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Review

Human Papillomavirus Accounts both for Increased Incidence and Better Prognosis in Tonsillar Cancer

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Abstract. *The aim of this review is to present the current knowledge on the status and significance of human papillomavirus (HPV) in tonsillar cancer. An increase in the incidence of tonsillar cancer has been reported and recent data suggest that this increase is due to an increased proportion of HPV in these tumours. Furthermore, patients with HPV positive cancer have been shown to have a lower risk of relapse and longer survival compared to patients with HPV-negative tonsillar cancer. Tailoring individual treatment in tonsillar cancer may be of importance in order to reduce patient suffering as well as to increase patient survival. Finally, the fact that the presence of HPV-type 16 E6 and E7 mRNA has been ascertained in tonsillar cancer suggests that HPV-16 indeed is an aetiological factor associated with the disease and that preventive vaccination for this patient group should be discussed.*

After years of development and anticipation, preventative vaccines against human papillomavirus (HPV) have now reached clinical practice. This has put even more focus on research regarding which types of cancers are associated with HPV infection, which groups of patients should be vaccinated and the possible beneficial effects of such vaccination (1, 2). The main goal of the two presently approved vaccines (Gardasil and Cevarix), both directed against HPV 16 and 18, is to protect against HPV-induced cervical cancer (3-5). In addition, Gardasil also aims to protect against HPV 6 and 11 and the development of genital warts (3). However, the presence of HPV is not only associated with cervical cancer but also with some types of head and neck, anogenital and non-melanoma skin cancer (6-12). In this review, we focus on the presence of HPV in tonsillar cancer and the increasing role of HPV in this tumour type, since this is the type of head and neck cancer where HPV is most commonly found (8).

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Tonsillar Cancer

Tonsillar cancer is the most common oropharyngeal cancer and tumours are mainly squamous cell carcinomas (8-10, 13). Unfortunately, when patients seek counselling for symptoms, such as pain related to swallowing or difficulties in swallowing, the tumour is fairly large, since small tumours do not present similar distress (9). Treatment with curative intent implies surgery and/or radiotherapy and in some cases also chemotherapy (10, 13). Radiotherapy can be given alone, before surgery *i.e.* preoperatively, or after surgery *i.e.* postoperatively. The primary goal for all treatment is to be radical, causing as limited functional and cosmetic damage as possible. The extent of the surgery depends on the size of the primary tumour, the presence of metastases to the neck lymph nodes and the response to the given radiotherapy. If a cure cannot be obtained, the patients receive palliative therapy in order to treat pain and discomfort.

Patients with stage I-II oropharyngeal cancer have a 5-year survival of around 60-70%, while patients with larger tumours, stage III-IV, have a 5-year survival of around 10-25%, while overall survival is around 25% (10, 13). Furthermore, despite similar histology and stage, as well as standardised treatment, it is difficult to predict the outcome of any individual case.

Recently, several reports describe the incidence of tonsillar cancer as increasing and indicate that patients with HPV-positive tonsillar cancer have a better clinical outcome than those with HPV-negative tonsillar cancer (10, 14-17).

Hence, both predictive and prognostic markers would be of significant clinical value in order to tailor treatment for tonsillar cancer patients.

Human Papillomavirus

There are more than 100 HPV types of which some (*e.g.* HPV 1, 2 and 4) are found in common warts, others (*e.g.* HPV6 and 11) have been demonstrated in *e.g.* chondylomas, while some (*e.g.* HPV16, 18, 31, 33) are associated with malignant tumours (4, 18-20). The correlation between HPV and cervical cancer is well established (19), however the association between HPV and head and neck cancer and other tumours is now also acknowledged (6-7, 9, 11, 21-23).

The HPV genome, enclosed in a 52-55 nm viral capsid, consists of double-stranded circular DNA arbitrarily divided into three regions: the non-coding region (NCR) and the early and late regions (18). The origin of viral DNA replication, a promoter and an enhancer region are located within the NCR. The early region-encoded proteins, E1-E2 and E4-E7, are important for pathogenesis and transformation (6, 18, 24). In so-called high risk HPVs, E6 and E7 are classified as oncogenes and regarded necessary for transformation. E6 binds to the p53 cellular protein and degrades it, while E7 binds to the retinoblastoma protein (Rb) and inhibits its function (6, 18, 24). This way, in oncogenic HPVs, the E6 and E7 proteins deregulate cell cycle control and contribute to tumour development. In non-oncogenic HPV types, these interactions occur less efficiently or are non-existent (24). The late region encodes L1 and L2, the two viral capsid proteins. The viral capsid consists of 360 L1 molecules and around 10 L2 molecules (6, 18). Under appropriate conditions, without assistance of viral genes, L1 self-assembles into virus-like particles (VLPs) that form the basis for the now approved vaccines against HPV infection (25).

HPV in Tonsillar Cancer

HPV DNA has been shown to be present in 45-100% of all tonsillar cancer (8, 10, 26-28). This variation depends partly on the methodology used for detection and the type of material that is available for analysis (27, 28). Recently, however, an evident increase in the proportion of HPV-positive tonsillar cancer has been reported (29).

Detection of HPV today is often based on polymerase chain reaction (PCR) technology, performed either as regular PCR or as quantitative PCR (27, 28, 30-32). Screening for HPV by PCR can initially be performed using general HPV PCR, with general primers for HPV *e.g.* GP5+/6+, or CPI/IIG allowing detection of several HPV types (30, 31). After general PCR screening, the PCR product can be HPV typed by a reverse hybridization technique called reverse line blot (RLB) or line probe assay depending on which primers were used for the initial HPV detection (32, 33). Alternatively, HPV typing may be performed by a type-specific PCR (34). HPV typing can also be performed by sequencing the general PCR product (27). To avoid false-negative results, the DNA is generally tested with regard to whether it is amplifiable by PCR with a control cellular gene (10). Another common HPV screening method is Hybrid Capture 2 (HC2), a method designed for detection of a selected combination of five low-risk and 13 high-risk HPV types (Digene Corporation, Gaithburg, Maryland USA) (35). The HC 2 test is the only FDA-approved HPV detection test available. The assay uses the fact that HPV DNA hybridizes with synthetic RNA probes complementary to DNA sequences to specific HPV types. The sensitivity of the HC2 test is lower than the sensitivity of PCR, equivalent to 1 pg HPV DNA (5000 copies of HPV genome) although a sensitivity down to 500 copies of HPV genome has been reported (36).

A New Epidemic – The Increase in the Incidence of Tonsillar Cancer Depends on an Increased Proportion of HPV-positive Tonsillar Cancer Cases

Recently, in the Stockholm area from 1970-2002, we disclosed a parallel three-fold increase in the incidence of tonsillar cancer and the proportion of HPV-positive tonsillar cancer cases, indicating a possible role of HPV infection in this disease (29). According to the Swedish Cancer Registry, the incidence of tonsillar cancer in the Stockholm area increased 2.8-fold (2.6 in men and 3.5 in women) between 1970 to 2002. During the same period, when examining 203 pretreatment paraffin-embedded tonsillar cancer biopsies from the same area for the presence of HPV DNA by PCR, we could demonstrate a 2.9-fold ($p<0.001$) increase in the proportion of HPV-positive tonsillar cancer cases. The distribution of HPV-positive tonsillar cancer was: 23% in the 1970s, 29% in the 1980s, 57% in the 1990s and 68% during 2000-2002.

We thus demonstrated a highly significant and parallel increase both in the incidence of tonsillar cancer and the proportion of HPV-positive tumours, suggesting that HPV may play an important role for the increased incidence of tonsillar cancer. Recently, D'Souza *et al.* (37) showed a similar tendency in the U.S. in finding that the HPV is present in 72% of all oropharyngeal cancer in the Baltimore area (Johns Hopkins Hospital and University). Furthermore, in both these studies, the majority of patients were men (29, 37). This should influence future preventive strategies as well as treatment for this type of cancer. In both studies, HPV 16 was most commonly found and was present in the majority of the cases (29, 37). Other HPV types (*e.g.* HPV-31, -33, -58, -59, -62 and -72), are less commonly found in tonsillar cancer and also show different geographical distribution (29, 37).

Expression of E6 and E7 mRNA in HPV-16-positive Tonsillar Cancer

The fact that HPV-16 is an aetiological agent for the development of tonsillar cancer is supported not only by its role in the increase of the incidence of tonsillar cancer but also by accumulating evidence of expression of HPV-16 E6 and E7 mRNA in tumours (17). Recently, expression of E6 and E7 mRNA was analyzed in 53 HPV-16-positive tonsillar cancer samples, where the expression housekeeping gene RNase P could successfully be detected. HPV-16 E6 mRNA and/or E7 mRNA expression was detected in 94% of these samples (17). In 79% of the samples, both E6 and E7 were detected, and in an additional 15% of the samples E7 was detected, while in the remaining 6% neither E6 nor E7 could be demonstrated. Hence, the fact that HPV-16 E6 and/or E7 mRNA is expressed in most HPV-16-positive cancers indicates an important role of HPV-16 in the carcinogenesis of tonsillar cancer.

HPV in Tonsillar Cancer – A Favourable Prognostic Factor

The presence of HPV DNA in tonsillar cancer has also been shown to be a favourable prognostic factor for clinical outcome (10, 17, 27, 38). In an early study with 52 patients, of the 27 patients with HPV-positive tumours, 52% were tumour-free 3 years after diagnosis as compared to 21% of the patients with HPV-negative tumours odds ratio (OR)=4.18, $p=0.025$ χ-test) (10). A significantly longer 5-year survival was moreover observed in patients with HPV-positive tumours 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, grade of differentiation and p53 immunohistochemistry (ICH) (10, 39). Since then, numerous reports on HPV as a favourable factor in tonsillar cancer have been added to the literature (17, 27, 38, 40). A study on 253 head and neck cancer patients, of whom 60 were patients with oropharyngeal cancer (mostly tonsillar cancer), reported improved disease-specific survival only for the HPV-positive oropharyngeal cancer group compared to the HPV-negative group and not for other cancer sites (38). In a similar way, the prognostic value of HPV did not hold for head and neck cancer in general, but specifically only for tonsillar cancer (9, 26, 38, 41). Another study demonstrated a significantly better survival for patients with tumours lacking pRb expression, however here 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 (42). Recently, in a larger study with 203 tonsillar cancer patients, 150 patients whom received treatment were evaluated for survival and the positive prognostic value of HPV was ascertained (17). It was in fact shown that 81% of patients with HPV-positive tumours had a 5-year survival rate as compared to 36% of patients with HPV-negative tumours ($p<0.001$) (17).

HPV Viral Load in Tonsillar Cancer Does not Affect Prognosis

The possible importance of HPV viral load on clinical outcome has also been evaluated (27, 17). In an early study, the presence of HPV was analysed by general and type-specific PCR and quantification was performed by a quantitative PCR (27). In this study, 11/22 analysed patients had HPV16-positive tonsillar cancer with a viral load between 10-15,500 HPV16 copies/cell (27). Moreover, patients with >190 HPV16 copies in their tumour cells had a significantly longer survival rate than patients with <60 HPV16 copies/cell ($p=0.039$). This tendency could however not be confirmed in a later larger study (17). Among 150 tonsillar cancer patients, the estimated viral load in the 86 tumour samples that were HPV-16 positive was between 0.08 and 130 copies/cell. Patients with HPV-positive samples were divided into groups using quartiles as cut-offs. When comparing these different groups, no significant differences between the groups were observed.

HPV and $p16^{INK4a}$ in Tonsillar Cancer in Relation to Response to Radiotherapy (RT)

Different studies have also been performed in order to examine whether the favourable clinical outcome of patients with HPV-positive tonsillar cancer was due to differences in radiosensitivity (17, 39). In one early study, 40 tonsillar cancer patients, 21 patients with complete response ((CR) *i.e.* no viable tumour) after RT and 19 non-CR patients were included (43). In another later study, the 150 tonsillar cancer patients, who have also been described above, were included (17). In the first study, no correlation was found between the presence of HPV in the tumours and response to RT. In the second study, there was a tendency for patients with HPV-positive tonsillar cancer to respond better to RT than those with HPV-negative tonsillar cancer, but these differences were not significant ($p=0.18$, Pearson's χ^2 test).

In a third study an additional approach was taken and $p16^{INK4a}$ over expression, analyzed by ICH, was correlated to HPV status in tonsillar carcinoma and to response to RT (44). Overexpression of $p16^{INK4a}$ was correlated to the presence of HPV in tonsillar carcinoma, which further showed that the presence of high-risk HPV was a risk factor for tonsillar carcinoma. Furthermore, almost all patients with a high-grade (2+ and 3+) $p16^{INK4a}$ staining had a CR to RT and both presence of HPV by PCR, as well as high-grade

$p16^{INK4a}$ IHC, were statistically significantly correlated to staying disease-free and having disease specific survival (44).

This latter result should be explored further with an improved staining method and the cut-off level of $p16^{INK4a}$ expression best suited for predicting response to RT should be investigated.

HPV in Tonsillar Cancer and Influence on the Cellular Genome

There are obvious differences with regard to clinical outcome in patients with HPV-positive and HPV-negative tonsillar cancer and differences in response to RT may not be the major explanation (39, 17). As a possible alternative explanation for this discrepancy in tonsillar cancer, the degree of DNA aberration (diploidy or aneuploidy) was analysed by Image Cytometry (ICM) (45) and the chromosomal setup was analysed by comparative genomic hybridisation (CGH) (40). In general, all tonsillar cancer was genetically unstable, but HPV-positive tonsillar cancer was possibly somewhat less unstable than HPV-negative tonsillar cancer when analysed both by *CGH* and *ICM* (40, 45).

The degree of DNA aberration was investigated by ICM to study whether HPV-positive and HPV-negative cancer differed in DNA content and whether the degree of DNA aberration influenced clinical outcome (45). The DNA content was estimated in 58 primary tonsillar tumours. Most of the tumours exhibited a high degree of aneuploidy and although HPV-positive tumours had a lower degree of aneuploidy than HPV negative tumours, this did not reflect on the prognostic value of HPV status. Hence, independent of DNA ploidy, patients with HPV-positive cancer were, to a higher degree, disease-free three years after diagnosis compared to patients with HPV-negative cancer (45).

In another study, where 25 tonsillar cancers were analysed by CGH, there were significant differences between the 15 HPV-positive and the 10 HPV-negative tumours (40). The mean number of chromosomal aberrations (ANCA) was 4.5 among the HPV-positive tumours and 6.1 for the HPV-negative tumours. A 3q24-increase was significantly higher ($p=0.049$) in HPV-positive tumours while a 7q11.2-q22 increase was present only in HPV-negative tumours ($p=0.017$). A better survival was also demonstrated for patients with HPV-positive tumours as compared to patients with HPV-negative tumours in this latter study ($p=0.002$) (40).

The fact that HPV-positive and HPV-negative tumours had a high DNA content and that few tumours were diploid was in concordance with that reported for cervical cancer (46-48). Nevertheless, in both studies a better clinical outcome was observed for patients with HPV-positive tumours compared to HPV-negative tumours, independent of the genetic instability and chromosomal set-up of the tumours (40, 45).

HPV in Tonsillar Cancer and Antivirus Vaccines

In conclusion, the presence of HPV in tonsillar cancer cases has been increasing over the past years and its presence seems to be a possible reason for the increased incidence of tonsillar cancer (29, 37). Moreover, the parallel increase of tonsillar cancer incidence and the increase in the proportion of HPV in tonsillar cancer cases, as well as the documented expression of E6 and E7 mRNA definitely support its aetiological role in this tumour. As a consequence of this piece of information, it is vital to monitor the effects of the present HPV vaccination, not only on the incidence of cervical cancer, but also on the incidence of tonsillar cancer. Moreover, even more importantly, since the majority of patients with tonsillar cancer are men, it is essential to further discuss if future vaccination against HPV infection should include both women and men.

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