

# Human Papillomavirus Type 16 Oropharyngeal Cancers in Lymph Nodes as a Marker of Metastases

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**Background:** Oropharyngeal squamous cell carcinomas (OSCCs) are associated with high-grade human papillomavirus (HPV) infection in 20% to 30% of cases. HPV-16 DNA has been detected in cervical lymph node metastases of HPV-16<sup>+</sup> OSCC. However, the meaning of HPV-16 DNA detection in lymph nodes remains controversial. Does the presence of HPV-16 DNA in lymph nodes correlate with their metastatic involvement, or is it just a consequence of the filter function of lymph nodes?

**Methods:** Viral load quantification using reverse transcriptase–polymerase chain reaction was retrospectively performed in primary tumors and in cervical lymph nodes, originating from levels IIa, IIb, and III, in 11 patients with HPV-16<sup>+</sup> OSCC and in 3 control patients with HPV-16<sup>-</sup> OSCC.

**Results:** A total of 45 lymph node levels were analyzed. HPV-16 DNA was not detected in HPV-16<sup>-</sup> OSCC lymph

nodes. No statistically significant difference was found between primary tumors and metastatic lymph nodes viral load ( $P > .01$ ). The viral load value was significantly higher in metastatic lymph nodes than in tumor-free lymph nodes ( $P < .01$ ). Among 27 tumor-free lymph node levels, the viral load value was undetectable in 16, low or medium ( $< 10^5$  copies per million cells) in 8, and high ( $> 10^5$  copies per million cells) in 3.

**Conclusions:** HPV-16 DNA detection in lymph nodes of patients affected with HPV-16<sup>+</sup> oropharyngeal cancer is indicative of metastatic involvement. Tumor-free lymph nodes with a high viral load value would suggest the presence of occult lymph nodes metastasis and the opportunity to use HPV-16 DNA as a metastatic marker. Further investigations are needed.

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**O**ROPHARYNGEAL SQUAMOUS cell carcinomas (OSCCs) occur frequently. They represent 25% of all upper aerodigestive tract cancers.<sup>1</sup> These cancers are associated with high-grade human papillomavirus (HPV) infection, particularly the oncogenic 16 genotype, in 20% to 30% of cases.<sup>2-4</sup>

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Oropharyngeal cancers are very lymphophilic. At the time of diagnosis, 70% of patients harbor cervical lymph node metastases,<sup>5</sup> and 20% to 30% of patients with clinically and radiologically N0 necks are affected with occult metastases.<sup>5,6</sup> Recent publications have shown that cervical nodal spreading was more frequent in HPV-16<sup>+</sup> cancers.<sup>3,7</sup> Cervical lymph node metastasis is a major prognostic factor and requires a precise evaluation to optimize treatment.

HPV-16 DNA has been detected, by non-quantitative methods, in cervical lymph node metastases of HPV-16<sup>+</sup> oropharyngeal

cancers.<sup>8-11</sup> However, few studies have been performed, and many questions remain unsolved, especially concerning the meaning of the presence of HPV-16 DNA among lymph nodes. Does the presence of HPV-16 DNA in lymph nodes correlate with their metastatic involvement, or is it just a consequence of the filter function of lymph nodes?

In the current study, viral load quantification was performed in the primary tumors and in cervical lymph nodes, regardless of their pathologic status. The aim was to evaluate the prevalence and the pattern of nodal spreading of HPV to the different cervical lymph node regions in patients with HPV-16<sup>+</sup> oropharyngeal cancer and to draw a parallel with tumoral diffusion.

## METHODS

Fourteen patients with OSCC of those referred to our institution were retrospectively included in the study from July 2007 to March 2010. Their mean age was 65 years (range, 47-85 years), and there were 9 men and 5 women. All patients were treated with oropharyngectomy and unilateral neck dissection. One patient underwent bilateral neck dis-

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section. A total of 15 selective neck dissections were performed, and 438 lymph nodes were obtained from the neck samples. The topographic classification by Robbins et al<sup>12</sup> was used by the surgeons (H.M., S.P., J.L.S.G.), the pathologist (M.L.), and the virologists (F.M., P.S.) as well to determine the involved neck territories. Lymph nodes originating from levels IIa, IIb, and III were submitted for pathologic and virologic analyses. A total of 45 lymph node levels were studied.

### PATHOLOGIC ANALYSIS OF LYMPH NODES

Lymph nodes were cut perpendicular to the long axis. Each lymph node half was sectioned at 3-mm intervals, and these 3-mm slices were fixed in formalin and paraffin embedded. Each 3-mm slice was analyzed in 4 additional 5- $\mu$ m sections. These 5- $\mu$ m sections were sectioned at 150- $\mu$ m intervals. The first 5- $\mu$ m section was used for hematoxylin-eosin-safran (HES) staining. If results from HES were negative, the 3 other 5- $\mu$ m sections were examined by immunohistochemical (IHC) analysis with an anticytokeratin cocktail (Cytokeratin AE1-AE3; Dako Corp, Glostrup, Denmark).

### HPV-16 DETECTION IN OSCC SAMPLES

All tumoral samples were screened by polymerase chain reaction (PCR) and hybridization using Inno-Lipa diagnostic assay according to the manufacturer's instructions (Innogenetics, Ghent, Belgium). DNA extraction and viral load quantification were performed in HPV-16<sup>+</sup> OSCC samples and in all lymph nodes, regardless of their primary tumor HPV-16 status.

### DNA EXTRACTION

The 5- $\mu$ m sections of paraffin-embedded samples were incubated for 3 hours at 65°C with lysis buffer (50-mM of Tris, 1mM of EDTA, and Tween 20, 0.5%). After 10 minutes of boiling followed by a centrifugation at 4°C, DNA extracts were diluted in a 1:10 solution before PCR analysis. In case of PCR inhibition, evidenced by a housekeeping gene misamplification, a second DNA extraction procedure using a spin column (Qiablood DNA extraction; Qiagen, Hilden, Germany) was performed from the first extract. DNA was then eluted in 50  $\mu$ L of water before PCR amplification.

### VIRAL LOAD QUANTIFICATION

To specifically quantify HPV-16, real-time quantitative PCR was performed using the 7500 Taqman System (Applied Biosystems, Carlsbad, California) with 5  $\mu$ L of DNA extracts. HPV-16 quantification was performed using 5'-GAGAACTGCAATGTTTCAG-GACC-3' forward and 5'-TGTATAGTTGTTTGAGCTCT-GTGC-3' reverse primers located inside the E6 HPV-16 gene (nucleotide 12-63). Amplification was revealed using 5'-FAM-CGACCCAGAAAGTTACCACAG-MGB-3' Taqman-MGB probe (Applied Biosystems). Absolute quantification was obtained using an E6 HPV-16 vector previously cloned using TOPO kit (Invitrogen, Carlsbad, California). Normalization, indicated by the absence of PCR inhibition, was evaluated using housekeeping Albumin gene amplification with 5'-GCT GTCATCTCTGTGGGCTGT-3' forward and 5'-AAACTCAT-GGGAGCTGCTGGTT-3' reverse primers, revealed with a Taqman 5'-FAM-CCTGTCTATGCCACACAAATCTCTC-MGB-3' probe. Results obtained from real-time PCR allowed evaluation of the relative HPV-16 viral load by 10<sup>6</sup> cells.

Statistical analysis was performed using Statview Software (version 5.0; SAS Institute Inc, Cary, North Carolina). Comparisons between continuous and dichotomous variables were based on the Mann-Whitney test. Comparisons between dichotomous vari-

ables were based on the Pearson  $\chi^2$  test or Fisher exact test.  $P < .01$  was considered to denote a significant difference.

## RESULTS

A total of 45 lymph node regions were analyzed: 9 regions in 3 patients with HPV-16<sup>-</sup> cancer and 36 regions in 11 patients with HPV-16<sup>+</sup> cancer.

### PATHOLOGIC RESULTS

Two of 3 patients with HPV-16<sup>-</sup> oropharyngeal cancer had cervical lymph node metastasis. Eight of 11 patients with HPV-16<sup>+</sup> oropharyngeal cancer had at least 1 cervical lymph node metastasis. Among the 36 lymph node regions analyzed in the patients with HPV-16<sup>+</sup> oropharyngeal cancer, 9 were metastatic, and 27 were tumor free. All HES<sup>-</sup> lymph nodes were confirmed by immunohistochemical analysis.

### VIROLOGIC RESULTS

HPV-16 DNA identification in each lymph node region was classified according to the HPV tumoral status; HPV-16 DNA was not detected in cervical lymph nodes of the 3 patients with HPV-16<sup>-</sup> oropharyngeal cancer. HPV-16 DNA was identified in the cervical lymph nodes of 10 of 11 patients with HPV-16<sup>+</sup> oropharyngeal cancer (in 20 of 36 lymph node regions).

Tumoral viral load quantification in patients with HPV-16<sup>+</sup> cancer (**Table 1** and **Table 2, Figure 1**): the viral load values ranged from  $7.5 \times 10^5$  to  $1.4 \times 10^8$  viral copies per million cells. The mean (median) value was  $3.5 \times 10^7$  ( $1.4 \times 10^7$ ) viral copies per million cells.

Viral load quantification in the lymph nodes of patients with HPV-16<sup>+</sup> cancer was performed according to the pathologic status (Table 1 and Table 2, **Figure 2**): HPV-16 DNA was detected in all metastatic lymph nodes. The viral load values of metastatic lymph nodes ranged from  $5.7 \times 10^5$  to  $8.4 \times 10^8$  viral copies per million cells with a mean (median) value of  $1.6 \times 10^8$  ( $4.1 \times 10^7$ ) viral copies per million cells. No statistically significant difference was found between the viral load values for the primary tumor and metastatic lymph nodes ( $P = .17$ ). Among 27 tumor-free lymph node levels, the viral load was undetectable in 16, low or medium ( $< 10^5$  copies per million cells) in 8, and high ( $> 10^5$  viral copies per million cells) in 3. Tumor-free lymph nodes viral load values ranged from 0 to  $3.2 \times 10^6$  viral copies per million cells with a mean (median) value of  $1.4 \times 10^5$  (0) viral copies per million cells. The viral load in metastatic lymph nodes was significantly higher than that of tumor-free lymph nodes ( $P = .004$ ).

Viral load quantification in the lymph nodes of patients with HPV-16<sup>+</sup> cancer was classified according to the lymph node level (Table 1 and Table 2, Figure 1) as follows: for the level IIa lymph nodes the viral load ranged from 0 to  $6.1 \times 10^8$  viral copies per million cells with a mean (median) value of  $5.6 \times 10^7$  ( $2.2 \times 10^4$ ) viral copies per million cells. For level IIb lymph nodes the viral load ranged from 0 to  $8 \times 10^8$  viral copies per mil-

**Table 1. Viral Loads (VLs) for Tumoral and Lymph Nodes According to the Pathological Status and Lymph Node Level<sup>a</sup>**

Patient No.	Tumor VL	Lymph Node					
		Level IIa		Level IIb		Level III	
		VL	Stage	VL	Stage	VL	Stage
1	$7.9 \times 10^7$	0	pN <sup>-</sup>	0	pN <sup>-</sup>	0	pN <sup>-</sup>
2	$1.2 \times 10^6$	0	pN <sup>-</sup>	0	pN <sup>-</sup>	0	pN <sup>-</sup>
3	$7.5 \times 10^5$	$8 \times 10^3$	pN <sup>-</sup>	0	pN <sup>-</sup>	0	pN <sup>-</sup>
4	$7.5 \times 10^7$	$2.2 \times 10^4$	pN <sup>-</sup>	0	pN <sup>-</sup>	0	pN <sup>-</sup>
5	$4.1 \times 10^7$	0	pN <sup>-</sup>	$6.1 \times 10^5$	pN <sup>+</sup>	0	pN <sup>-</sup>
6	$1.3 \times 10^8$	$1 \times 10^3$	pN <sup>-</sup>	$1.1 \times 10^4$	pN <sup>-</sup>	$4 \times 10^4$	pN <sup>-</sup>
7	NA	$1.2 \times 10^7$	pN <sup>+</sup>	$1.4 \times 10^4$	pN <sup>-</sup>	$6.9 \times 10^4$	pN <sup>-</sup>
8	$1.6 \times 10^7$	0	pN <sup>-</sup>	$8.4 \times 10^8$	pN <sup>+</sup>	0	pN <sup>-</sup>
9	$2.9 \times 10^6$	$6.1 \times 10^8$	pN <sup>+</sup>	0	pN <sup>-</sup>	$3.2 \times 10^6$	pN <sup>-</sup>
11	$1.9 \times 10^6$	$4.1 \times 10^7$	pN <sup>+</sup>	$4.1 \times 10^7$	pN <sup>+</sup>	$9.3 \times 10^4$	pN <sup>-</sup>
12 <sup>b</sup>	$1.4 \times 10^7$	$7.5 \times 10^7$	pN <sup>+</sup>	$6.1 \times 10^5$	pN <sup>-</sup>	0	pN <sup>-</sup>
	$1.4 \times 10^7$	$4.8 \times 10^6$	pN <sup>+</sup>	$6.2 \times 10^5$	pN <sup>-</sup>	$5.7 \times 10^5$	pN <sup>+</sup>

Abbreviation: NA, not available.

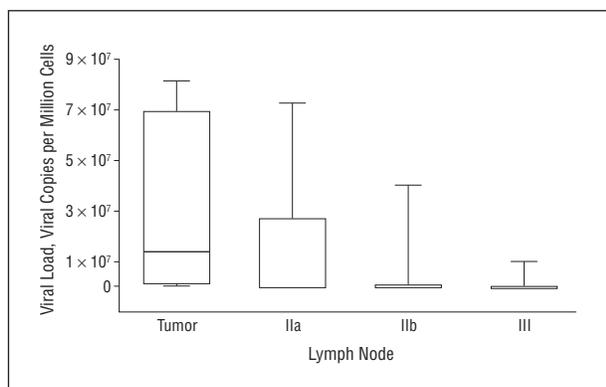
<sup>a</sup>Viral copies per million cells.

<sup>b</sup>Patient 12 underwent a bilateral neck dissection.

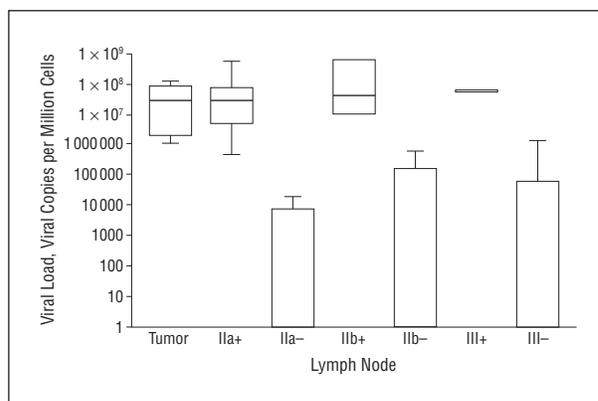
**Table 2. Viral Loads (VLs) for Tumoral and Lymph Nodes<sup>a</sup>**

Viral Load	Tumor VL	Lymph Node VL				
		Level IIa	Level IIb	Level III	Stage N <sup>+</sup>	Stage N <sup>-</sup>
Minimum	$7.5 \times 10^5$	0	0	0	$5.7 \times 10^5$	0
Maximum	$1.3 \times 10^8$	$6.1 \times 10^8$	$8.0 \times 10^8$	$5.0 \times 10^7$	$8.4 \times 10^8$	$3.2 \times 10^9$
Mean	$3.5 \times 10^7$	$5.6 \times 10^7$	$7.4 \times 10^7$	$3.3 \times 10^5$	$1.6 \times 10^8$	$1.4 \times 10^9$
Median	$1.4 \times 10^7$	$2.2 \times 10^4$	$1.4 \times 10^4$	0	$4.1 \times 10^7$	0

<sup>a</sup>Viral copies per million cells.



**Figure 1.** Distribution of the viral load of tumoral and lymph node levels (viral copies per million cells), regardless of the pathologic status. The bottom and top of each box represent the 25th and 75th percentiles, the bands inside the box represent the 50th percentile (the median), and the brackets represent the 10th and 90th percentiles.



**Figure 2.** Distribution of the viral load of tumoral and lymph node levels (viral copies per million cells), according to the pathologic status. The bottom and top of each box represent the 25th and 75th percentiles, the bands inside the box represent the 50th percentile (the median), and the brackets represent the 10th and 90th percentiles.

lion cells with a mean (median) value of  $7.4 \times 10^7$  ( $1.4 \times 10^4$ ) viral copies per million cells. For level III lymph nodes, the viral load ranged from 0 to  $5.7 \times 10^7$  viral copies per million cells with a mean (median) value of  $3.3 \times 10^5$  (0) viral copies per million cells.

### COMMENT

Few studies have focused on the presence of HPV-16 DNA in cervical lymph nodes of patients affected by OSCC.

Most of these studies have used HPV-16 DNA as a possible diagnostic tool to predict the site of tumor origin in cases of carcinoma of unknown primary.<sup>8-11,13</sup>

Available data come mainly from studies in patients with cervical cancer.<sup>14-21</sup> The association between high-risk HPV (HR-HPV) infection and the development of cervical cancer has been well established.<sup>22,23</sup> HR-HPV infections are strictly epitheliotropic viruses. Their DNA is located in the nucleus of tumoral cells. Because metastatic cells originate from a unique tumoral clone, many

authors wonder about the possibility of using HR-HPV DNA as a diagnostic marker of metastases, especially in lymph nodes.<sup>14,21</sup> Analyses<sup>14,21</sup> of pelvic lymph nodes in women with cervical cancer have shown the presence of HR-HPV DNA regardless of their pathologic status. This has been demonstrated by several studies, but the significance of HR-HPV DNA presence in lymph nodes remains controversial. Some authors<sup>16-19</sup> believe that HR-HPV DNA represents microscopic undetected metastatic cells that predispose patients to a higher risk of locoregional recurrence, particularly those who do not receive adjuvant therapy. However, HR-HPV DNA in lymph nodes could represent viral particles internalized by scavenger lymphocytes or macrophages and would not have any prognostic value.<sup>14,15,20</sup>

Publication heterogeneity in terms of population, methods, and virologic analyzing techniques explains this lack of consensus. Moreover, the currently available data are mostly based on nonquantitative methods,<sup>3,14,15</sup> despite the importance of this information in understanding the significance of viral presence in lymph nodes. Viral load measurement enables researchers to determine the number of viral copies per cell. In the present study, viral load was measured by reverse transcriptase PCR (RT-PCR) in tumors and in different lymph node levels regardless of the pathologic status of the lymph nodes. The aim of this systematic approach was to quantify the viral presence so as to establish a mapping of viral spread and to compare virologic and pathologic results.

HPV-16 DNA was not detected in lymph nodes of patients with HPV-16<sup>-</sup> oropharyngeal cancer. These patients were tested as a control group because analyses of healthy oropharyngeal mucosa have shown HPV infection in 5% to 10%<sup>3</sup> of cases.

Viral load is maximal in the tumor and decreases progressively in the different lymph node levels. The viral load decreases in lymph nodes the further they are from the primary tumor. Level IIa, which is theoretically the first step of lymphatic drainage for OSCC,<sup>6</sup> has the highest viral load.

In our study, comparison between metastatic and tumor-free lymph nodes revealed that HPV-16 genome has been identified in all metastatic lymph nodes. This finding contrasts with those of many cervical cancer publications in which HR-HPV detection in metastatic lymph nodes ranged from 42% to 100%,<sup>14,21</sup> even if all cervical cancers were virtually associated with HR-HPV.<sup>14,21,22</sup> These variations are probably related to the heterogeneity of the virologic techniques used to detect HR-HPV genome and attest to the high sensitivity of viral load measurement by RT-PCR.

The viral load of metastatic lymph nodes is close to the primary tumors viral load. No statistically significant difference was found between them ( $P > .05$ ). The viral load of metastatic lymph nodes is significantly higher than in tumor-free lymph nodes ( $P < .05$ ). These observations indicate a close relation between the viral load value and the presence of tumoral cells.

Among tumor-free lymph nodes, viral load is variable. In most cases, it is undetectable or low, but in rare occasions it can reach very high values—close to those

tumoral lymph nodes (eg, in patients 9 and 12). What is the explanation of high values? Is there a correlation with occult lymph node metastases? This question is particularly interesting because recent reports have shown that 5% to 20% of patients affected with upper aerodigestive tract squamous cell carcinoma harbored occult lymph nodes metastases.<sup>15,24,25</sup> These metastases are not identified by routine pathologic examination but can be detected by other methods, such as immunohistochemical analysis or molecular biology.<sup>15,24,25</sup> Several authors have detected HR-HPV DNA in 14% to 60% of tumor-free pelvic lymph nodes of women affected by cervical cancer.<sup>7,14,21</sup> However, no exhaustive quantitative analyses were performed, and the meaning of HR-HPV qualitative detection remains controversial. In the current study, measurement of the viral load of tumor-free lymph nodes emphasizes the existence of 2 populations. There was a group of lymph nodes with a high viral load ( $>10^5$  viral copies/million cells), which could be related to the presence of occult metastases. This could explain the importance of locoregional recurrences in some patients whose cancer was classified as stage pN<sup>-</sup> who did not receive adjuvant therapy, as reported by several studies of cervical cancers<sup>16-19</sup> and head and neck cancers.<sup>25</sup> In these patients with a high viral load, adjuvant therapy should be discussed, as suggested by Rolla et al,<sup>21</sup> and for detection of early neck cancer recurrence, at least close monitoring is required. However, there was a second group of lymph nodes in which the viral load was undetectable or had a low to medium value ( $<10^5$  viral copies/million cells). In this group, the viral load can represent rare metastatic cells or viral particles internalized by scavenger lymphocytes or macrophages.

In conclusion, in patients affected with HPV-16<sup>+</sup> OSCC, there is an important correlation between HPV-16 DNA detection in lymph nodes and the presence of metastatic involvement. Viral load quantification in tumor-free lymph nodes highlights 2 distinct groups according to the viral quantification. High viral load values strongly suggest the presence of occult metastases and the opportunity to use HPV-16 DNA as a metastatic marker. Further investigations are needed.

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