Abstract. The purpose of this article is to review the current knowledge on the status and significance of human papillomavirus (HPV) in tonsillar cancer. Current data in scientific reports and data from the Karolinska Hospital and Karolinska Institute, Sweden, demonstrate that approximately half of all tonsillar cancer is HPV-positive. Moreover, patients with HPV-positive cancer have a lower risk of relapse and longer survival compared to patients with HPV-negative tonsillar cancer. The favourable outcome for patients harbouring HPV-positive tonsillar cancer cannot be attributed to increased radiosensitivity, since there is no significant difference in sensitivity to radiotherapy between HPV-positive and -negative tonsillar cancer. However, HPV-positive cancer exhibits less genetic instability i.e. shows a lower degree of aneuploidy and a tendency to have fewer chromosomal aberrations, when compared to HPV-negative tonsillar cancer.

The fact that preventive vaccines against human papillomavirus (HPV) may soon reach clinical practice has put more focus on research regarding infections with HPV and the association between HPV and cancer (1,2). The best-known example of HPV infection and its correlation to cancer is in cervical cancer, where HPV is present in almost all cases (3). However, there is also an association between the presence of HPV and cancer in other regions, such as some types of anogenital cancer, non-melanoma skin cancer and head and neck cancer (4,5). In this article we would like to focus on head and neck cancer and, in particular, tonsillar cancer, since this is the cancer within the head and neck cancer region where HPV is most commonly found (6).

Head and neck cancer

Head and neck cancer includes cancer of the lip, the oral cavity, the nose and sinuses, the nasopharynx, the oropharynx, the hypopharynx, the larynx, the esophagus, the salivary glands, as well as the soft tissues of the neck and the ear (7-9). Tonsillar cancer is the most common tumour in the oropharynx (6). Essential functions such as breathing, eating and speaking may be greatly affected in patients with advanced tumours in the aerodigestive tract. Further distress may be added due to cancer growth in the face and the neck. In addition, surgery and radiotherapy treatment can be disabling and disfiguring. Therefore it is of utmost importance to optimise individual therapy, i.e. to give the most efficient treatment with the lowest possible impact on function and form.

Treatment. Head and neck cancer treatment with curative intent implies surgery and/or radiotherapy (8,9). Radiotherapy can be given alone, before surgery, i.e. preoperatively, or after surgery, i.e. postoperatively. The radiotherapy dose ranges from 50-68 Gray (Gy) which, at the Karolinska Hospital, Sweden, is delivered as fractionated radiotherapy 5 days a week (9). The primary goal for surgery is to be radical and, with an adequate safety margin, to excise all tumour tissue with as limited functional and cosmetic damage as possible. If cure cannot be obtained, the patients receive palliative therapy, in order to treat pain and discomfort. Palliative therapy may include radiotherapy, chemotherapy, optimal pain relief, nutrition aid and psychological support.

Incidence and prevalence. Cancer in the head and neck region comprises 3-4% of all cancer in Sweden, which is
slightly lower than that observed in other developed countries (9,10). Approximately 1000 new cases of head and neck cancer are diagnosed annually in Sweden, excluding esophageal cancer (9). The incidence is almost twice as high in men as in women (9). In Europe, the Latin countries have a higher prevalence compared to Northern Europe (10,11). In other areas of the world, head and neck cancer is much more common and, for example, in Bombay, India, it accounts for nearly 50% of all cancer cases (11).

Prognosis. The five-year survival is approximately 30-40% for all patients with head and neck cancer. Eighty percent of the relapses occur within 1.5 years (8,9). No major changes in survival for patients with head and neck cancer have been noted in the last few decades.

Aetiology. The large global differences in the incidence of head and neck cancer imply that exogenous factors are of importance for its development. As much as 80-90% of all head and neck cancer may be attributed to known risk factors, such as smoking and alcohol abuse (11,12) and there is a dose-response relationship between tobacco exposure and risk of head and neck cancer (13). Viruses have also been proposed to be connected with head and neck cancer (14). EBV was the first virus reported to be associated with head and neck cancer, more specifically to nasopharyngeal cancer in Southern China (15). Later, the first reports appeared on HPV in head and neck cancer (16-19). Accumulating molecular and epidemiological data indicate that high-risk types of HPV, similar to those observed in cervical cancer, are associated with a subset of head and neck cancer (6,20-23). The prevalence of HPV in head and neck cancer varies significantly between different reports. This discrepancy can probably be explained by differences in tumour site and the method used for analysis, as well as the number of cases included. The strongest association so far has been found for oropharyngeal cancer, especially tonsillar cancer, where HPV has been found to be present in between 45-70% of all cases (6,8,21,22,24,25).

Human papillomavirus

There are more than 100 HPV types (3,26). Some HPV types are associated with common warts (e.g. HPV1, 2 and 4), while others e.g. HPV6 and 11, are more commonly found in condylomas and papillomas (27). Finally there are HPV types such as HPV16, 18, 31, 33 and others that are associated with malignant tumours (28). The best known association is the correlation between oncogenic HPVs and cervical cancer, however more recently a connection between oncogenic HPVs and other tumours of the anogenital region and of the head and neck have been acknowledged (29-31).

The genome of all HPVs is similar and consists of a double-stranded circular DNA, enclosed in a 52-55nm viral capsid (26). It is divided into three regions: the non-coding region (NCR) and the early and late regions (Figure 1). The NCR consists of the origin of viral DNA replication, a promoter and an enhancer region. The early region encodes the early proteins E1, E2 and E4-7, which are important for pathogenesis and transformation ability (3,32). E1 is necessary for viral replication. E2 is important for the regulation of viral transcription. E6 and E7 are classified as oncogenes in oncogenic HPVs and are considered to be necessary for transformation. E6 binds to the cellular protein p53 and degrades it, while E7 binds to the retinoblastoma protein Rb and abrogates its function (3,32). Through these interactions, in oncogenic HPVs, the E6 and E7 proteins enhance the inhibition of cell cycle control and thus contribute to tumour development. In the non-oncogenic HPV types, the same interactions occur less efficiently or not at all (32).

The proteins of the late region L1 and L2 code for the viral capsid proteins. L1 is the major capsid protein and the
capsid consists of 360 L1 molecules and 10 L2 molecules (3). Similar to other related viruses (polyomaviruses) (33), the major capsid protein L1 can form virus-like particles (VLPs) without the help of other viral genes (34). This is one of the properties of L1 which allows it to be used for vaccine production with regard to preventive vaccines against HPV (34).

Tonsillar cancer

Tonsillar cancer is the most common oropharyngeal cancer and most tumours are squamous cell carcinomas (6-9). Small tumours in the tonsils do not usually give rise to any discomfort. Thus, unfortunately, the patients may not seek counselling until the tumour is fairly large and presents symptoms, such as pain related to swallowing or difficulties in swallowing (9). Other common initial symptoms are pain in the ear, or a lump in the neck due to tumour spreading to the lymph nodes (9). Tonsillar cancer in Sweden is generally treated with (pre-operative) full-dose radiotherapy (64 Gy) against the primary tumour and the neck. The extent of the surgical treatment depends on the size of the primary tumour, the presence of metasteses to the neck lymph nodes and the response to the given radiotherapy. In general, patients with limited tumours (stage I-II) oropharyngeal cancer have a 5-year survival of 60-70%, while patients with larger tumours, stage III-IV oropharyngeal cancer, have a 5-year survival of 10-25% and the overall survival for patients with oropharyngeal cancer is only slightly more than 25% (8,9). However, despite similar histology and stage, as well as standardised treatment, it is not easy to predict the outcome of the individual case. Hence, both predictive and prognostic markers would be of significant clinical value in order to tailor treatment for tonsillar cancer patients.

Tonsillar cancer and HPV

HPV DNA has been shown to be present in 45-70% of all tonsillar cancer (8,21,24,35,36). The variation depends mainly on the methodology used for detection and the type of material that is available for analysis (35,36). In the studies performed at the Karolinska Institute and Hospital, Sweden, some material had been stored for several years and could thus be compared with more recent fresh-frozen tumour material (8,35,36). It was easier to detect HPV in fresh-frozen (-70°C) tumours compared to formalin-fixed and paraffin-embedded tumours, due to the fact that the DNA was degraded in formalin-fixed and paraffin-embedded material and even more so in material that had been stored for several years (35,36).

The most common and sensitive technique for detection of HPV today is based on polymerase chain reaction (PCR) technology and is performed either as a regular PCR or as a quantitative PCR (35,36). In the past, less sensitive techniques such as Southern blots or in situ hybridisation techniques were used (36). Screening for HPV by PCR analysis is usually initially performed using a general HPV PCR, with general primers for HPV e.g. GP5+/6+, or CPI/IIG allowing for detection of several HPV types (37,38). If HPV DNA is found, the next step is usually to perform a type-specific PCR for typing followed by DNA sequencing.

The presence of HPV was examined in pre-treatment tumour biopsies taken from 84 patients that were diagnosed with tonsillar cancer at the Karolinska Hospital, during the years 1984-1999 (8,35,36). DNA was extracted from the biopsies by routine procedures and a general HPV-PCR was performed with primers GP5+/6+. HPV- negative samples were also run in a PCR with CPI/IIG primers (34,35). The HPV PCR-positive samples were then tested for HPV-type by HPV-type-specific PCRs and sequencing. To avoid false-negative results and to determine whether the DNA was amplifiable, a PCR was also performed on a control cellular gene (HLA DQ) (8). The frequency of HPV in our material was 46% (39/84) (36). As expected the detection level of HPV was higher in the fresh-frozen material (55%) as compared to the paraffin-embedded material (43.5%) (8,35,36). The majority of the HPV-positive material (37/39) was HPV16-positive, one sample had a double infection with both HPV16 and 33 and one sample was only HPV33-positive (35). Both findings, i.e. the frequency of HPV in tonsillar cancer as well as the clear dominance of HPV-type 16, were in line with other reports (6,21,23-25,39-41). Consequently, the typical finding is that 90-100% of the HPV-positive biopsies in tonsillar cancer are HPV-16 and 0-7% are HPV-33, while HPV-31, HPV-59 or non-typeable HPV (HPV-X) are found more rarely.

HPV in tonsillar cancer - a favourable prognostic factor

One aim was to investigate whether HPV was a favourable prognostic factor (8). In this study 52 out of 60 patients could be evaluated for clinical outcome. Twenty-three of these 52 patients were HPV-positive and 52% of the patients with HPV-positive tumours were tumour-free 3 years after diagnosis as compared to 21% of the patients with HPV- negative tumours (OR=4.18, p=0.025, χ²-test) (Figure 2). Patients with HPV-positive tumours also exhibited a significantly longer 5-year survival compared to patients with HPV-negative tumours (53.5% compared to 31.5%, p=0.047). HPV was a favourable prognostic factor independent of tumour stage, age, gender and grade of differentiation (8). However, note that the prognosis for stage I was good for all patients (Figure 2). In another study, p53 immunoreactivity was also analysed by immunohistochemistry (IHC) (45). Independently of p53 IHC, HPV was a favourable factor for clinical outcome.
Additional reports on HPV as a favourable factor have since then been added to the literature (8,21,24,35,42,43). In a study by Gillison et al. (21), including 253 head and neck cancer patients, of which 60 were patients with oropharyngeal cancers (mostly tonsillar cancers), disease-specific survival was significantly improved for the HPV-positive oropharyngeal cancer group compared to the HPV-negative group. In contrast, among patients with cancer at sites other than the oropharynx, the disease-specific survival was similar regardless of HPV status (21). Accordingly, the prognostic value of HPV did not seem to hold for head and neck cancer in general, but for tonsillar cancer more specifically (7,21,25,44). Another study presented a survival analysis on 31 patients with tonsillar cancer according to the pRb expression of the cancers and demonstrated a significantly better survival for patients with tumours lacking pRb expression (24). HPV presence and survival were not analysed separately, but only indicated since there was a significant correlation between lack of pRb expression and presence of HPV (24). A third study, of 52 patients with tonsillar cancer, reported a survival advantage in patients with HPV-positive tonsillar cancer (42). However, in this study, the HPV-positive patients were substantially younger than the HPV-negative patients were and the survival advantage was thus not statistically significant when adjusting for age in multivariate analysis (42).

In a subsequent study, the possible importance of HPV viral load on clinical outcome was evaluated (35). The presence of HPV was analysed by general and type-specific PCR and quantification was performed by a quantitative PCR. Eleven of the 22 analysed patients had HPV16-positive tonsillar cancer and the viral load ranged from 10-15,500 HPV16 copies/cell. Our estimation of viral load in tonsillar cancer was thus in line with the study of Klussman et al. (40), in which six tonsillar cancers and their metastases were analysed and the viral copy number per β-actin varied between 5.8 and 152.6. Interestingly, in our study, patients with >190 HPV16 copies in their tumour cells had a significantly longer survival rate than patients with ≤60 HPV16 copies /cell (p=0.039) as shown in Figure 3. It is possible that a stronger immune response is generated against tumour cells that contain a high viral content, which may explain why patients with a large viral load have a better survival.

**HPV in tonsillar cancer - in relation to radiosensitivity**

A separate study was performed in order to examine whether the favourable clinical outcome of patients with HPV-positive tonsillar cancer was due to differences in radiosensitivity (45). Forty patients with tonsillar cancer (21 patients with tumours that had a complete remission (CR) after radiotherapy and 19 patients who did not have a complete remission (non-CR), i.e. no response, a partial response or progressive disease) were included in this study (46). The tumours were analysed by PCR for the presence of HPV DNA and by immunohistochemistry for the presence of mutated p53. The evaluation of the radiotherapy outcome was performed one month after completion of radiotherapy by clinical examination and, when required, also with a biopsy at the primary tumour site. When no evidence of tumour could be observed, the outcome was classified as CR and, when there was viable tumour left, the outcome was classified as non-CR. Among the 40 patients, 34 had tumours with amplifiable DNA and could be

![Kaplan-Meier graph showing significantly better disease-specific survival in patients with tumours with ≥HPV16 copies/β-actin compared to patients with tumours with ≤HPV16 copies/β-actin in tonsillar cancer](image)

**Figure 3.** Kaplan-Meier graph showing significantly better disease-specific survival in patients with tumours with ≥HPV16 copies/β-actin compared to patients with tumours with ≤HPV16 copies/β-actin in tonsillar cancer (p=0.039, log rank test, n=11). Reproduced from Mellin et al. (35) with permission from Wiley.

<table>
<thead>
<tr>
<th>HPV status</th>
<th>CR</th>
<th>Non-CR</th>
<th>Total No</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV+</td>
<td>8</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>HPV-</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

1 HPV DNA-positive according to PCR data, with GP5+/6+ primers.
2 Response evaluated one month after completed radiotherapy.
Reproduced from Mellin et al. (35) with permission from Anticancer Research.

Table I. HPV status according to complete (CR) response to radiotherapy.
evaluated for HPV status. There were no significant differences in radiotherapy response between patients with tumours with different HPV status or p53 status; in fact 57% of the patients with HPV-positive tumours and 50% of the patients with HPV-negative tumours had CR (Table I).

### HPV in tonsillar cancer and influence on the cellular genome

There are differences with regard to clinical outcome between patients with HPV-positive and -negative tonsillar cancer and this appears, primarily, not to be due to differences in radiosensitivity (45). To find a possible explanation for this discrepancy, a number of tonsillar cancer biopsies were examined with regard to their genetic instability (43,47). The degree of DNA aberration (diploid or aneuploid) was analysed by Image Cytometry (ICM) (47) and the chromosomal set-up was analysed by "comparative genomic hybridisation" (CGH) (43). All tonsillar cancer was genetically unstable, but HPV-positive tonsillar cancer was generally somewhat less unstable than HPV-negative tonsillar cancer when analysed both by ICM and CGH (47,43).

Using image cytometry (ICM), the degree of DNA aberration was investigated in order to study whether HPV-positive and -negative cancers differ in DNA content and whether the degree of DNA aberration also influences clinical outcome (47). The DNA content was estimated in 58 primary tonsillar tumours. The normal diploid cell nuclear DNA content was indicated as the 2c value (c is the haploid genome equivalent). The fraction (percent) of cancer cells exceeding 2.5c was referred to as the 2.5c exceeding rate (2.5c ER), while the percent of cancer cells exceeding 5c was referred to as the 5c exceeding rate (5cER). Cancer cells with a DNA content value above 2.5c were considered as either proliferating diploid cells or aneuploid cells, whereas cells with a DNA content value above 5c ER were considered to be aneuploid (hyperploid). A lesion was classified as diploid if none of the cells exceeded 5c ER and less than 35% of the cells were between 2.5-5c ER.

Most of the analysed tumours exhibited a high degree of aneuploidy, harbouring a mean of 17.5% of the cells in 5cER while only 7 (12%) of the tumours were found diploid. Patients with a cancer with a 5c ER below the mean value were, to a higher degree, disease-free after 3 years and displayed a better survival compared to patients harbouring tumours with a 5c ER above the mean value. These differences were, however, not statistically significant. HPV-positive tumours had a tendency to have a lower mean 5c ER, 13%, as compared to 22% for the HPV-negative tumours ($p=0.066$, $\chi^2$ test). Furthermore, significantly fewer HPV-positive tumours had a 5c ER above the mean value compared to the HPV-negative tumours ($p=0.026$, $\chi^2$ test).

Nonetheless, independent of DNA content, patients with HPV-positive cancer were, to a higher degree, disease-free three years after diagnosis compared to patients with HPV-negative cancer (Table II).

Of the 25 tumours that were analysed by CGH, 15 were HPV-positive and there were significant differences between HPV-positive and negative tumours (43). The average number of chromosomal aberrations (ANCA) among the HPV-positive tumours was 4.5 while the corresponding figure was 6.1 for the HPV-negative tumours ($p=0.066$, $\chi^2$ test). Furthermore, significantly fewer HPV-positive tumours had a 5c ER above the mean value compared to the HPV-negative tumours ($p=0.026$, $\chi^2$ test).

Nonetheless, independent of DNA content, patients with HPV-positive cancer were, to a higher degree, disease-free three years after diagnosis compared to patients with HPV-negative cancer (Table II).

### Table II. HPV status and mean value of 5c ER correlated to disease-free 3 years after diagnosis.

<table>
<thead>
<tr>
<th>HPV status</th>
<th>2.5c ER ≥ 17.5%</th>
<th>2.5c ER ≤ 17.5%</th>
<th>5c ER ≥ 17.5%</th>
<th>5c ER ≤ 17.5%</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV+</td>
<td>2/3 (67%)</td>
<td>9/15 (60%)</td>
<td>2/12 (17%)</td>
<td>2/12 (17%)</td>
<td>15/42 (36%)</td>
</tr>
<tr>
<td>HPV-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. All HPV+ tumours were HPV-16 +, except for one tumour which contained both HPV-16 and -33.
2. Mean value of 5c ER was 17.5%.

### Table III. Differences of gains and losses of HPV+ positive and negative tonsillar cancer.

<table>
<thead>
<tr>
<th>ANCA</th>
<th>+3q</th>
<th>+7q</th>
<th>-11q</th>
<th>+3q/-11q</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>15 (60%)</td>
<td>4 (16%)</td>
<td>11 (44%)</td>
<td>9 (36%)</td>
</tr>
<tr>
<td>HPV+ cases</td>
<td>11 (73%)</td>
<td>0 (0%)</td>
<td>7 (47%)</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>HPV- cases</td>
<td>4 (40%)</td>
<td>4 (40%)</td>
<td>4 (40%)</td>
<td>3 (30%)</td>
</tr>
</tbody>
</table>

1. HPV DNA-positive according to PCR data, with GP5+/6+ or CPI/IIG primers. Reproduced from Dahlgren et al. (35) with permission from Wiley.
chromosomal set-up, in both our studies a better clinical outcome was observed for patients with HPV-positive tumours compared to HPV-negative tumours (43,47).

**HPV in tonsillar cancer and anti-virus vaccines**

In conclusion, HPV is present in approximately half of all tonsillar cancer and is a prognostic favourable factor for clinical outcome (35). This appears not to be due to differences in radiosensitivity between HPV-positive and HPV-negative tonsillar tumours (43). However, in general, HPV-positive tumours are somewhat less genetically unstable compared to HPV-negative tonsillar tumours (43,47). The fact that patients with a high HPV viral load in their tumours have longer survival than patients with a lower viral load suggests that there could be an immune response against the virus, which contributes to the better clinical outcome (35). If so, it could be important to enhance this anti-viral-immune response. Clinical trials using HPV16-specific peptides to boost the immune system against HPV infection are presently ongoing in the Netherlands for patients with cervical cancer (C. Melief, personal communication). It would be of interest to proceed with similar studies in patients with HPV16-positive tonsillar cancer. Alternatively, for prevention of HPV infection and, hence, in the long run HPV-associated cancer including e.g. both cervical cancer and some types of tonsillar cancer, the use of virus-like particles seem to be possible candidate vaccines.

**Acknowledgements**

This work was supported in part by the Swedish Cancer Foundation, the Stockholm Cancer Society, the Stockholm City Council and the Karolinska Institutet, Sweden.

**References**


