 (13%) | |
| – Other sites in the oropharynx[d] | 2 (8%) | 8 (36%) | 10 (21%) | |
| Origin tested FFPE material | | | | |
| – Tonsils | 12 (48%) | 9 (41%) | 21 (45%) | 0.361 |
| – Base of the tongue | 6 (24%) | 3 (14%) | 9 (19%) | |
| – Neck dissection preparation | 5 (20%) | 2 (9%) | 7 (15%) | |
| – Other sites in the oropharynx[e] | 2 (8%) | 8 (36%) | 10 (21%) | |
| Histological tumor differentiation | | | | |
| – Poor | 2 (8%) | 4 (18%) | 7 (15%) | |
| – Moderate | 19 (76%) | 14 (64%) | 33 (70%) | |
| – Well | 3 (12%) | 1 (5%) | 3 (6%) | |
| – Unknown | 1 (4%) | 3 (14%) | 4 (9%) | 0.718 |

[a] Presence of HPV defined as both HPV PCR and p16 IHC positive in FFPE material.
[b] Absence of HPV defined as both HPV PCR and p16 IHC negative in FFPE material.
[c] Staging by American Joint Committee for Cancer (AJCC) TNM-staging oropharyngeal cancer, 7th edition, 2010.
[d] Other primary tumor sides: Soft palate 3 (67%), unknown location oropharynx 3 (6%), Vallecula 4 (8%).
[e] Other locations derived FFPE material: Soft palate 3 (6%); Vallecula 4 (8%); Unknown location oropharynx 3 (6%).

**Table 2**
Cytological scraped material characteristics.

| Variables | HPV positive (%)[a] N = 25 | HPV negative (%)[b] N = 22 | Total (%) N = 47 |
|---|---|---|---|
| Neck level lymph node aspiration | | | |
| – Level 2 | 14 (56%) | 17 (77%) | 31 (66%) |
| – Level 3 | 0 (0%) | 2 (9%) | 2 (4%) |
| – Level 4 | 2 (8%) | 0 (0%) | 2 (4%) |
| – Other[c] | 9 (36%) | 3 (13%) | 12 (26%) |
| Ultrasound guided FNA cytology | | | |
| – Yes | 23 (92%) | 22 (100%) | 45 (96%) |
| – No | 0 (0%) | 0 (0%) | 0 (0%) |
| – Unknown | 2 (8%) | 0 (0%) | 2 (4%) |
| Tumor cell count cytological slides | | | |
| – Sufficient | 21 (84%) | 20 (91%) | 40 (85%) |
| – Low tumor cell count[d] | 4 (16%) | 3 (9%) | 7 (15%) |
| Cytological staining | | | |
| – Giemsa | 25 (100%) | 17 (77%) | 42 (89%) |
| – Papanicolaou | 0 (0%) | 5 (23%) | 5 (11%) |

[a] Presence of HPV defined as both HPV PCR and p16 IHC positive in FFPE material.
[b] Absence of HPV defined as both HPV PCR and p16 IHC negative in FFPE material.
[c] Other locations FNAC: Level 1: 1 (2%); Level 5: 2 (4%); Unknown level: 9 (10%).
[d] Low tumor cell count, but found sufficient for analysis. Screened by a pathologist.

**Table 3**
HPV Discrepancy analysis.

| | HPV status SPF-10 FFPE | HPV Status SPF-10 FNAC | Tumor cell count FNA biopsy sample | Origin FFPE | After retesting SPF-10 FFPE | After retesting SPF-10 FNAC |
|---|---|---|---|---|---|---|
| Case 21 | HPV 16 | HPV 16/33 | Sufficient tumor cell count | Base of the tongue | HPV 16 | HPV 16 |
| Case 23 | HPV 16 | – | Sufficient tumor cell count | Base of the tongue | No 2nd available | – |
| Case 42 | – | HPV 33 | Sufficient tumor cell count | Base of the tongue | No 2nd available | HPV 33 |
| Case 48 | – | HPV 33 | Sufficient tumor cell count | Tonsil | – | HPV 33 |

In four cases, there was a mismatch in HPV detection or classification. All samples had a sufficient tumor cell load and all FFPE materials were derived from the primary tumor location.

primary tumor site could be identified despite extensive work-up and they remained CUP. In 40 cases both histological data from the primary location and cytological material from metastatic lymph nodes were available. In seven cases histology was available from the neck dissection specimen. The characteristics of the cytological material are summarized in Table 2.

### 3.2. HPV analysis

HPV-DNA analysis on cytological material matched with the HPV status in 24 of the 25 cases positive for HPV analyzed on FPPE material, resulting in sensitivity of 96%. Case 21 contained an additional HPV type (HPV 33) in the FNAC. Retesting this sample showed only positivity for HPV 16 (Table 3). One case (case 23) in the FFPE HPV positive group was HPV negative on FNAC testing and remained negative after retesting the sample (Table 3). This sample contained sufficient tumor cell for analysis.

None of the 22 p16 IHC and FFPE HPV negative cases were found positive for HPV 16 in the FNAC (100%). So there were no false positive tests on FNAC, resulting in a specificity of 100%. Agreement between both test assays was high. ($\kappa = 0.806$; $p < 0.001$).

## 4. Discussion

In this study the test characteristics of HPV analysis in cytological material obtained by FNA from a metastatic lymph node in patients with HNSCC by using the HPV status on histological material as a gold standard were tested. With a sensitivity of 96% and specificity of 100% and equally strong PPV and NPV (92%, 100% respectively), this test proves to be useful in the management of patients with cervical lymph node metastasis from an OPSCC or from a CUP. In the latter cases this is particularly relevant as the possibility of HPV analysis on cytological material would be the only option to establish the HPV status in the absence of biopsy material from a primary tumor. Depending on HPV status, and therefore the likelihood of an occult oropharyngeal origin, decisions on the primary treatment modality (surgery or radiotherapy) and inclusion of the ipsilateral oropharynx in the radiation target volume, can de made for those patients. Moreover, similar to patients with OPSCC, it is likely to give prognostic information.

Previous studies have shown that determination of HPV status based on FNAC can indicate the primary site of origin [15,16]. These studies showed that the presence of HPV in HNSCC material points towards a primary tumor location in the oropharynx. It has also already been demonstrated that HPV-DNA is present in the metastases of HPV-related primary tumors. This can be explained by the fact that HPV-DNA is incorporated in human host DNA, before the malignant process is activated [2]. However, these studies did not validate the HPV analysis on cytological material of lymph node metastasis using traditional HPV testing on histological biopsy material as a gold standard as was the goal of the current study.

Different PCR assays are available to determine HPV status in oropharyngeal carcinomas. Commonly used assays are the GP5+/GP6+ PCR assay and the broad-spectrum SPF-10 PCR assay. Even though both tests have high test qualities, there is a marked difference in sensitivity with the SPF-10 test used in this study having a higher sensitivity [14,17]. This might lead to different analytical and clinical sensitivity and specificity of the test [18].

Although it could be expected that material derived from metastatic lymph nodes would only contain HPV from a metastatic process and therefore contains only the HPV DNA from the original primary tumor, one FNAC sample (case 21) contained also HPV 33. As this HPV type was not apparent in the retest run, the viral load was probably very low (high analytical sensitivity of this PCR assay) and may imply a non-clinically relevant infection [17]. The same was found in two OPSCC cases HPV negative in the testing on FFPE (cases 42 and 48) that were found positive for HPV 33 in the FNAC analysis.

An algorithm including both staining for p16 and detection of HPV is often used to establish HPV status and the use of p16 IHC as first step in the screening of HPV presence has been proposed [13,19,20]. Studies of Schache et al. and Smeets et al. [13,19] have shown that this algorithm has a very high sensitivity and specificity (sensitivity 97% and 100%, specificity 94% and 100% respectively). In the current study, cytological cell blocks were not available for further p16 IHC staining. However, a similar algorithm could be feasible in analysis of cytological samples obtained from cervical neck nodes, at first doing p16 IHC on histologically processed cytological material and thereafter, in case of p16 expression, further evaluation by PCR.

Besides diagnostic and prognostic benefits, determining the HPV status in cytological material could be used for treatment stratification depending on HPV status with the objective to obtain optimal oncologic outcome paired with decreased treatment related toxicity and morbidity. Various studies have retrospectively shown relatively better locoregional control rates with (chemo)radiotherapy in patients with HPV positive tumors as compared to HPV negative cases [21,22]. Balaker and coworkers concluded in their systematic review that in patients with CUP of the head and neck, there is no difference in overall survival between patients who underwent surgery and radiotherapy and those who had primary radiation or chemoradiation therapy [23]. Other studies support these findings [22,24]. Given the relatively low incidence of CUP, randomized trials to determine optimal treatment will be difficult to conduct. However, testing on HPV in these patients could guide the choice of treatment. In patients with HPV positive CUP it is likely that the response on treatment will be similar to that of HPV positive OPSCC and it would be suggestive for an occult oropharyngeal origin. These patients, based on HPV testing on cytological material of the nodal metastasis, could be selected for primary radiotherapy on the neck as well as the ipsilateral oropharynx analogous to the treatment algorithms used for primary OPSCC.

Of future interest is whether reducing the intensity of (chemo-)radiotherapy regimens is feasible in patients with HPV-related HNSCC. However, there is a delicate balance between lowering the intensity of treatment with the objective to reduce toxicity and the likelihood of locoregional recurrence or survival [25]. For this reason, it is important that future prospective trials on OPSCC or CUP

of the head and neck stratify for HPV-status. In this perspective the possibility of HPV testing on FNAC is highly relevant.

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## Ehtical approval

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## Conflict of interest

There are no conflicts of interest for all the authors.

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