

## HPV16 DNA in Histologically Confirmed Tumour-free Neck Lymph Nodes of Head and Neck Cancers

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**Abstract.** *Background:* Human papillomavirus (HPV) has been demonstrated in lymph node neck metastases (NM) of HPV-positive squamous cell carcinomas of the head and neck (HNSCC), underscoring the possible role of HPV for HNSCC progression. Reports on HPV infections in histopathologically tumour-free lymph-nodes of the SCC of the uterine cervix developing higher rates of lymph-node metastases and recurrences later in the survey of the patients was the starting point of the present study. *Materials and Methods:* The presence of HPV-DNA in primary tumours (PT, n=45), NM (n=45) and histologically confirmed tumour-free neck lymph-nodes (LN, n=102) of HNSCC from 60 patients was analysed by PCR and Southern blot hybridisation. *Results:* A highly positive correlation of simultaneous HPV-DNA detection in PT and NM was demonstrated. In the case of HPV-positivity of PT and/or NM [24/60 cases (40%)], 11/24 (45.8%) LN contained HPV-DNA, as well. Accepting HPV demonstration as a marker for the presence of micro-metastasis, HPV analysis would result in an upstaging of the N category in 4 out of these 11 patients. *Conclusion:* Considering the high agreement of HPV-DNA detection in PT and simultaneous HPV-DNA demonstration in the draining NM corroborating the monoclonal character of the tumour cells, the HPV-DNA presence in LN seems to be indicative of micro-metastasis in these lymph nodes. Thus, HPV analysis might be another

powerful tool for the definition of the N-status of HPV-positive HNSCC.

Squamous cell carcinomas of the head and neck (HNSCC) show an HPV-prevalence between 20% to 30% for oropharyngeal, hypopharyngeal as well as laryngeal squamous cell carcinomas and over 50% for squamous cell carcinomas of the Waldeyer's tonsillar ring (1-4). In a subset of these tumours, viral activity could be established by demonstration of viral mRNA presence, down-regulation of pRb- and cyclin D1-expression as well as by the up-regulation of p16 expression (3-8). We and others reported HPV-positive HNSCC presenting more aggressive tumour behaviour when compared to HPV-negative cases. The HPV-positive cases present with a higher lymph nodal status at time of first diagnosis, which is statistically the strongest predictor for decreased survival times of both HPV-positive as well as HPV-negative patients (1, 3, 5, 9). Vice versa, we demonstrated HPV-DNA positivity in lymph-node neck metastases of HNSCC in about 63% of all analysed cases independently of the primary tumours' anatomical location [22/35 investigated cases (9)]. However, HPV positivity is associated with better recurrence-free and overall survival times of patients with similar nodal status, attributed to a better response of the HPV-positive group toward therapy, especially radiotherapy (1, 5, 6, 10). Thus, the potential significance of high risk HPV infection in a subset of HNSCC showing progression behaviour comparable to that of squamous cell carcinomas of the uterine cervix is corroborated by the above described findings (1-10).

In carcinomas of the uterine cervix, HPV infection represents the main carcinogenic risk factor (11, 12). The draining lymph nodes and metastatic tissues are HPV-positive, as well. In a number of cases, even the tumour-free lymph nodes have been shown to harbour HPV-positive tumour-derived cells. In those cases, the tumour behaviour is more aggressive, including the development of metastases and recurrences (13, 14). However, it is still unclear whether

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HPV-DNA demonstration in tumour-free lymph-nodes of HPV-positive squamous cell carcinomas of the uterine cervix is an indicator for presence of micro-metastasis of the tumour (15, 16).

In HNSCC with a lymph node status of N0 by intensive sectioning and standard H&E staining as well as immunohistochemical staining on neck dissection specimens, micro-metastases have been detected in up to 8% of investigated cases resulting in upstaging (17, 18). Instead of the T-category of the primary tumours, the anatomical site of the oral cavity and the pharynx as well as the maximum depth of invasion of the tumour were shown to be strong predictive factors for metastasis, thus treatment (*i.e.*, elective neck dissection on N0-patients, for instance) of patients showing these features was recommended.

Data on the HPV-DNA presence in histologically confirmed tumour-free lymph-nodes of HNSCC are lacking. In the present study, the HPV presence in primary tumours, lymph node neck metastases as well as in histologically confirmed tumour-free lymph nodes corresponding to HNSCC from 60 patients treated at our department was analysed as a contribution to the discussion regarding the possible use of HPV positivity as indicator for the presence of micro-metastases in HNSCC.

In addition to fresh shock-frozen tumour-tissue, archival paraffin-embedded material collected from patients with positive HPV-status of their tumours was also analysed. This was not only to enlarge the investigation group of HPV-positive cases, but also to investigate the two materials in terms of reliability and comparability of the given results.

## Materials and Methods

*Analysis of shock frozen (fresh) tissue specimens.* Various tissue samples of histopathologically confirmed HNSCC were obtained during surgery from 46 patients (40 male; 6 female; 39 to 90 yrs, mean  $59.1 \pm 11.1$  yrs) treated at the Department of Otorhinolaryngology, Head and Neck Surgery of the Christian-Albrechts-University, Kiel, Germany. Written informed consent from all patients was obtained prior to surgery. The specimens were immediately stored at  $-80^{\circ}\text{C}$  for subsequent molecular analysis.

Altogether, 63 tissue specimens from tumour-free lymph nodes of 46 patients were tested for HPV-DNA presence. In addition, tissue samples from primary tumours and lymph node neck metastases were available from 17 patients, primary tumour samples from 15 patients and metastasis specimens from 14 patients. An overview of investigated tissue specimens and clinicopathological features is given in Table I.

Intra-operatively, the tissue specimens from clinically identifiable metastases were obtained out of the centre of tissue samples in order to save resection margins. Histopathological confirmation of the true presence of squamous cell carcinoma (SCC) in primary tumour and neck metastasis as well as the absence of squamous cell carcinoma in neck lymph nodes, respectively, was given subsequently.

The anatomical location of the primary tumours of the investigated tissue samples was as follows: hypopharynx (n=4), larynx (n=22), oral cavity (n=4) and Waldeyer's tonsillar ring [n=13; (palatine tonsil, n=7; lingual tonsil, n=5; pharyngeal tonsil, n=1)]. The tumours derived from the Waldeyer's tonsillar ring (nasopharyngeal, palatine, and lingual tonsil) were analysed as a separate group of squamous cell carcinomas. Though the Waldeyer's tonsillar ring has substantial anatomical overlap especially with the oropharynx, this division was preferred, because there the squamous cell carcinomas of the Waldeyer's tonsillar ring seem to constitute a distinct subgroup of HNSCC most likely due to the lymphoepithelial character of the tissue (2, 19). Additionally, 3 cases of lymph node metastases of the neck of a carcinoma with unknown primary tumour (CUP-syndrome) were investigated.

*Analysis of paraffin-embedded (archival) tissue specimens.* Paraffin-embedded tissue specimens were collected from patients with HPV-positive primary tumours. The HPV-DNA analysis of the fresh frozen primary tumour specimens had been performed previously with a combination of type-specific (for HPV6, 11, 16 and 33) and consensus primer PCRs and has been published (9, 20).

Altogether, 39 tissue specimens from histologically confirmed tumour-free neck lymph nodes of 14 patients were tested for HPV-DNA presence. In 13 cases, the results could be compared to the results of the investigation of paraffin-embedded specimens of primary tumours and neck metastases and in one case to the results of neck metastases, only. An overview of investigated tissue specimens and clinicopathological features is given in Table II.

The anatomical location of primary tumours of the investigated tissue samples was as follows: hypopharynx (n=2), larynx (n=5), oropharynx (n=1), oral cavity (n=1) and Waldeyer's tonsillar ring [n=6; (palatine tonsil, n=3; lingual tonsil, n=3)]. Additionally, tissue specimens of a CUP-syndrome were investigated.

*PCR-amplification and confirmatory Southern blot hybridisation.* PCR was either carried out on DNA extracted out of 25-mg frozen tissue samples or out of 5- $\mu\text{m}$  sections of paraffin-embedded tumour material, each prepared as described (9, 20, 21). The presence of amplifiable DNA in every individual sample was confirmed by PCR applying  $\beta$ -globin primers. The HPV-DNA type-specific diagnosis was performed with type-specific primer PCR for HPV16 and consensus primer PCRs and subsequent Southern blot hybridisation of the PCR products with corresponding probes as described previously (20, 21).

*Survival analysis.* The impact of HPV infections on survival the applying the Kaplan-Meier method could not be analysed due to the relatively small number of patients per group. The Fisher's exact test was used to test for differences between the groups.

## Results

*Results of analysis on fresh frozen material.* The HPV-DNA analysis was performed altogether in 128 tumour specimens, including 63 tumour-free lymph nodes, 32 primary tumours as well 33 neck metastases [patients investigated: n=46 (see Table I)].

Table I. Clinicopathological features of patients, combination of investigated tissue specimens, and results of analysis of fresh frozen HNSCC tumour samples.

#	Age	Sex	PT <sup>(1)</sup> location	TNM	Available tissue specimens	HPV-positive tissue specimens	HPV-type
1	54	m <sup>(2)</sup>	palatine tonsil	T2N2M0	PT, NM <sup>(4)</sup> , tfLN <sup>(5)</sup>		
2	67	f <sup>(3)</sup>	palatine tonsil	T2N2M0	PT, tfLN (2x)	PT	HPV16
3	56	m	palatine tonsil	T2N0M0	PT, tfLN		
4	52	m	palatine tonsil	T3N2M0	PT, NM, tfNLN (3x)		
5	60	m	palatine tonsil	T3N2M0	PT, NM, tfLN (2x)	PT, NM	HPV16
6	54	m	palatine tonsil	T4N2M0	NM(2x), tfLN		
7	43	m	palatine tonsil	T1N2M0	NM, tfLN		
8	58	m	lingual tonsil	T3N1M0	PT, NM, tfLN (2x)		
9	50	m	lingual tonsil	T4N2M0	PT, NM (2x), tfLN		
10	57	m	lingual tonsil	T2N2M0	PT, NM, tfLN	PT, NM	unidentified
11	49	m	lingual tonsil	T2N0M0	PT, tfLN	PT, <b>tfLN</b>	HPV16
12	52	m	lingual tonsil	T3N2M0	PT, NM, tfLN	PT, NM, <b>tfLN</b>	HPV16
13	68	m	pharyngeal tonsil	T1N2M0	NM, tfLN		
14	55	m	oral cavity	T3N2M0	PT, NM, tfLN		
15	57	f	oral cavity	T1N2M0	NM, tfLN		
16	49	m	oral cavity	T4N2M0	PT, NM, tfLN		
17	61	m	oral cavity	T1N1M0	NM, tfLN (2x)	NM, <b>tfLN</b>	HPV16
18	62	m	larynx	T3N2M0	PT, NM, tfLN		
19	55	m	larynx	T3N0M0	PT, tfLN (2x)		
20	63	m	larynx	T3N0M0	PT, tfLN (2x)		
21	39	m	larynx	T2N0M0	PT, tfLN (2x)	PT	HPV16
22	59	m	larynx	T2N1M0	NM, tfLN		
23	78	m	larynx	T4N0M0	PT, tfLN		
24	55	f	larynx	T4N2M0	PT, NM, tfLN (2x)	PT	HPV16
25	49	m	larynx	T4N2M0	PT, NM, tfLN (2x)	PT, NM	HPV16
26	68	m	larynx	T4N0M0	PT, tfLN (2x)		
27	44	m	larynx	T4N0M0	PT, tfLN (2x)		
28	61	m	larynx	T4N1M0	PT, tfLN		
29	59	f	larynx	T1N0M0	PT, NM, tfLN		
30	46	m	larynx	T3N2M0	PT, tfLN		
31	58	m	larynx	T3N0M1	PT, tfLN (2x)	PT	HPV16
32	66	m	larynx	T3N0M0	PT, tfLN		
33	55	m	larynx	T4N3M0	PT, NM, tfLN		
34	57	m	larynx	T3N0M0	PT, tfLN (2x)		
35	72	m	larynx	T4N2M0	PT, tfLN	PT	HPV16
36	60	m	larynx	T3N2M0	PT, NM, tfLN		
37	55	m	larynx	T3N2M0	NM, tfLN		
38	90	f	larynx	T4N1M0	NM, tfLN		
39	86	m	larynx	T4N0M0	NM, tfLN		
40	52	m	hypopharynx	T2N2M0	NM, tfLN (2x)		
41	45	f	hypopharynx	T4N2M0	PT, NM, tfLN		
42	77	m	hypopharynx	T2N1M0	PT, NM, tfLN	NM	HPV16
43	64	m	hypopharynx	T4N1M0	NM, tfLN		
44	56	m	CUP	TxN2M0	NM, tfLN		
45	60	m	CUP	TxN1M0	NM, tfLN		
46	86	m	CUP	TxN1M0	NM, tfLN		

(1) PT: primary tumour, (2) m: male, (3) f: female, (4) NM: lymph node neck metastases, (5) tfLN: histologically tumor-free neck lymph node.

a) *HPV in primary tumours.* Out of the 32 investigated primary tumours from 32 patients, HPV16 DNA could be detected in 9 cases. Consensus primer PCR and Southern hybridisation revealed one additional HPV-positive case. HPV-analysis on the single consensus primer positive but

HPV16-negative case, searching for HPV6, 11, 18 and 33, remained negative (Table I, case #10).

Concerning the anatomical location of the primary tumour, the distribution of the HPV-positive cases was as follows: Waldeyer's tonsillar ring [5/10 (50%); including

Tabel II. Clinicopathological features of patients, combination of investigated tissue specimens and results of analysis of paraffin-embedded HNSCC tumour samples.

#	Age	Sex	PT <sup>(1)</sup> location	TNM	Available tissue specimens	HPV-positive tissue specimens	HPV-type
1 <sup>a</sup>	67	m <sup>(2)</sup>	palatine tonsil	T1N2bM0	PT, NM <sup>(4)</sup> , tFLN <sup>(5)</sup>	-	-
2	64	m	palatine tonsil	T3N2aM0	PT (2x), NM, tFLN (2x)	PT, NM, <b>tFLN</b>	HPV16
3	64	m	lingual tonsil	T2N2bM0	PT, NM (3x), tFLN (4x)	PT, NM, <b>tFLN</b>	HPV16
4	74	m	lingual tonsil	T2N1M0	PT (2x), NM, tFLN	PT, NM	HPV16
5	65	m	oral cavity	T1N2bM0	PT (3x), NM (2x), tFLN (5x)	PT, NM, <b>tFLN</b>	HPV16
6 <sup>b</sup>	60	m	oropharynx	T2N1M0	PT, NM, tFLN (3x)	-	-
7	36	f <sup>(3)</sup>	larynx	T4N2bM0	PT, NM, tFLN (4x)	PT, NM, <b>tFLN</b>	HPV16
8	55	m	larynx	T3N3M0	PT (2x), NM (2x), tFLN (2x)	PT, tFLN	HPV16
9	73	m	larynx	T3N2bM0	PT, NM (3x), tFLN	NM	HPV16
10 <sup>c</sup>	55	m	larynx	T3N1M0	PT (3x), NM, tFLN	-	-
11	45	m	larynx	T4N2bM0	PT (2x), NM (2x), tFLN	PT, NM, tFLN	HPV16
12	57	m	hypopharynx	T2N1M0	PT (2x), NM, tFLN (6x)	PT	HPV16
13	65	m	hypopharynx	T3N2bM0	PT, NM (2x), tFLN (2x)	tFLN	HPV16
14	76	m	CUP	TxN1M0	NM (4x), tFLN (6x)	NM, tFLN	HPV16

(1) PT: primary tumour, (2) m: male, (3) f: female, (4) NM: lymph node neck metastases, (5) tFLN: histologically tumor-free neck lymph node.

<sup>a</sup>Analysis on fresh frozen tissue specimens had shown infection with an unidentified HPV type.

<sup>b</sup>Analysis on fresh frozen tissue specimens had shown infection with HPV16.

<sup>c</sup>Analysis on fresh frozen tissue specimens had shown infection with HPV33.

lingual tonsil (3/5; 60%), palatine tonsil (3/5, 40%)] and larynx [5/18 (27.8%)]. HPV was not detected in the SCC of the soft palate (n=1), floor of mouth (n=1) or hypopharynx (n=2).

*b) HPV in lymph node neck metastases.* From the 33 investigated neck metastases (patients, n=31) tested, HPV16 DNA could be detected in 5 cases. Consensus primer PCR and Southern hybridisation revealed one additional HPV-positive metastasis. HPV-analysis on this case excluded HPV6, 11, 18 and 33 positivity, therefore the HPV-DNA remained undetermined. This neck metastasis corresponds to the only undetermined HPV-positive PT described above (Table I, case #10).

From the 6 cases with HPV-positive neck metastases, 3 (50%) were associated with squamous cell carcinomas of the Waldeyer's tonsillar ring (lingual tonsil, n=2; palatine tonsil, n=1), one with squamous cell carcinoma of the floor of mouth, larynx, and hypopharynx, respectively.

In 4 out of 5 (80%) cases with an HPV-positive primary tumour, the related neck metastases were HPV-positive. Likewise, in 11 out of 12 (91.7%) cases with HPV-negative primary tumours, the neck metastasis were negative.

*c) HPV in histologically confirmed tumour-free neck lymph nodes.* After testing 51 histologically confirmed tumour-free lymph nodes taken from 46 patients, HPV16 DNA could be detected in 3 cases. Consensus primer PCR and Southern hybridisation did not demonstrate any additional HPV-positive cases.

Altogether, 3 lymph nodes classified histopathologically as tumour-free, and belonging to different patients, were HPV-positive.

Out of the 12 patients with an HPV-positive primary tumour and/or HPV-positive neck metastasis, 3 (25%) also contained HPV-DNA in a corresponding tumour-free lymph node, (Table I, cases #11, #12, #17). Assuming HPV detection in the tumour-free lymph nodes as a sign of the presence of tumour cells (see Discussion), the demonstration of HPV-DNA led to an upstaging from N1 to N2 and from N0 to N1, in 2 out of 3 of these cases, respectively.

With regard to the anatomical location of the related primary tumour, HPV-positive tumour-free lymph nodes were derived from an SCC of the floor of mouth in a single case and in 2 additional cases from the lingual tonsil. Thus, in 2 (40%) out of the 5 patients with SCC of the lingual tonsil investigated, HPV-DNA could be demonstrated in their corresponding tumour-free lymph nodes. In the case of HPV-positive SCC of the lingual tonsil, 2/3 cases (66.7%) were also HPV-positive in the tumour-free lymph nodes.

Thus, HPV-DNA could be detected in 12/46 (26.1%) HNSCC cases in at least one of the investigated tissue specimens, *i.e.*, either in primary tumour, neck metastases or in both. For detailed information on the investigated cases and the results, see Table I.

*Results from analysis of archival material.* Paraffin-embedded tissue specimens obtained from of patients with histologically confirmed HNSCC which tested positive for

HPV infection from 1994 to 2002 were included in this part of analysis. HPV16 DNA had been demonstrated in 12 cases, HPV33 in one case, and in an additional case, the HPV type remained undetermined [Table II (9, 21)].

Here HPV-DNA analysis was performed in a total of 86 paraffin-embedded tumour specimens, including 39 histologically confirmed tumour-free lymph nodes, 22 primary tumours and 25 neck metastases [patients investigated: n=14 (see Table II)].

a) *HPV in primary tumours and lymph node neck metastases.* All 47 tumour specimens obtained from 14 patients (22 primary tumours and 25 related neck metastases) were studied by type-specific PCR and subsequent Southern hybridisation with type-specific internal probes for the detection of HPV6, 11, 16 and 33. The negative samples were subjected to consensus-primer PCR and Southern hybridisation. Twenty-four out of the 47 (57.4%) tissue specimens were HPV-positive and HPV16 was identified in 23/24 HPV-positive cases (95.8%). The demonstration of HPV-DNA in paraffin-embedded tissue specimens of an HPV33-positive and of a consensus primer positive case failed. These results are summarised in Table II.

Of the 13 patients from whom both the primary tumour and the neck metastases were available for analysis, 6 (46.2%) were HPV-positive in both samples, 4 (30.8%) were HPV-negative in both samples, in one patient (7.7%) the primary tumour was negative and the neck metastases HPV16 positive and in another case (7.7%), HPV16 DNA could be detected in the primary tumour, but not in the neck metastases. HPV16 was also demonstrated in the one case in which only neck metastases could be analysed. In summary, out of the 14 investigated patients, 10 (71.4%) were HPV-positive in at least one of the investigated paraffin-embedded tissue specimens derived from primary tumours and neck metastases. Three patients were HPV16 positive.

b) *HPV in tumour-free neck lymph nodes.* In 39 of the histologically confirmed tumour-free lymph nodes derived from 14 patients, HPV16 DNA could be detected in 13 cases (33.3%). Further analysis using Consensus primer PCR and Southern hybridisation did not demonstrate any additional HPV-positive cases. In total, 8/14 (57.1%) patients showed HPV-DNA in paraffin-embedded tumour-free lymph nodes (Table II). HPV detection in these tumour-free lymph nodes led to upstaging in two cases (N1 to N2 and N2a to N2b), considering HPV-DNA presence as marker for micro-metastasis. All but one of the HPV-positive tumour-free lymph nodes could be assigned to patients already demonstrated to contain HPV-DNA in the paraffin-embedded tissue specimens of the corresponding primary tumour and/or neck metastases.

*Statistical analysis.* When comparing the number of HPV positives between those materials derived from paraffin-embedded tissue (11/14) and fresh frozen samples (12/46), the difference was highly significant ( $p=0.001$ ). In addition, when comparing the number of tumour-free neck lymph nodes detected positive *vs.* the number of tested HPV positives in each group - paraffin or fresh, the difference was significant at the 5% level ( $p=0.039$ ) - but not significant at the 1% level. Therefore we conclude that it is legitimate to use paraffin material for this type of analysis, without any apparent disadvantages in comparison with fresh material.

When comparing the overall HPV positivity according to the tumour localisation, no significant difference could be detected ( $p>0.5$ ). Even when comparing the 2 largest groups, tonsillar carcinomas in all sub - localizations *vs.* laryngeal carcinomas, no significant difference in HPV positivity could be found. Since in general the frequency of HPV positives is usually highest in tonsillar carcinomas, this is probably an effect of the particular population sample available for this study.

No significant difference could be found between the number of tumour-free neck lymph-nodes detected HPV-positive *vs.* the overall HPV positives by localisation ( $p=0.465$ ). This means that, at least for the sample analysed, there is no higher proportion of HPV-positive tumour-free neck lymph-nodes in any particular localisation.

## Discussion

In the present study, we simultaneously analysed the HPV-DNA presence in different tissue specimens of HNSCC, *i.e.*, in primary tumours (n=45), lymph node neck metastases (n=45) and histologically confirmed tumour-free neck lymph nodes (n=102) from 60 patients. The study design included 2 groups of analysed tissue specimens: in 46 cases tissue specimens were collected prospectively and stored as fresh frozen material until analysis; in 14 cases archival (paraffin-embedded) tissue specimens were collected from HNSCC cases with previously demonstrated HPV-DNA presence in the primary tumours (9, 20). We demonstrate, for the first time to our knowledge, the presence of HPV16 DNA in tumour-free lymph nodes of HNSCC.

We and others have already reported the presence of HPV-DNA in lymph node neck metastases of HPV-positive HNSCC thus strengthening the possible influence of HPV on HNSCC progression (9, 22). These results were in accordance with data shown for carcinomas of the uterine cervix, where a high correlation of HPV presence in primary tumours and the simultaneous presence of HPV-DNA in the draining lymph nodes and even their distant metastases was first demonstrated (13, 14). Reports on HPV infections in histopathologically tumour-free lymph nodes of these

cervical carcinomas developing higher rates of lymph node metastases and recurrences later in the survey of the patients was the starting point of the present study (14).

The analysis of fresh frozen HNSCC tissue specimens taken from 46 patients revealed that the detected HPV prevalence of 26.1% is within the range that should be expected when a comparable study population employing identical methodical standards is investigated (3, 9, 20). In this study section, with 4 out of 5 cases presenting with HPV-DNA in neck metastases when the primary tumour was HPV-positive, and *vice versa* with 11 cases not showing HPV-DNA in metastasis when the primary tumour was HPV-negative, the hypothesis of the monoclonal character of the tumour cells is corroborated. The data are in agreement with our earlier observations investigating primary tumours and lymph node neck metastases of 35 HNSCC patients with the demonstration of a 78% agreement of simultaneous HPV-DNA presence in the primary tumour and the draining lymph node neck metastases (9). Out of those 35 patients with advanced stage HNSCC, since they already presented with clinically identifiable neck metastases at the time of the first diagnosis, 22 (62.9%) had shown HPV infection in at least one of the investigated tissue specimens (primary tumour and/or neck metastasis). In comparison to studies exclusively focussing on primary tumours without taking into account the lymph nodal status and including approximately 20-30% HPV-positives (3, 9, 20), the HPV prevalence in that study was strikingly high. The high HPV prevalence together with the finding that 93% of analysed HPV-positive HNSCC-patients (1) developed lymph node neck metastases in the course of tumour disease, whereas the HPV-negative HNSCC did so in only 63% of the investigated cases, support the promoting influence that HPV infections seem to have in the carcinogenesis of HNSCC. We obtained comparable results by investigating archival material, with a HPV prevalence of 57.1% (8/14) in the neck metastases of HPV-positive advanced stage HNSCC.

With 3 (25%) histologically confirmed tumour-free lymph nodes from 12 HPV-positive HNSCC containing HPV-DNA upon investigation of fresh frozen material and 8 (57.1%) HPV-positive tumour-free lymph nodes from 14 HPV-positive HNSCC upon investigation of archival material, the HPV prevalence in tumour-free lymph nodes of HPV-associated HNSCC diverges among the 2 study groups. However, both groups cannot be directly compared, since up to 6 different tissue specimens deriving from tumour-free lymph nodes were available for archival material investigation, in comparison to only 2 (in a single case 3) different tissue specimens available for the analysis of the fresh frozen material (see Tables I and II). Therefore, the true HPV prevalence in tumour-free lymph nodes of HPV-positive HNSCC can be assumed to be in the upper prevalence rate region, as shown for the paraffin-embedded

archival material. The reliability of the results is supported by preceding investigations of clinically healthy as well as different benign and malignant lesions of the mucosa of the upper aerodigestive tract, including fresh frozen and archival tissue specimens (23). The results from these studies demonstrated that HPV contamination due to contaminated archival material on the one hand, or false-positive HPV-results due to mispriming in the case of absent HPV-DNA on the other, seems to be unlikely when employing the methodical standards described. The statistical analysis in the present study showed that it is basically legitimate to use paraffin material for this type of analysis, without any apparent disadvantages in comparison with fresh material.

It is controversial whether or not HPV-DNA demonstration in tumour-free lymph nodes of HPV-positive SCC can be considered as an indicator for the presence of micro-metastasis of the tumour (15, 16), since a morphological proof of metastatic tissue cells is not possible. Yet, in light of the assumed monoclonal character of tumour cells this hypothesis seems to be plausible, especially since intensive sectioning, standard H&E staining and immuno-histochemical staining on neck dissection specimens of HNSCC with a lymph nodal status N0 after routine patho-histological examination led to the detection of micro-metastases in approximately 8% of cases (17, 18). Considering the fact that in this study only 13 patients with a lymph nodal status of N0 were investigated, the comparably higher micro-metastasis rate of 18.3% (11/60 patients) was expected.

In HNSCC, the exact evaluation of the correct lymph nodal status is of high priority since the N-status of the tumour stage has strong impact on the decision and indication of tumour therapy management. The absence of lymph node neck metastases corroborates the decision for "wait and see"-strategies in tumour therapy management. Recently, the statistical analysis of 73 patients suffering from HNSCC had shown: (i) a correlation between decreased survival and increased lymph node status, (ii) an association between HPV positivity and higher lymph node status at initial presentation and (iii) a better survival of HPV-positive patients compared to HPV-negative patients given the same initial nodal category (1). The better course of patients with HPV-positive lymph node neck metastases could be the result of better responsiveness to radiotherapy, since all neck metastasis-positive patients had postoperative irradiation (1, 6). Therefore, assuming HPV-DNA detection in lymph nodes as sign for micro-metastasis, HPV analysis on neck lymph nodes of HPV-positive HNSCC patients might be a valuable instrument for defining the N-status of these patients. In light of the recent development of minimal invasive strategies to further determine the N-status in cases of a clinical lymph nodal status N0 by

performing sentinel lymph node biopsies (24, 25), HPV-DNA analysis might be of special interest. Since it is unclear whether sentinel lymph node histology is the accurate instrument to reliably exclude tumour cell presence in the sentinel lymph nodes (25), HPV analysis could contribute to this issue. In the case of HPV-positivity of the lymph nodes, additional treatment of the patients should be recommended, with HPV analysis of the neck lymph nodes thus becoming of decisive importance for the clinical course of the HPV-positive patients.

The anatomical location of the tonsils is frequently associated with HPV infections, with the corresponding consequences at the molecular as well as the epidemiological level (1-7, 9, 10, 19, 22), the present study being no exception. Prospectively investigating fresh frozen tissue specimens from this anatomical localisation, we determined 5/10 HPV-positive primary tumours, 3/6 HPV-positive lymph node neck metastases and even 2/3 HPV-positive tumour-free lymph nodes, therefore confirming once more the Waldeyer's tonsillar ring as clearly prominent in this respect.

In conclusion, the analysis of the HPV-DNA presence in lymph nodes of HNSCC, even when histopathologically confirmed as tumour-free, should be assessed in a larger study population to further define the role of HPV infections in metastasis and to evaluate the statistical impacts on the clinical course of the patients regarding the early metastatic spread and early recurrence of disease. Furthermore, the question should be addressed as to whether or not HPV-DNA in the lymph nodes represents micro-metastases, leading to introduction of HPV analysis in the determination of the lymph nodal status of HPV-positive cases, in general and of sentinel lymph nodes in particular.

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