Prevalence of human papillomavirus types, viral load and physical status of HPV16 in head and neck squamous cell carcinoma from the South Swedish Health Care Region

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Incidence of human papillomavirus (HPV)-positive head and neck squamous cell carcinoma (HNSCC) is rising in several countries. Intriguingly, large variations of HPV16 viral load and different proportions of the physical viral status among HNSCC have been reported. We analysed fresh biopsies of 275 HNSCC patients from the South Swedish Health Care Region for HPV types with modified general primer PCR and Luminex. Seventy-eight HPV16-positive HNSCC cases were further investigated for viral DNA load and physical status using quantitative PCR for HPV E2 and E7 genes. Presence of intact E2 gene, as a surrogate marker for episomal HPV, was investigated with conventional PCR. Fifteen different HPV types were detected in HNSCC cases and HPV16 was present in 74 % of the HPV-positive cases. HPV was detected in 65 % (92/141) and 11 % (15/134) of oropharyngeal and non-oropharyngeal carcinomas, respectively (P<0.0001). HPV was detected in 73 % (75/103) of tonsillar carcinomas. The median load of HPV16 was 13 copies cell\(^{-1}\) (range 0.003–1080). Among HPV16-positive patients with oropharyngeal carcinoma, metastases to regional lymph nodes were observed in 100 % (17/17) and 68 % (40/58) for those with <1 HPV16 copy cell\(^{-1}\) and >1 HPV16 copy cell\(^{-1}\), respectively (P=0.007). Among HPV16 cases, purely integrated HPV16 was found in 6 %, whereas entirely episomal and mixed virus was detected in 51 and 42 % of cases, respectively. Conclusively, HPV16 viral DNA load demonstrated a large diversity among HNSCCs. Although integration of HPV16 is common (48 %), the episomal HPV16 is salient (93 %) among HPV16 HNSCCs. In addition, low amount of HPV16 was associated with lymph node metastases among oropharyngeal carcinomas.

INTRODUCTION

Among head and neck squamous cell carcinomas (HNSCCs), human papillomavirus (HPV) is the primary cause of an increase in the number of oropharyngeal carcinomas in several countries (Gillison et al., 2015). Also, a significant increasing trend of HPV-positive oropharyngeal tumours over time has been demonstrated (Stein et al., 2015).

The present prevalence of HPV, from the year 2000, in oropharyngeal SCC shows large variations between different countries, with the lowest and highest values from Spain (6 %) and Taiwan (75 %), respectively (Stein et al., 2015). In Sweden, a mean HPV prevalence of about 70 % has been reported for these lesions (Stein et al., 2015). HPV has also been detected at other sites of HNSCC, and meta-analysis reported HPV in 31 and 28 % of laryngeal and oral cavity cancerous sites, respectively (Saulle et al., 2015).

Among HNSCCs, HPV16 predominates with a proportion of about 82 % of HPV-positive cases (Gillison et al., 2015). An intriguing large variation, up to a million-fold, of viral DNA load in HPV16-positive HNSCC cases has been reported in several studies (Badaracco et al., 2007; Deng et al., 2013;
Holzinger et al., 2012; Klussmann et al., 2001; Koskinen et al., 2003). Among cervical uteri carcinomas, integration of HPV is common, although with large variations of the integration frequencies in different studies (mean 78%, range 30–100%), but also the physical form of only episomal HPV has been demonstrated (mean 38%, range 0–70%) (Arias-Pulido et al., 2006; Badaracco et al., 2002; Das et al., 1992; Guo et al., 2007; Ho et al., 2006; Kristiansen et al., 1994; Matsukura et al., 1989; Park et al., 1997).

Furthermore, a mixed HPV status with co-existence of both episomal and integrated HPV is also commonly detected (mean 39%, range 5–92%) among cervical uteri carcinomas (Arias-Pulido et al., 2006; Badaracco et al., 2002; Das et al., 1992; Guo et al., 2007; Ho et al., 2006; Kristiansen et al., 1994; Matsukura et al., 1989; Park et al., 1997). Although integration of HPV16 is frequent in HNSCC, it is common to find a mixed viral status of HPV16 in the lesions (Deng et al., 2013; Koskinen et al., 2003). The aims of the present study were to determine the prevalence of HPV types in HNSCC from the region of Southern Swedish Health Care Region, to analyse the viral DNA load and the physical viral status of the HPV16-positive cases, as well as to study the clinical characteristics of HPV-positive patients.

RESULTS

Clinical characteristics of patients and HPV prevalence

Among the 275 study subjects, 70% were males and median age of study population was 63 years (Table 1).

The patients could be divided almost equally into oropharyngeal (51%) and non-oropharyngeal (49%) cancer site groups. Overall, HPV positivity was associated with the younger age group [odds ratio (OR)=1.90, 95% confidence interval (CI) 1.16–3.11, P=0.013] and with oropharyngeal site (OR=18.11, 95% CI 9.49–34.57, P<0.0001) (Table 1).

HPV was found in 73% of tonsillar carcinoma cases, 56% of base of tongue cancer cases, 27% of oral cavity cancers, 14% of laryngeal cancers and no HPV in any hypopharyngeal cancers (Table 2).

<table>
<thead>
<tr>
<th>Total</th>
<th>HPV+</th>
<th>%</th>
<th>HPV−</th>
<th>%</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
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</tr>
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<td>275</td>
<td>107</td>
<td>38.9</td>
<td>168</td>
<td>61.1</td>
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<td></td>
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<tr>
<td>Female</td>
<td>193</td>
<td>80</td>
<td>41.5</td>
<td>113</td>
<td>58.5</td>
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<td></td>
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<tr>
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<td>82</td>
<td>27</td>
<td>32.9</td>
<td>55</td>
<td>67.1</td>
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<tr>
<td>≤62</td>
<td>63</td>
<td>130</td>
<td>61</td>
<td>46.9</td>
<td>69</td>
<td>53.1</td>
<td></td>
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<tr>
<td>&gt;62</td>
<td>145</td>
<td>46</td>
<td>31.7</td>
<td>99</td>
<td>68.3</td>
<td>1.90</td>
<td>1.16–3.11</td>
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<tr>
<td>Cancer diagnosis</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Oropharyngeal carcinoma</td>
<td>141</td>
<td>92</td>
<td>65.2</td>
<td>43</td>
<td>30.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-oropharyngeal carcinoma</td>
<td>134</td>
<td>15</td>
<td>11.2</td>
<td>127</td>
<td>89.8</td>
<td>18.11</td>
<td>9.49–34.57</td>
</tr>
</tbody>
</table>

HPV type distribution, viral DNA load and physical status

Altogether 15 different HPV types were found, including five low-risk types HPV6, 11, 13, 43 and 86, in the HNSCC patients (Tables 2 and S1, available in the online Supplementary Material). HPV16 was present in 29% (80/275) of the HNSCCs and comprised 74% (79/107) of the HPV-positive cases. Among tonsillar cancers, HPV16 was detected in 60% (62/103) and comprised 83% (62/76) of the HPV-positive cases. Overall, HPV33 was detected in 3% (8/275), each of HPV6, 18, 35 and 45 in between 1 and 2%, and each of HPV11, 13, 31, 43, 45, 52, 68, 86 and 73 were detected in <1% of the patients (Table 2). HPV11, 13, 31, 43, 45, 52, 68 and 73 were only detected in non-oropharyngeal sites (Table 2). Duplicate HPV types were detected in 1.8% (5/275) of the samples. Overall, high-risk and low-risk HPV types were detected in 37% (103/275) and 2.5% (7/275) of the samples, respectively.

Seventy-eight HPV16 HNSCC samples were further investigated for viral DNA load and physical status (Table 3). The majority of these patients were males (78%), below median age of 63 years (60%), smokers (69%), with oropharyngeal carcinoma (96%), alive at the last follow-up (91%), with lymph nodes metastasis (74%) and with relatively small tumours (61.5%) (Table 1). The median viral DNA load of HPV16 was 13 copies cell⁻¹ (range 0.003–1079 copies cell⁻¹) (Fig. 1, Table S1).

Among the HPV16-positive patients with oropharyngeal carcinoma, metastases to regional lymph nodes were observed in 100% (17/17) of those with a viral DNA load <1 HPV16 copy cell⁻¹ and 68% (40/58) of those with >1 HPV16 copy cell⁻¹ (P=0.007). Distal metastasis was present in one patient with HPV16-positive tonsillar carcinoma with mixed viral status and 59.6 viral copies cell⁻¹. Large tumours (T3 and T4) and small tumours (T1 and T2)
### Table 2. HPV type distribution among HNSCC patients

<table>
<thead>
<tr>
<th>Tumour site</th>
<th>N</th>
<th>HPV+ n (%)</th>
<th>Single HPV type</th>
<th>&gt;1 HPV type</th>
<th>6 LR</th>
<th>11 LR</th>
<th>13 LR</th>
<th>16 HR</th>
<th>18 HR</th>
<th>31 HR</th>
<th>33 HR</th>
<th>35 HR</th>
<th>43 HR</th>
<th>45 HR</th>
<th>51 HR</th>
<th>52 HR</th>
<th>68 HR</th>
<th>86 HR</th>
<th>73 HR</th>
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<tr>
<td>Oropharyngeal</td>
<td>141</td>
<td>92 (65)</td>
<td>89</td>
<td>3</td>
<td>1</td>
<td>77</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Tonsil</td>
<td>103</td>
<td>75 (73)</td>
<td>74</td>
<td>1</td>
<td>1</td>
<td>61+1+51</td>
<td>3</td>
<td>6</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1+16</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Base of tongue</td>
<td>27</td>
<td>15 (56)</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>13+1+86</td>
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<td>1</td>
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<td>1+16</td>
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<tr>
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<tr>
<td>Non-oropharyngeal</td>
<td>134</td>
<td>15 (11)</td>
<td>13</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Oral cavity</td>
<td>55</td>
<td>5 (27)</td>
<td>4</td>
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<td>Tongue</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>1+52</td>
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<td>Floor of mouth</td>
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<td></td>
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<td>1+45</td>
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<tr>
<td>Other oral cavity (gingiva and bucca)</td>
<td>11</td>
<td>1 (9)</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Hypopharynx</td>
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<td>1 (9)</td>
<td>0</td>
<td>0</td>
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<td></td>
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<tr>
<td>Larynx</td>
<td>74</td>
<td>10 (14)</td>
<td>9</td>
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<tr>
<td>Supraglottis</td>
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<td>2 (20)</td>
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<td></td>
<td></td>
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<tr>
<td>Glottis</td>
<td>64</td>
<td>8 (13)</td>
<td>7</td>
<td>1</td>
<td>1+45</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2+1+31</td>
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<td></td>
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</tr>
<tr>
<td>Total</td>
<td>275</td>
<td>107 (39)</td>
<td>102</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>80</td>
<td>5</td>
<td>1</td>
<td>8</td>
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<td>1</td>
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<td>1</td>
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</tr>
</tbody>
</table>

HR, high risk.
LR, low risk.
*HPV68 and HPV73 were classified here as high-risk types despite the exact classification as a probable and a possible high-risk type, respectively (Arbyn et al., 2014).
†Double HPV positive for marked type.
showed similar distribution of the physical HPV16 status (Fig. 2).

Overall, among the HPV16-positive HNSCCs, purely integrated virus was detected in 6% of cases (5/78), mixed status in 42% (33/78) and pure episomal status in 51% (40/78). The proportions of the physical viral states stratified by viral DNA load are shown in Fig. 3. The E2/E7 ratio and viral DNA load per cell stratified according to the viral physical status are shown in Table 4. We compared the groups with each other to analyse statistical differences of E2/E7 ratio and viral DNA load between the groups. Among the HPV16 cases, as compared with the pure episomal subgroup, the E2/E7 ratios were significantly lower among the mixed subgroup (\(P<0.0001\)) and integrated subgroup (\(P<0.001\)). In the series of viral DNA loads, as compared with the integrated subgroup, the viral DNA loads were significantly higher among the episomal subgroup (\(P=0.040\)) and the mixed subgroup (\(P=0.015\)) (Table 4).

**DISCUSSION**

Among fresh HNSCC samples from the South Swedish Health Care Region, the HPV prevalence (65%) in the oropharyngeal carcinomas was in accordance with previous data from Sweden (Hansson et al., 2005; Stein et al., 2015). Hansson et al. (2005) reported an overall HPV prevalence of 54% by the use of nested PCR on three different samples (cotton swabs from tumour and tonsillar fossa as well as a mouthwash sample), collected from September 2000 to January 2004 within our catchment region. Based on seven studies from 2000 to 2014, Stein et al. (2015) describe in a review an overall HPV prevalence of about 70% among oropharyngeal carcinomas in Sweden. Our HPV prevalence of the tonsillar and base of tongue carcinomas (69%, 90/130) was also similar to that of a recent study from Stockholm county (74%, 186/252), which analysed paraffin-embedded biopsies collected during 2008–2012, a study period comparable to our own (Näsmann et al., 2015).

Moreover, our findings agree with reports that oropharyngeal cancers are significantly more HPV-related than the non-oropharyngeal carcinomas (Gillison et al., 2015). Furthermore, it has been reported that HPV16, 18, 31 and 33 account for 99% of HPV-positive HNSCC cases (Kreimer et al., 2005). In our series of HPV-positive samples, we observed that three of these HPV types (16/18/33) were associated with the oropharyngeal site of HNSCC (oropharyngeal tumours: 94%, 87/92 and non-oropharyngeal tumours: 40%, 6/15) (\(P=0.0001\)). In fact, the five high-risk HPV types 16, 18, 33, 35 and 51 were present in 99% (91/92) of our HPV-positive oropharyngeal tumours. Twelve per cent of HPV-positive cases harboured types such as HPV6, 11, 13, 35, 43, 45, 51, 52, 68, 73 and 86, out of which 3.7% (4/107) had a single low-risk type of HPV6 or HPV13 (one case). Notably, 40% (6/15) of the HPV types in the present study, HPV11, 13, 31, 45, 52 and 68, were only detected in non-oropharyngeal sites. However, to the best of our knowledge we have detected the largest spectrum hitherto of 15 HPV types among HNSCCs. Previous studies of HNSCC, using a similar approach of wide-spectrum PCR, reported fewer HPV types, between 5 and 8 (Badaracco et al., 2000; Deng et al., 2013; Koskinen et al., 2003).

Thus, the current use of PCR with modified general primers followed by Luminex appears to improve the rate of false-
negative results. This might have clinical implications, since HPV is a favourably prognostic factor, not limited only to HPV16 (Snow & Laudadio, 2010). We also observed four cases with single low-risk HPV types (HPV6 and HPV13) and it remains to be clarified if these HPV types are drivers in the carcinogenesis of HNSCC since Badaracco et al. (2007) reported viral transcription in only one out of three HPV6-positive HNSCCs. We also noted that the proportions of HPV16 (84 %), HPV33 (8 %) and HPV35 (3 %) of our HPV-positive tonsillar and base of tongue samples were very similar to those from the Stockholm area for HPV16 (87 %), HPV33 (7 %) and HPV35 (3 %) (Näsman et al., 2015).

To the best of our knowledge, the series of 78 HPV16-positive HNSCC samples is the second largest hitherto analysed for viral DNA load (Holzinger et al., 2012). Interestingly, a higher viral DNA load of HPV16 has been associated with improved survival and better prognosis among tonsillar and oropharyngeal carcinoma patients (Cohen et al., 2008; Holzinger et al., 2012; Mellin et al., 2002). We observed a large variation of up to 100 000-fold for the HPV16 viral DNA load in different samples. Large variations in HPV16 viral DNA load among HNSCC samples have also been reported in several other studies (Badaracco et al., 2007; Deng et al., 2013; Holzinger et al., 2012; Klussmann et al., 2001; Koskinen et al., 2003). A limitation of our study was that we analysed whole sample DNA preparations and the viral DNA load might have varied between samples due to dilution by HPV-negative cells (e.g. connective tissue and lymphocytes), as suggested by Klussmann et al. (2001). However, we found a link between a low amount of HPV16 in primary oropharyngeal tumours and metastases to regional lymph nodes. Although additional experimental studies have to be performed in order to support our observation, it is tentative to speculate that a low amount of HPV16 in primary oropharyngeal tumours may lead to sustained infections promoting decreased activation of cellular immune response against the HPV-infected cells. It is also possible that low viral DNA load of HPV16 in oropharyngeal tumours represents lesions without viral aetiology. Transcription activity analysis of HPV16 mRNA could have given information if these lesions were driven by HPV. However, our study was only designed for analysis of HPV DNA, and thus samples were not processed for preservation of RNA.

More than 80 % of recurrences of oropharyngeal carcinomas are detected within 2.5 years after diagnosis (Regional Cancer Center West Sweden, 2014). In our series, fewer than 10 % of patients with HPV16 had a recurrence within our follow-up period (mean follow-up 26.5 months, range 1.3–68.3 months), which did not allow a statistical analysis of the relation between viral DNA load and survival.

Concerning the viral physical status, only a small proportion of HPV16-positive cases (6 %) manifested purely integrated HPV16, with mixed (42 %) and purely episomal (51 %) forms being more common. Interestingly, Deng et al. (2013) reported the same low proportion (6 %) of integrated HPV among 18 fresh biopsies from oropharyngeal carcinomas with HPV16; 72 % had mixed status, but only 22 % demonstrated purely episomal HPV16 by the E2/E6 ratio method (Peitsaro et al., 2002). In contrast, Koskinen et al. (2003) reported a correspondingly higher integration rate of 48 %, mixed status in only 17 % and that 34 % had a pure episomal form among 23 HPV16-positive paraffin-embedded HNSCC samples, as determined by the use of an E2/E6 ratio PCR method. However, recently Parfenov et al. (2014) reported a higher integration rate of 71 % for HPV among HNSCCs, as well as integration breakpoints across the entire genome of HPV, by the use of next-generation sequencing (Parfenov et al., 2014). Thus, the next-generation sequencing appears to yield a higher HPV integration rate than the E2/E6 ratio method used, since it is affected by limitations such as decreased detection of HPV integration when large excess (at least 10-fold) of episomal HPV16 is simultaneously present (Ruutu et al., 2008). However, at the time of the design of our study, we chose to use the surrogate marker for integration and episomal state of HPV16 by the use of our modified version of the previously described E2/E7 ratio PCR method.

Fig. 2. Tumour size (T1 to T4) and physical status of HPV16 in 78 HNSCC cases.

Fig. 3. Proportion of HPV16 physical status stratified by viral DNA load among 78 HNSCC cases.
Table 4. Ratio of E2/E7 genes and viral DNA load per cell stratified according to HPV16 physical status

<table>
<thead>
<tr>
<th>HPV16 viral physical status</th>
<th>Median E2/E7 ratio (mean)</th>
<th>Median viral DNA load per cell (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total n=78 (100 %)</td>
<td>0.87 (0.9)</td>
<td>12.85 (46.5)</td>
</tr>
<tr>
<td>Range</td>
<td>0.001–2.2</td>
<td>0.003–1079.6</td>
</tr>
<tr>
<td>Episomal n=40 (51 %)</td>
<td>1.09 (1.2)</td>
<td>12.85 (30)</td>
</tr>
<tr>
<td>Range</td>
<td>0.9–2.2</td>
<td>0.003–228.8</td>
</tr>
<tr>
<td>Mixed n=33 (42 %)</td>
<td>0.65 (0.5)</td>
<td>28.82 (73.4)</td>
</tr>
<tr>
<td>Range</td>
<td>0.2–0.8</td>
<td>0.008–1080</td>
</tr>
<tr>
<td>Integrated n=5 (6 %)</td>
<td>0.60 (0.5)</td>
<td>0.46 (0.9)</td>
</tr>
<tr>
<td>Range</td>
<td>0–0.8</td>
<td>0.004–2</td>
</tr>
</tbody>
</table>

*P-value* represents difference of E2/E7 ratios between groups with different status of HPV16.
†P-value represents difference of viral DNA load per cell between groups with different status of HPV16.

(Letsolo et al., 2016). In addition, we used a supplementary test, for amplification of the complete E2 ORF of HPV16 by PCR, to further support the presence of the episomal state of HPV16 (Letsolo et al., 2016).

We appreciate that the predominance of the episomal form of HPV among tonsillar carcinomas was highlighted in 2004 in a review by Syrjän (2004). In our series of HNSCCs and that of Deng et al. (2013), a strikingly high proportion of episomal HPV16 (93 %) was present (mixed and purely episomal status) with an intact HPV16 E2 gene, which potentially could repress the viral oncogene transcription via E2–protein binding sites in the upper regulatory region of HPV16 (Doeberitz & Vinokurova, 2009). Interestingly, methylation of E2-binding sites of HPV16 appears to be a possible mechanism for inactivation of the repressive function of E2 protein in oropharyngeal cancer (Reuschenbach et al., 2015).

The strength of the present study was the use of fresh biopsies that were sent immediately for HPV analyses. We chose not to use stored paraffin-embedded samples, since they are not designed for HPV analysis by molecular biology techniques, and because we have observed a false-positive rate of 8 % (6/76) among negative controls of 'blank' paraffin-embedded blocks sectioned between cervical cancer samples (Lotten Darlin, personal communication). A limitation of our approach is that it does not allow for verification of tumour cells in the biopsies that were used for HPV analysis. However, all biopsies were collected from visible viable tumour tissues diagnosed as HNSSCC by histology.

Interestingly, we demonstrated that the E2/E7 ratio method varied by 10 % from the theoretical ratio of 1, and therefore 0.9 was set as the cut-off for pure episomal HPV16. Using this cut-off, 12 % (9/78) of HPV16-positive samples were classified as pure episomal HPV16 instead of mixed status, but we cannot completely exclude the possibility that integrated HPV16 existed in this subgroup. However, an advantage of our study compared to that of Peitsaro et al. (2002) was the use of modified forward primer and probe for the E2 PCR to accommodate for the mismatches within some HPV16 sequences (GenBank: FJ610146, FJ610152 and U89348). In addition to the E2/E7 ratio assay, we performed analysis for the presence of the complete E2 gene with the assumption that it represented the episomal form of HPV16, and that its absence indicated a purely integrated form of HPV16.

In summary, the observed HPV prevalence in oropharyngeal carcinomas in South Sweden was within the expected range for Sweden. We found large variations of HPV16 viral DNA load among HNSSCC samples and that low viral DNA load was linked to regional node metastasis. In addition, the episomal HPV16 was the predominating form in these lesions. Longer follow-up studies are required to investigate the impact of HPV16 viral DNA load and viral physical status on the clinical outcome among our HPV-positive HNSSCC patients.

**METHODS**

**Cases.** The catchment area of the South Swedish Health Care Region comprises 1.8 million people. Patients suspected of having head and neck carcinoma are referred to one of nine Ear, nose and throat (ENT) clinics where the diagnostic work-up is done. This includes ordinary ENT examination, with biopsy and/or fine needle aspiration if indicated/possible. If necessary, it is followed by clinical examination/endoscopy under anaesthesia, with biopsies of the epipharynx, base of tongue and tonsillectomy. Relevant computed tomography and magnetic resonance imaging scans are performed. Once the diagnosis is established, the patient is referred to the region's joint ENT-Oncology multidisciplinary therapy conference at the University Hospital of Lund and re-examination is performed under anaesthesia for assessment of resectability. Tumour boundaries are tattooed, since primary treatment almost always is radiotherapy with curative intention, with subsequent salvage surgery as on option.
Fresh biopsies were collected during November 2006 to April 2013 from 275 HNSCC patients. The inclusion criteria were HNSCC within the International Classification of Diseases 10th revision (ICD-10, http://apps.who.int/classifications/icd10/browse/2010/en). Oropharyngeal tumours (n=141) included tonsillar carcinoma (n=103), base of the tongue cancers (n=27) and additional not-specified oropharyngeal cancers (n=11). Non-oropharyngeal tumours (n=134) consisted of hypopharyngeal tumours (n=5) and oral carcinomas (n=55), which were subdivided into carcinomas of the tongue (n=32), floor of mouth (n=12) and not-specified oral cavity (gingiva and buccae) (n=11) carcinomas. Laryngeal carcinomas (n=74) were divided into supraglottic (n=10) and glottic (n=64) sites.

The Lund University regional ethical review board approved the study (LU 376-01).

Sample preparation. During the re-examination under anaesthesia at the ENT clinic in Lund, a sample was taken with a biopsy forceps (Trucut) from the viable tumour tissue for analysis of HPV, and if necessary for complementary routine histopathology in cases where tumour samples from the referring ENT clinic were not satisfactory. Thus, all samples were confirmed to be HNSCC by histology. Tissue samples for histopathology were placed immediately in buffered formalin and sent within 24 h to the pathology laboratory. The 2-3 mm biopsy for HPV analysis was immersed in 1 ml saline solution at room temperature and immediately sent to the Microbiology Laboratory in Malmö by the Hospital postal service. The laboratory received the sample within 24 h, and it was further processed on the same day.

The saline was removed and the biopsy was digested in lysis buffer and the DNA extracted with the Total Nucleic Acid NA kit (Roche) using Magna Pure LC (Letsolo et al., 2016). Sample adequacy was assessed by PCR for the human β-globin gene (Sturegaard et al., 2013). Detection of genital HPV types was carried out by modified general primer PCR, amplifying a segment of the HPV L1 gene, followed by Luminex analysis for identification of 39 HPV types simultaneously (Letsolo et al., 2016; Schmitt et al., 2006; Soderlund-Strand et al., 2009).

Viral DNA load. Briefly, the number of viral genomes per cell was quantified by carrying out two separate real-time PCR tests, including appropriate standard curves, to amplify the HPV16 E7 gene and the human β-globin gene as described by Letsolo et al. (2016). The samples were analysed in duplicate.

Physical status of HPV16. As a marker for episomal HPV16, the samples were analysed for integrity of complete E2 ORF by PCR adapted from Collins et al. (2009), as described by Letsolo et al. (2016). If ampli-
cions were absent, the sample was subjected to a further twofold dilution and PCR was repeated. In addition, the ratio of E2/E7 gene copy numbers was determined to investigate the presence of integrated, mixed or episomal forms of HPV16. HPV16 was classified as integrated when complete E2 ORF was absent and E2/E7 ratio was <0.9, mixed status with the presence of complete E2 ORF and E2/E7 ratio of <0.9, and pure episomal status with the presence of complete E2 ORF and E2/E7 ratio of ≥0.9. The E7 copy numbers were retrieved from the viral DNA load measurement as described above. For quantification of the E2 copy numbers we used a modified version (Letsolo et al., 2016) of the PCR described by Peitsaro et al. (2002) where E2/E6 ratios >1 indicate pre-
domination of the episomal form. Quantification was extrapolated from a linear regression standard curve that was included in each batch assay, as described previously (Letsolo et al., 2016). The samples were analysed in duplicate.

Intra-assay variation of the E2/E7 ratio method for episomal HPV16. To investigate variation of the E2/E7 ratio method for pure episomal HPV16, we performed quantitative PCR for the E2 and E7 genes as described above, by the use of 50 000 to 50 copies per PCR of HPV16 plasmid DNA in a background of 25 ng human DNA. The PCR was repeated 10 times for each input HPV16 quantity. The E2/E7 ratio method demonstrated a mean coefficient of variation of 10 % (Fig. 4), and 0.9 was set as the cut-off for predominance of the episomal form.

Statistical analysis. GraphPad Prism software was used for the statistical analysis. A two-tailed Fisher’s exact test was performed to deter-
mine the associations between clinical characteristics and HPV status. OR with a 95 % CI and P-values were calculated. A Kruskal–Wallis test was applied to viral physical status data and a Dunn’s multiple compar-
test along with P-values was applied to express differences of viral DNA loads and E2/E7 ratios between episomal, mixed and integrated physical status groups.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Cancer Society, Laryngofonden, the King Gustaf V Jubilee Fund, Governmental funding (ALF), Region of Scania, the Foundation of the University Hospi-
tal of Lund, the Gunnar Nilsson Cancer Foundation and the Berta Kamrad Foundation for Investigation and Control of Cancer Dis-
cases, and the Cancer Foundation of the University Hospital of Malmö. Funding sources had no influence on the study design or the interpretation of results. We thank Lena Nilsson and Herman Palm (Department of Medical Microbiology, Laboratory Medicine Skane) for technical assistance.

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