

DEPARTMENT OF ONCOLOGY-PATHOLOGY
KAROLINSKA INSTITUTET, STOCKHOLM, SWEDEN

**STUDIES ON HUMAN PAPILLOMAVIRUSES IN
HEAD AND NECK CANCER**

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To my family

Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. The traditional risk factors for HNSCC are smoking and alcohol. However, recently IARC has also recognized human papillomaviruses (HPV) as an etiological factor for oropharyngeal cancer, a subset of head and neck cancers. Among oropharyngeal cancer, tonsillar and tongue base cancer dominate, both often associated with HPV. The aim of the present study was to examine the involvement of human papillomavirus (HPV) in two subtypes of HNSCC, tonsillar and hypopharyngeal cancer. For tonsillar cancer the purpose was to evaluate the prevalence of HPV over time and in relation to clinical outcome. In addition we wanted to evaluate if EGFR or phosphorylated EGFR were useful as markers, together with HPV, to predict response to treatment. For hypopharyngeal cancer, the aim was to analyze the prevalence of HPV and if HPV was a risk factor for this tumor type.

In the first paper, we found a 7-fold increase in the incidence of HPV positive tonsillar cancer, between 1970 and 2006, in the County of Stockholm, highlighting HPV as the causative factor for the increased incidence of this tumor type. In addition we found a decline in the incidence of HPV negative tonsillar cancer.

In the second paper, we found a high 5-year disease specific survival for HPV positive tonsillar cancer (81%), as compared to 36% for patients with HPV negative tonsillar cancer. HPV E6 and/or HPV E7 RNA were present in 94% of the samples analyzed, demonstrating the involvement of HPV in carcinogenesis.

In the third paper, we analyzed the presence of HPV in HNSCC from Greece and found that HPV is common in tonsillar carcinoma also from this country.

In the fourth paper, the presence of HPV and overexpression of p16 in hypopharyngeal cancer from patients in Stockholm, was evaluated. Only 6% were HPV positive, indicating that HPV is not an important risk factor for this disease.

In the fifth paper, overexpression of EGFR and presence of phosphorylated EGFR in tonsillar cancer, were evaluated in relation to tumor HPV status and clinical outcome. We found a correlation between the presence of phosphorylated EGFR and HPV, but not between phosphorylated EGFR and clinical outcome, when HPV positive and negative tumors were evaluated separately.

Our studies revealed HPV as a major factor behind the increased incidence of tonsillar cancer in the Stockholm area and an important prognostic factor for this disease, while HPV was not an important risk factor for hypopharyngeal cancer in this area.

List of publications

- I. Näsman A., Attner, P., Hammarstedt, L., Du, J., Eriksson, M., Giraud, G., Ahrlund-Richter, S., Marklund, L., **Romanitan, M.**, Lindquist, D., Ramqvist, T., Lindholm, J., Sparén, P., Ye, W., Dahlstrand, H., Munck-Wikland, E. and Dalianis, T. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int. J. Cancer*, 2009; 125(2):362-6.
- II. Lindquist, D.*, **Romanitan, M.***, Hammarstedt, L., Näsman, A., Dahlstrand, H., Onelov, L., Ramqvist, T., Munck-Wikland, E., Ye, W. and Dalianis, T. Human papillomavirus is a favourable prognostic factor in tonsillar cancer and its oncogenic role is supported by the expression of E6 and E7. *Molecular Oncology*, 1:350-355, 2007.
- III. **Romanitan, M.***, Näsman, A.*, Ramqvist, T., Polykretis, L., Vogiatzis, P., Vamvakas, P., Tasopoulos G., Vavanis, C., Arapantoni-Dadioti, P., Banis, K. and Dalianis, T. Human Papillomavirus frequency in oral and oropharyngeal cancer in Greece. *Anticancer Res.* 28:2077-2080, 2008
- IV. Wendt, M.*, **Romanitan, M.***, Näsman, A., Dalianis, T. Hammarstedt, L., Marklund, L., Ramqvist, T. and Munck-Wikland, E. Presence of human papillomaviruses and p16 expression in hypopharyngeal cancer. Submitted.
- V. **Romanitan, M.**, Näsman, A., Munck-Wikland, E., Dalianis, T. and Ramqvist T. Epidermal growth factor receptor (EGFR) and phosphorylated EGFR in relation to human papilloma virus (HPV) status and clinical outcome in tonsillar squamous cell carcinoma. Submitted.

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Abbreviations

aa	amino acid
bp	base pair
EGFR	epidermal growth factor receptor
FFPE	formalin-fixed paraffin embedded
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma viruses
HPV+	HPV positive
HPV-	HPV negative
HR-HPV	high-risk human papilloma virus
IARC	International Agency for Research on Cancer
ICD	International Classification of Diseases
IHC	immunohistochemistry
ISH	in situ hybridization
LCR	long control region
LR-HPV	low-risk human papilloma virus
MFI	Median Fluorescent Intensity
MHC	major histocompatibility complex
NCR	non-coding region
ORF	open reading frame
p16	p16 ^{INK4A}
PCR	polymerase chain reaction
pEGFR	phosphorylated epidermal growth factor receptor
pRB	retinoblastoma protein
qPCR	quantitative PCR
qRT-PCR	quantitative reverse transcriptase PCR
SCC	squamous cell carcinoma
TSCC	tonsillar squamous cell carcinoma
UICC	International Union Against Cancer
URR	upper regulatory region
VLP	virus-like particle

1. Introduction

In 2008, Harald zur Hausen was awarded the Nobel Prize for his discovery concerning the role of Human Papillomaviruses (HPV) in cervical cancer. The suggestion of a causal relationship between HPV and cervical cancer was made already in the 1970's although it was only gradually accepted by the scientific community[1]. Today, these viruses are recognized as carcinogenic infectious agents, not only in cervical cancer but also in a proportion of anogenital and head and neck cancer [2-4]. The focus of the present thesis is on HPV in head and neck cancer, specifically in head and neck squamous cell carcinoma (HNSCC) with special emphasis on tonsillar squamous cell carcinoma (TSCC).

1.1 Human Papillomaviruses

1.1.1 Taxonomy

HPV are non-enveloped, epitheliotropic, double-stranded DNA viruses, which are able to infect mucosal and cutaneous epithelia [5]. They belong to the Papillomaviridae family and today there are more than 150 different known types of HPV [6]. However, this number is steadily increasing and it is difficult to estimate how many HPV types there are that remains to be identified.

All papillomaviruses share a common genetic structure and the taxonomic classification is based on the sequence of the L1 open reading frame (ORF) [7-8]. They are divided into "families", "genera", "species", "types", "sub-types" and "variants" depending on the similarity of the L1 sequence [7]. Different genera have less than 60% nucleotide sequence identity in the L1 ORF, while species within a genus share between 60-70% nucleotide sequence identity. Between types the difference is >10%, while the difference between subtypes is 2-10% and between variants less than 2%. With regard to cancer caused by HPV, the HPV type is the most important taxonomic unit, although differences between variants may be of importance for their cancer promoting potential [9-10].

HPV are small non-enveloped DNA double stranded viruses [11]. They can be divided in **cutaneotropic** and **mucosotropic**, depending on the type of tissue they have been isolated from. Cutaneotropic have mainly been isolated from cutaneous and plantar warts, from cutaneous lesions in patients with verruciform epidermodisplasia or from immunosuppressed patients. Mucosotropic have mainly been isolated from mucosal epithelia from both benign and malignant lesions of the anogenital area. Moreover, the HPV types can be also divided in **high risk HPV** (HR-HPV), that are more likely to be associated with cancer development and **low risk HPV** (LR-HPV) that rarely or never cause cancer. HR-HPV types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 [12]. In addition, there is more

limited evidence for the carcinogenicity of HPV 26, 53, 66, 67, 68, 70, 73, and 82 [12]. These HR-HPV types can cause growths that are usually flat and not that easily detected, as compared to the genital warts caused by some low-risk types e.g. HPV6 and HPV11 [13]. In the present thesis the focus will be on mucosal HR-HPV types, since these are the types involved in head and neck cancer.

1.1.2 Genomic organization and viral proteins

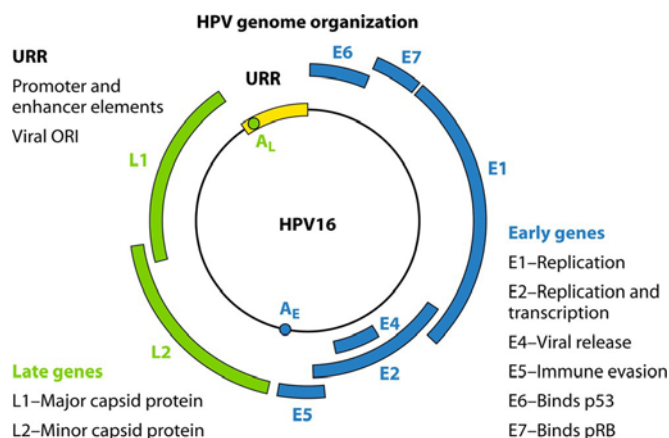


Figure 1. Organization of the HPV16 genome [14].

HPV viral particles have a single double-stranded of about 8000 base pairs (bp) that can be divided into three regions as determined by their functions; the long control region (LCR), sometimes denoted the non-coding region (NCR) or upper regulatory region (URR), and two coding regions, the early (E) and the late (L) coding regions, Figure 1, [8, 14]. The early region encodes for regulatory proteins E1, E2, E4-7 necessary for viral replication and the late region encodes for the structural proteins L1-L2 involved in virion assembly.

E1

The E1 viral protein is a 68 kDa protein that is necessary for DNA replication [8]. The size of the E1 protein ranges from 593 (HPV48) to 681 (HPV10) amino acids (aa). It is both the largest and the most highly conserved among the viral proteins and is essential for replication of the HPV genome [15]. The C-terminal enzymatic domain has helicase and adenosine triphosphatase activity, while the N-terminal region is involved in DNA replication.

E2

E2 is a 50 kDa protein, regulating viral transcription from the early promoter and is essential for viral replication [8]. Disruption of the E2 viral protein, often occurring during integration, causes increased levels of the E6 and E7 transforming proteins, thus promoting carcinogenesis. The E1 and E2 proteins form a complex that binds to sequences at the viral origin of replication.

E4

The 17 kDa E4 protein has a role in the latest phase of viral life cycle, when viral particles are produced and released, and is expressed together with the capsid proteins in the upper layers of the epithelium [8, 16]. It is probably important in viral release and assembly, as well as in interacting with and destroying the keratin cytoskeleton and induces G2 arrest. It may also have a role in regulating gene expression.

E5

E5 is, together with E6 and E7, one of the transforming proteins of HPV [17]. E5 contains three membrane-spanning domains but although it has a capacity in transformation, E5 is considered to have a weaker transforming capacity than E6 and E7 [8, 18]. The expression of E5 is often lacking in cervical carcinoma cells, due to deletions in the open reading frame, indicating that it is not necessary for transformation. It is expressed late in the virus life cycle and can enhance the immortalization capacity of E6 and E7. It has been shown that E5 increase the activation of epidermal growth factor receptor (EGFR) and also inhibits the localisation of the major histocompatibility complex (MHC) class I and II proteins to the plasma membrane [19]. In addition, E5 is proposed to be involved in the formation of tetraploid cells, which are frequently found in precancerous cervical lesions.

E6

Together with E7, E6 is one of the main transforming proteins [8, 20]. It is a 151 aa protein with two zinger-finger domains. E6 is expressed early in the viral cycle and binds directly to E6AP, a cellular ubiquitin ligase, causing the degradation of p53, preventing cell death, apoptosis and promoting the replication of viral DNA, Figure 2. In addition E6 has other, p53-independent functions, also involved in cellular immortalization or transformation. It has been demonstrated that expression of HPV16 E6 in the skin of transgenic mice can cause the development of malignant skin tumors, both in mice with p53 and in mice lacking p53. E6

also induces telomerase activity which requires E6AP. The E6/E6AP complex targets proteins containing a PDZ domain. Several other p53-independent functions/targets of E6 have been suggested, e.g. the inhibition of histone acetyltransferase activity of the important coactivator p300 [21-22].

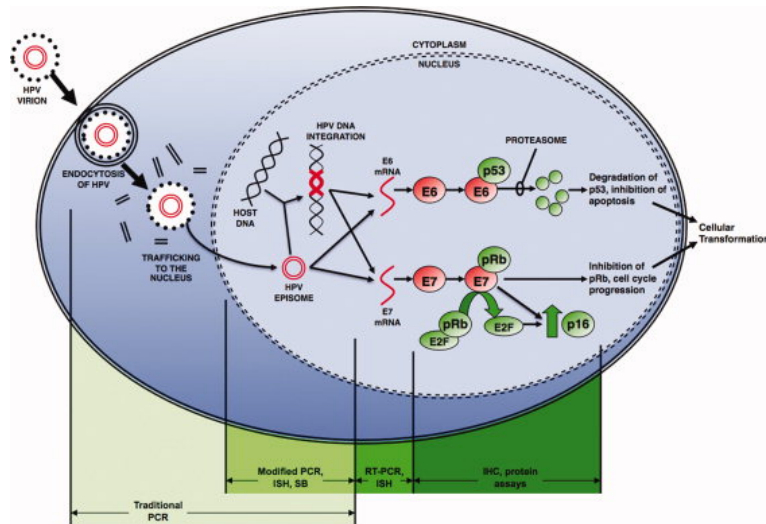


Figure 2. Schematic view of HPV infection of a mucosal cell [23].

E7

The second major transforming protein E7, is a small, 98 aa, protein with casein kinase II phosphorylation sites with a role both in immortalization and cellular transformation [8, 20]. One of the most studied mechanisms is the binding to the underphosphorylated form of the retinoblastoma protein (pRb) as well as other “pocket proteins” including p107 and p130. When E7 binds to Rb, the transcription factor E2F is released and this induces the transcription of the necessary cyclins and cdks for G1-S phase transition, Figure 2. Functional inactivation of pRb by E7 also induces an upregulation of p16^{INK4A} (p16) expression. As is described below, overexpression of p16 has often been used as an important marker for HPV E7 activity. In addition, E7 can also interact with cyclin A, cyclin E and histone deacetylases.

L1

L1 is the major capsid protein L1 and builds up the viral capsid together with L2, each capsid containing 360 L1 molecules [8]. It is a protein that is very conserved between different HPV types. L1 is expressed in terminally differentiated epithelial cells and self-assembles into pentamers, with 72 pentamers forming the viral capsid. L1 produced e.g. by yeast or in a baculovirus system can, even in the absence of L2 or viral genomes, self-assemble into virus like particles (VLP). Such VLPs form the basis for the HPV vaccines as described below.

L2

L2 codes for the minor capsid protein and is only expressed in terminally differentiated epithelial cells [8]. L2 is less abundant than L1 with around 12 copies per virion. Although it is not necessary for viral assembly, it has an important function during infection of cells by HPV.

1.1.3 HPV infection and replication

The primary route of mucosal HPV infection is the transmission through a mucosal injury [8, 14, 24]. HPV needs the availability of a wounded skin or mucosa, a metaplastic epithelium or a squamocolumnar junction. The virus infects basal and parabasal multilayered epithelial cells Figure 3. Early on there is a round of viral DNA replication, amplifying the viral genome to 50-100 copies per cell. During this early phase the expression of HPV proteins, including E6 and E7, is low, but when the host cell stops dividing and starts to differentiate into mature keratinocytes, the expression of several early genes, E1, E2, E5, E6 and E7, is increased. Especially E1 and E2 are necessary for genome amplification and the result is a replication of the viral genome to a high amount, >1000 copies per cell. In the latest step of HPV infection, where viral particles are formed, mainly E4 and the capsid proteins L1 and L2 are expressed.

It should be noted that HPV infection, at least for anogenital infection, mainly is a sexually transmitted disease, spread by both men and women. The most important risk factors for genital HPV infection and cervical cancer are all related to individual's sexual behavior e.g. early age of first sexual contact, high number of sexual partners and sexual contact with high risk individuals.

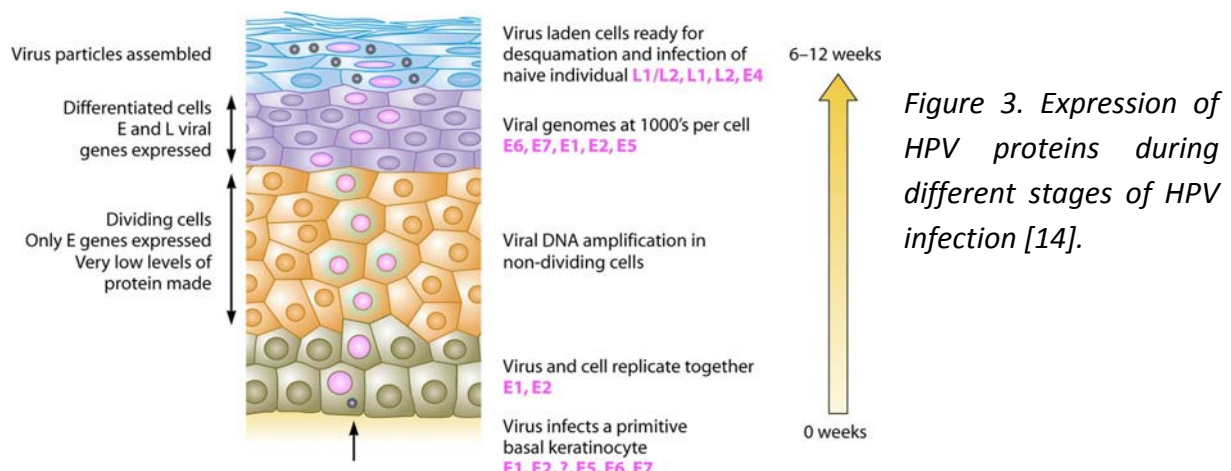


Figure 3. Expression of HPV proteins during different stages of HPV infection [14].

1.1.4 HPV proteins in tumor development.

The major transforming proteins E6 and E7 works together in causing HPV induced tumors [8, 20]. As described above, The E7 protein binds and inactivates the retinoblastoma tumor suppressor gene product pRB, releasing the E2F transcription factor, causing the cell to enter S-phase. In a cell with functional p53, such forced entry into the S-phase may cause the cell to go into apoptosis. An important function of p53 is to arrest cells in G1 to allow host DNA to be repaired or, alternatively, to induce apoptosis. However, since the E6 protein induces degradation of p53, E6 expressing cells are not capable of such p53-mediated apoptosis and the cell can continue to divide in spite of the activity of E7.

The levels of E6 and E7 are regulated by E2. In many tumors, the viral DNA genome is integrated within the host cell genome, causing a disruption in the E2 gene and leading to increased levels of E6 and E7 and a more malignant phenotype. Integration is not necessary, and there are both cervical and head and neck cancers where the HPV genome is episomal [25]. E6 and E7 have the ability to immortalize human keratinocytes, but E5 seems to be important in the early course of infection, although the E5 gene is frequently deleted when the HPV genome is integrated during malignant progression [26]. E5 can protect cells from apoptosis and might potentiate the transforming activity of E7 [27].

It is important to note that the presence of E6 and E7 in a cell is not enough to cause the development of cancer. Additional changes in cellular genes are needed and this is the reason for the long delay, often several decades, between HPV infection and the appearance of malignant tumors. The normal epithelium is gradually transformed to hyperplasia, dysplasia, carcinoma *in situ* and invasive cancer.

1.2 HPV and cancer

Human Papillomavirus is considered a causative factor in a subset of human malignancies and benign lesions. The implication of HPV in cervical cancer is today acknowledged and accepted, but the virus is also involved in several other non-cervical malignancies including vulvar, vaginal, penile, anal, and oropharyngeal cancer [3, 28]. In a study by Parkin et al from 2006, 5.2% of all cancer worldwide was estimated to be caused by HPV [28]. In Figure 4, a summary of the involvement of HPV types in tumors from different sites is presented. It is important to note that while the estimate for the proportion of HPV in cervical cancer is consistently around 98-100%, the values of HPV prevalence in e.g. oropharyngeal cancers vary a lot [29]. Thus, values for HPV caused cancer from other sites than the cervix should be treated with caution.

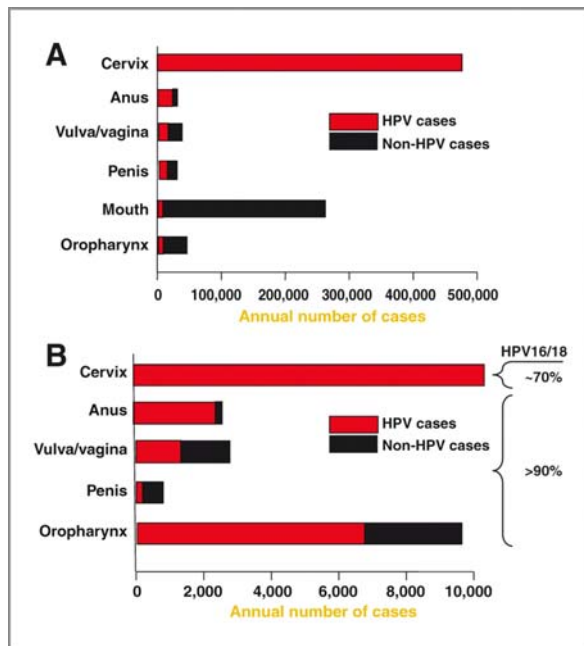


Figure 4. Worldwide incidence and distribution of HPV-associated cancer [3].

1.2.1 Assessment of the involvement of HPV in cancer

Despite more than 30 years of research on HPV and cancer and while it is now recognized that virtually all cervical cancers are caused by HPV, there is for other cancer types often a debate on whether and to what degree they are caused by HPV [3, 28]. At present such discussions are especially prominent with regard to HPV in e.g. lung cancer and oesophageal cancer, where values of HPV prevalence varies widely between different reports, and where some researchers believe that many tumors of this type are caused by HPV and while others believe that HPV is not involved in tumors of this type at all [30-31]. For many cancer types where it is established that HPV is involved, there is often a disagreement on how many are really caused by HPV. One reason for these discrepancies is that HPV prevalence is measured in different ways. Often it is measured by the presence of HPV DNA in tumor samples. This is usually performed either in a PCR based assay or by in situ hybridization (ISH) [32]. In a PCR based assay, the presence of HPV in the sample, and sometimes also the type, is evaluated. This does not show if HPV is actually in the tumor cells or not, and often no quantitative assessment is made. In contrast, ISH is performed directly on the tumor tissue and it is possible to see if the HPV is in the tumor cells or not. A drawback is that this technique is less sensitive than PCR and HPV positive tumors can be missed [33].

To establish that HPV is really active in the tumor cells mainly two different methods have been used; detection of HPV E6 and/or E7 RNA or overexpression of the cellular p16 protein [32, 34]. Presence of HPV DNA together with expression of HPV E6 and/or E7 RNA has often been regarded as the “golden standard” to evaluate if a tumor is caused by HPV or not [35]. Since mRNA analysis is not always easy to perform an alternative has been analysis of p16

expression in the tumor cells by immunohistochemistry (IHC) [34]. As described above, inactivation of pRb by E7 induces upregulation of p16 expression. Thus, overexpression of p16 can be used as a marker for the presence of E7 in the cells. Since p16 is easy to evaluate in a pathology unit, p16 has also been as a surrogate marker for the presence of HPV in e.g. oropharyngeal cancer [36-37]. However, since p16 can be overexpressed in a subset of HPV negative tumors (10-15% of HNSCC cancer), this is not to be recommended [38-39]. Instead, a combination of the analysis of the presence of HPV DNA and overexpression of p16 is now often suggested as a good marker for the active involvement of HPV in a tumor [34-35]. It can be noted that in Paper I and II of the present thesis, HPV DNA together with E6 and/or E7 RNA (for a portion of the samples) was analyzed, while in Paper IV, analysis of HPV DNA was combined with the analysis of p16 overexpression.

1.3 Head and neck cancer

Head and neck cancer refers to malignancies of the lip, oral cavity, oropharynx, hypopharynx, nasopharynx, larynx and sinonasal tract. The most common histological type is squamous cell carcinoma. Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer worldwide and the eighth most common cause of cancer death, causing approximately 300 000 deaths/year [40]. The main etiological factors are smoking and alcohol abuse [41]. Betel quid and areca nut chewing are also important risk factors for oral cavity cancers in India and Taiwan. In the last years, also HPV has been acknowledged by the International agency for research on cancer (IARC) as a risk factor for oropharyngeal cancer, especially for tonsillar cancer [41]. Since the focus of this thesis is HPV in oropharyngeal and hypopharyngeal cancer, the focus of this introduction will be on these head and neck subsites.

1.3.1 Classification of head and neck cancer

In the diagnosis of HNSCC, it is classified according to the TNM-system designed by the International Union Against Cancer (UICC) [42]. The TNM-system is based on the size of the primary tumor (T), the presence, size, number and localization of regional metastasis (N) and the presence of distant metastasis (M). UICC classifications for cancer, from different head and neck subsites, are provided below.

Primary tumor (T)

Oropharynx:

- TX Primary tumor cannot be assessed
- T0 No evidence of primary tumor
- Tis Carcinoma in situ
- T1 Tumor \leq 2 cm in greatest dimension
- T2 Tumor $>$ 2 cm but \leq 4 cm in greatest dimension
- T3 Tumor $>$ 4 cm in greatest dimension or extension to lingual surface of the epiglottis
- T4a Moderately advanced local disease
 - Tumor invades the larynx, deep/extrinsic muscle of the tongue, medial pterygoid, hard palate, or mandible
- T4b Very advanced local disease
 - Tumor invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases the carotid artery

Hypopharynx:

- TX Primary tumor cannot be assessed
- T0 No evidence of primary tumor
- Tis Carcinoma in situ
- T1 Tumor limited to 1 subsite of the hypopharynx and/or \leq 2 cm in greatest dimension
- T2 Tumor invades more than 1 subsite of the hypopharynx or an adjacent site or measures $>$ 2 cm but \leq 4 cm in greatest dimension without fixation of the hemilarynx
- T3 Tumor $>$ 4 cm in greatest dimension or with fixation of the hemilarynx or extension to the esophagus
- T4a Moderately advanced local disease
 - Tumor invades thyroid/cricoid cartilage, hyoid bone, thyroid gland, esophagus, or central compartment soft tissue (including prelaryngeal strap muscles and subcutaneous fat)
- T4b Very advanced local disease
 - Tumor invades prevertebral fascia, encases carotid artery, or involves mediastinal structures

Regional lymph nodes (N)

NX	Regional nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node ≤ 3 cm in greatest dimension
N2	Metastasis in a single ipsilateral lymph node > 3 cm but ≤ 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none > 6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none > 6 cm in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node > 3 cm but ≤ 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none > 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none > 6 cm in greatest dimension
N3	Metastasis in a lymph node > 6 cm in greatest dimension

Distant metastasis (M)

M0	No distant metastasis
M1	Distant metastasis

Tumor stage

Stage is calculated from a combination of the score for T, N and M as described below:

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
IVB	T Any	N3	M0
	T4b	N Any	M0
IVC	T Any	N Any	M1

1.3.2 Oropharyngeal cancer

Tonsillar and base of tongue cancer are the two most frequent subtypes of oropharyngeal cancer and 90% belong to these two types and the background below mainly concerns these two types .

1.3.2.1 Histopathology of tonsillar cancer

The most frequent tonsillar cancer type is squamous cell carcinoma (SCC), and mostly these are moderately to poorly differentiated [43]. Rarer variants of SCC are basosquamous carcinoma, nonkeratinizing carcinoma and undifferentiated carcinoma. Tonsillar cancer caused by HPV are all SCC. In contrast, the second most frequent tonsillar tumor type, lymphoma of the tonsil, is not caused by HPV. Other types of tonsillar tumors, leiomyosarcomas, salivary gland tumors and sarcomas are extremely rare.

1.3.2.2 Symptoms of tonsillar cancer

Unfortunately, patients with tonsillar cancer are, since they are usually asymptomatic in the beginning of the disease, mostly diagnosed in the later stages of the disease. The clinical complaints are a sore throat, a swollen lymph node, pain that radiates on the ipsilateral side with the affected tonsil, swallowing difficulties, a lump in the neck and fatigue. Tonsils have a high amount of lymphatics that provide the neoplasm the possibility to metastasize to neck nodes. For this reason many patients are presenting a lump in the neck at the time of diagnosis.

1.3.2.3 Treatment of oropharyngeal cancer

Treatment of oropharyngeal cancer patients differs both between different countries, hospitals and over time. Patients with oropharyngeal cancer, included in the present study, were treated at the Karolinska University Hospital. During this period the main treatment was for patients with localized disease only radiotherapy. In case of regional lymph nodes metastasis, radiotherapy targeting the neck region was performed, followed by neck dissection. If radiotherapy was unsuccessful, the treatment was complemented with surgery.

Today, the treatment at many hospitals, including the Karolinska University hospital is more intensified, with oncological treatment, radiotherapy with or without chemotherapy [44]. External radiotherapy is performed by conventional fractionated radiotherapy and/or hyperfractionated, accelerated radiotherapy. Brachytherapy is also an option nowadays, as a sole modality of treatment or more frequently performed with radiotherapy with or without concomitant chemotherapy. Chemotherapy can be added to the treatment as induction

chemotherapy before radiotherapy, as concomitant chemotherapy during radiotherapy, or as adjuvant chemotherapy after surgery in case of residual tumor. Surgical treatment of the primary tumor is performed only in the case of residual tumor and might affect the quality of life of these patients, by affected speech, eating and drinking. Neck dissection is sometimes also performed.

1.3.2.4 HPV and oropharyngeal cancer

For a long time smoking, alcohol and smokeless tobacco (snuff and betel nut) were considered the main causes for tonsillar cancer [45]. However, many tonsillar cancer patients have no history of tobacco or alcohol habits.

Already in the early 1980's Syrjänen et al. provided some data on the possible involvement of HPV in the aetiology of a proportion of oral, laryngeal, benign sino-nasal papillomas and SCCs [29, 46-47]. Since then, gradually, more and more evidence has accumulated that show an association between HR-HPV and oropharyngeal cancer, especially for tonsillar and tongue base cancer [29, 48-50]. Particularly during the last decade, a number of studies on this topic have been published and in 2007 HPV was acknowledged by the International Agency for Research against Cancer (IARC), as a risk factor for oropharyngeal cancer [41]. In accordance with this, several studies has shown that sexual behavior, e.g. a high number of sexual partners, is a risk factor for tonsillar cancer [51-52]. Furthermore, individuals with a defective immune defense, e.g. organ transplant patients and human immunodeficiency virus (HIV) patients, as well as husbands of women with in situ cervical cancer, belong to the tonsillar cancer risk group [51-52]. In several countries, a decrease in smoking has caused a decline in the incidence of head and neck cancer in general [53-55]. In contrast, the incidence of HPV associated oropharyngeal cancer, specifically tonsillar and base of tongue cancer, has increased in many countries in the Western world, e.g. Sweden, USA, Netherlands, Finland and UK [56-63]. Thus the age-standardized incidence of tonsillar cancer in Stockholm increased from 1.3-3.6/100 000 person-years between 1970 and 2002 [56]. Similarly, the incidence of base of tongue cancer in Sweden increased from 0.15/100 000 person-years during 1970-1974 to 0.47/100 000 person-years during 2005-2007 [64]. It should be noted that although oropharyngeal cancer is more common in men than in women an increase in the incidence can be seen both among women and men [57, 65].

The prevalence of HR-HPV in oropharyngeal cancer varies between different studies and regions with figures between 20 and 80%. This large variation is probably partly due to differences in e.g. smoking habits between different regions and during which time period the patients were treated. However, part of this variation may also be due to methodological differences, e.g. differences in the method used for detection of HPV, in the definition of HPV positive samples (e.g. HPV DNA and/or RNA or HPV DNA + p16) or in which

subsites of oropharyngeal cancer was included [35]. In contrast to the many studies showing a high or relatively high HPV prevalence in oropharyngeal cancer, a very low HPV prevalence (4.4 %) in oropharyngeal cancer was found in a recent study on a head and neck tumors from Central Europe and Latin America [66]. The reason for this much lower prevalence is not known, but differences in the methodologies may play a role.

It should be noted that there is a subgroup of non-tonsillar, non-base of tongue cancer with much lower HPV prevalence (17%) and it is likely that HPV is not the causative agent in these tumors since there is no correlation between the presence of HPV DNA and p16 [67]. There is also a major difference between the high HPV prevalence in the base of tongue in comparison to the much lower prevalence in the mobile tongue [68].

In contrast to the variation in HPV prevalence in oropharyngeal cancer between different countries and studies, there is much more agreement with regard to the dominance of HPV16 among the HR-HPV present in these tumors. Thus, around 90% of the HR-HPV positive tumors are HPV16 positive, while the remaining 10% are positive for HPV18, 26, 31, 33, 45, 52, 58 or 59 [35, 48, 56, 59, 69]. In addition the LR-HPV types 6 and 11 have rarely been found [69]. The number of samples with a specific type other than HPV16 is often just one or two samples. While it is thus difficult to distinguish the order in the prevalence of these other types, it can be noted that HPV33 seems to be more frequent while HPV18 is very rare, especially in comparison to the prevalence of HPV18 in cervical cancer (Figure 6).

1.3.2.5 Prognosis of tonsillar and tongue base cancer

In general, the prognosis for head and neck cancer patients, including those with cancer of the oropharynx, is most influenced by TNM stage and extension of the primary tumor. In contrast, several studies, including Paper II in the present thesis, have indicated that HPV+ tonsillar and base of tongue cancer patients have a better prognosis than HPV- patients, even for tumors with a high TNM stage [49, 64, 68, 70-76]. In line with the result for TSCC presented in Paper II, similar results have been shown for base of tongue cancer where patients with HPV+ base of tongue cancer has a 5-year OS of 77% as compared to 40% for those with HPV- base of tongue cancer [64]. As a result of studies from our group and other groups, HPV is now considered to be an independent prognostic factor for oropharyngeal cancer. As noted above there is a subgroup of non-tonsillar, non-base of tongue cancer with much lower HPV prevalence (17%) and where it is likely that HPV is seldom the causative agent. For these tumors the overall survival is around 50% regardless of the presence of HPV [67]. The high survival for patients with HPV+ base of tongue cancer can also be compared to the much lower survival for patients with tongue cancer, a head and neck subsite that is mostly HPV negative [68]. The age standardized relative survival rate for men diagnosed in

Sweden 2000-2004 was 44% [77]. Similar to the results by our group, concerning oropharyngeal cancer patients from the Karolinska University hospital ([64]and Paper II in the present thesis), a study from the US showed that the 5-year overall survival rate was improved by 100% among patients with HPV+ base of tongue cancer and 28–60% among those with HPV+ tonsillar cancer [78]. Also when HNSCC was evaluated for overexpression of p16, as a surrogate marker for HPV, the presence of p16 was found to be a favorable prognostic factor. Thus the 5-year overall survival rate was 62% for patients with p16 positive HNSCC as compared to 26% for those with p16 negative tumors treated with radiotherapy [76].

1.3.2.6 HPV and oral infection

Since it has been established that HPV is a risk factor for oropharyngeal cancer the question of oral HPV infection is naturally of importance. In contrast to the vast number of studies on prevalence of different HPV types in the anogenital area and especially in the cervix, there are relatively few corresponding studies on the HPV prevalence in the oral area. The HPV prevalence in these studies are usually lower than in samples from the cervix with <10% of samples positive for any HPV [79-80], but there are also studies with values up to 30% [81]. This variation may partly be due to differences in sampling technique e.g. mouth wash or scraping from the buccal mucosa, but may also be due to differences in the sampled population, sample preparation and analysis. Common to all studies are that HPV16 is the most common type also in the oral cavity as in the cervix. This is in line with the predominance of HPV16 in oropharyngeal cancer. Noteworthy is that also the majority or all other mucosal HR-HPV can also be found in oral samples, even though many of these are very rare or so far nonexistent in oropharyngeal cancer. In a study from our group it was also shown that there is a concordance between the HPV types found in the cervix and the oral cavity [79].

1.3.3 Hypopharyngeal cancer

Hypopharyngeal cancer represents 3-5 % of all head and neck malignancies. It is less frequent than tonsillar cancer and is characterized by a worse prognosis [82].

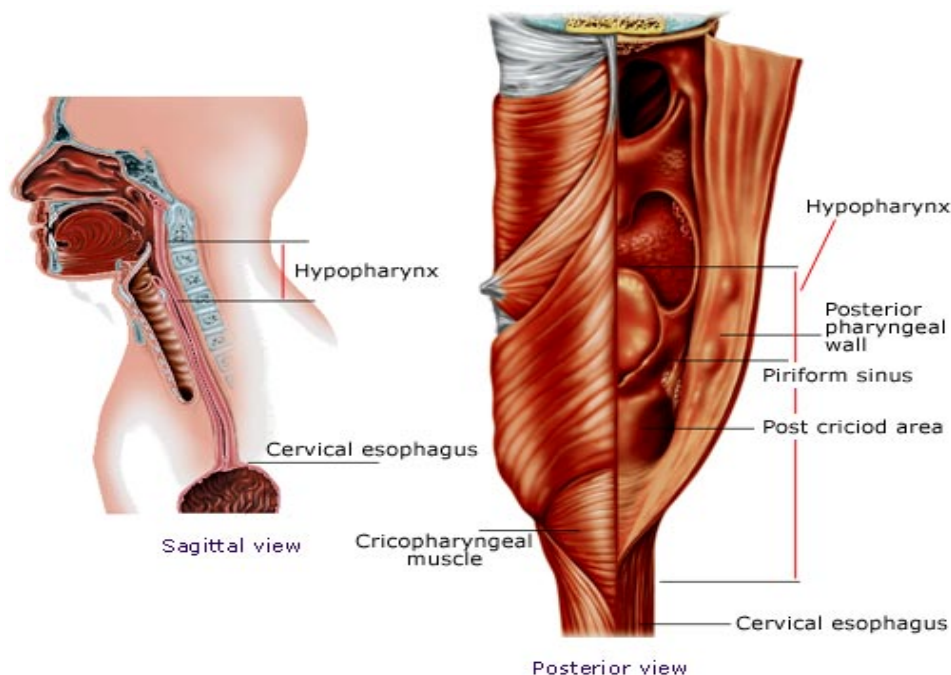


Figure 5. The location of the hypopharynx in the head and neck region. By Pierre Delaere, with permission.

The hypopharynx, also called laryngopharynx, is the lower part of the pharynx and is situated between the oropharynx above and esophagus below, Figure 5. It includes three sub-sites: the piriform sinus (most of the cancers arise in this area), the postcricoid area and the posterior pharyngeal wall. This anatomy is important in understanding the future symptomatology of these patients. It should be noted that the larynx is not included in the hypopharynx.

Unfortunately, symptoms for patients with hypopharyngeal cancer usually appear when the tumor is large and most of the patients have advanced stage disease at presentation [82-84]. Symptoms vary from dysphagia, chronic sore throat, foreign body sensation in the throat and otalgia to weight loss, hemoptysis, laryngeal stridor and hoarseness which are usually later signs in the disease. A metastatic node in the neck can also be the sole symptom and first later, after examination, a primary tumor in the hypopharynx is diagnosed.

1.3.3.1 Histopathology of hypopharyngeal cancer

Most hypopharyngeal carcinomas are epithelial-type squamous cell carcinomas [82, 85]. More rarely they are basaloid squamoid carcinomas, spindle-cell carcinomas, small-cell carcinomas, nasopharyngeal-type undifferentiated carcinomas (lymphoepitheliomas) or carcinomas of the minor salivary glands. Carcinomas of the hypopharynx are frequently poorly differentiated.

1.3.3.2 Treatment and prognosis of hypopharyngeal cancer

Treatment is based on surgery and irradiation, either alone or in combination. Induction and concurrent chemotherapy with radiotherapy has been also proposed with the aim to avoid 'mutilating surgery' and to improve the quality of life, since more organ function is preserved this way [86]. At the Karolinska University Hospital patients are treated with external radiotherapy locally and regionally with or without chemotherapy. When there are metastases, neck dissection is carried out 4-6 weeks after completion of radiation therapy. Patients in good general health and where the tumor is considered resectable, are usually treated with primary local resection with or without neck dissection, in combination with postoperative radiotherapy administered locally and regionally. Salvage surgery for treatment failures after radiotherapy is also considered. Hoffman et al. reviewed in his study the treatments of hypopharyngeal SCC in the USA during the 1980s and 1990s and pointed out a superior survival for primary surgery only (50.4%) and combined primary surgery with radiotherapy (48%), with radiotherapy only (25.8%) [87].) However, as noted below this figure is usually lower.

Patients with hypopharyngeal cancer have one of the worst prognosis among head and neck cancer patients, since these patients are usually asymptomatic at early stages. The 5-year survival rate is 15-30% [82, 85]. It is influenced by many factors such as the stage of the cancer or if the patient smokes during radiation therapy. The poor overall survival rate of the disease has not changed over the years and different treatment modalities are discussed to improve the survival of hypopharyngeal cancer. At the Karolinska University Hospital, the 3-year survival rate is approximately 22% while the 5-year survival rate is 17%.

1.3.3.3 Risk factors for hypopharyngeal cancer

Alcohol ingestion and tobacco use are the traditional etiological factors for hypopharyngeal cancer [88]. In addition, poor diet and Plummer-Vinson syndrome, a genetic disorder that causes a long-term iron deficiency, has also been described as being involved in hypopharyngeal carcinogenesis. Gastroesophageal or laryngotracheal reflux of the gastric contents has been considered putative factors for the development of tumors in the posterior cricoid region of the hypopharynx.

1.3.3.4 HPV and hypopharyngeal cancer

There are very few studies with a focus on HPV in hypopharyngeal cancer. In most studies where this has been investigated, hypopharyngeal cancers were included among tumors from several different head and neck subsites [89-93]. Thus, the number of included hypopharyngeal cancer samples was often too low for a proper assessment of the

prevalence of HPV in this cancer type. Nevertheless, these studies gave rather moderate values (0-29%) of the fraction of hypopharyngeal cancers that are HPV positive. In contrast to this, one major study has been performed on HPV in hypopharyngeal cancer specifically [94]. In this study, a very high prevalence of HPV was reported in that 82% were found to be positive for HR-HPV. It should be noted that only a few (11%) of these were also p16 positive, indicating that few of the cancers were actually caused by HPV.

1.3.4 EGFR and head and neck cancer

The epidermal growth factor receptor (EGFR) is a transmembrane 170-kd glycoprotein that constitutes one of four members of the erbB family of tyrosine kinase receptors and consists of an extracellular receptor domain, a transmembrane region, and an intracellular domain with tyrosine kinase function [95]. EGFR dimerization stimulates its protein-tyrosine kinase activity and induces the autophosphorylation of several tyrosine residues as Tyr992, Tyr1045, Tyr1068, Tyr1148 and Tyr1173 [96-97]. This in turn leads to the activation of downstream pathways, principally the MAPK and PI3K/Akt pathways leading to DNA synthesis and cell proliferation.

Overexpression and amplification of EGFR is frequently observed in tumors of epithelial origin including HNSCC [98-100]. Thus EGFR expression is elevated in over 80% of invasive HNSCC [101]. Overexpression of EGFR is in many studies considered as a negative prognostic factor for patients with HNSCC and has been correlated with resistance to radiotherapy, poor local control and survival [99, 101-105]. Mutated p53 in combination with moderate-to-high levels of EGFR in HNSCC has also been associated with a shorter disease free survival and time to treatment failure [106]. In contrast, other studies have not shown a correlation between EGFR expression and clinical outcome in HNSCC [107-108]. Also when phosphorylated EGFR has been analyzed the result has been contradictory. Thus some studies has found a correlation between the presence of e.g. Tyr1068 and treatment outcome while other have not found such a correlation [109-111].

For most studies on EGFR in HNSCC the HPV status of the tumors has not been taken into account. However, in two earlier studies on oropharyngeal cancer where the HPV status was included in the evaluation a negative correlation between the presence of HPV and overexpression of EGFR was found [112-113]. In both of these studies the EGFR expression was also correlated to an improved prognosis.

1.4 HPV and cervical cancer

Cervical cancer is the second most common cancer worldwide and it accounted for an estimated 273 000 deaths in the world in 2002 [114]. The association between HPV and cervical cancer was discovered by Harald zur Hausen already in the 1970's and is now recognized as a fact. In contrast to the situation for HPV and HNSCC, nearly all cervical cancer worldwide is caused by HPV [115]. An estimation of overall HPV prevalence published in 2010 after an analysis of 30,848 invasive cervical cancers worldwide, showed an increase from 85.9% in studies published from 1990 to 1999 to 92.9% in studies published from 2006 to 2010 although in many publications a figure of 98-99% is given [116].

In contrast to HPV in HNSCC, more HR-HPV types (e.g. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) are considered as causative of cervical cancer [12]. HPV16 is, as for oropharyngeal cancer, the most common, but in contrast to its rare occurrence in oropharyngeal cancer, HPV18 is relatively common in cervical cancer, Figure 6. In Europe and the US, HPV18 is the second most common type in cervical cancer which is the reason for its inclusion in both HPV vaccines. While virtually all HPV induced oropharyngeal cancers are SCC, only around 80% of cervical cancers are SCC while 15% are adenocarcinoma and the latter ones are usually caused by HPV18.

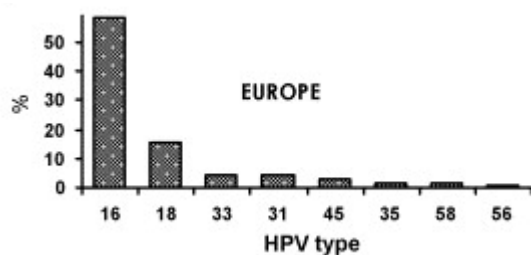


Figure 6. The eight most common HPV types in cervical cancer in Europe [117].

It should be noted that although HPV is considered a “necessary cause” for the development of cervical cancer it is not a sufficient cause since large number of infections by HPV of cervix are resolved spontaneously (>90%) [118].

1.4.1 Screening and treatment of cervical cancer

An important difference between the oropharyngeal and cervical cancers is that, while there is no screening program for oropharyngeal cancer, and these often are discovered late, there are efficient screening programs for cervical cancer. In Sweden, the national organized screening program to prevent cervical cancer was introduced in the 1960s and covers approximately 80% of women in the screening ages of 23–65 years [119]. Currently the preferred method of diagnosis of the disease is both visual examination the cervix

(colposcopy) and histological examination of a biopsy but more recently the addition of tests for the presence of HPV DNA and/or RNA has been evaluated.

The primary aim of cervical screening is to detect pre-cancerous changes in the epithelium of the cervix. Treatment of these pre-cancerous changes is important in preventing progression of the disease. A secondary aim of cervical screening is the early detection of invasive disease as this may improve prognosis. In spite of this, almost 500 women in Sweden are diagnosed with cervical cancer every year [119].

Cervical cancer is mainly treated surgically (hysterectomy), chemotherapy (mainly Cisplatin) and radiation therapy (external and brachytherapy). These treatments are given in different combinations dependent on diagnosis stage, i.e. early or advanced stage, invasive or non-invasive, etc. [120]

1.5 Vaccines against HPV

An important achievement of HPV research is the development of two prophylactic vaccines against HPV: Cervarix (GlaxoSmithKline) and Gardasil (Merck). While Cervarix contains virus-like particles (VLPs) of recombinant L1 capsid protein from HPV16 and HPV18, Gardasil also contains VLPs of the non-oncogenic types HPV6 and 11 [121-122]. While HPV16 and 18 are the most prevalent HR-HPV types in cervical cancer HPV6 and 11 are implicated in about 80-90% of genital warts and also in laryngeal papillomatosis. Both vaccines use adjuvants to boost the immune reaction against the antigens. Both Cervarix and Gardasil have been demonstrated to be very efficient for protection both against genital HPV infection and against the development of precancerous cervical lesions [121-122]. These vaccines are only for prophylactic use; they are not therapeutic vaccines, and cannot be used to treat an already existing HPV infection or existing cancer lesions. Although they both have been on the market for several years, public vaccination of young girls in Sweden did not start until 2012, mainly due to legal disputes.

2 Aim of the thesis

- To study the incidence of HPV positive and negative tonsillar cancer in Stockholm area between 1970 and 2007
- To study the oncogenic and prognostic role of HPV in tonsillar cancer
- To analyze the presence of human papillomavirus (HPV) in oral and oropharyngeal cancer from patients diagnosed during the years 1986-2007 in Greece
- To investigate the prevalence of HPV in hypopharyngeal cancer from patients in the Stockholm area, in correlation to overexpression of p16 and clinical outcome
- To correlate overexpression of EGFR and presence of EGFR phosphorylated at tyrosine 1068 and 1148 in tonsillar cancer with presence of HPV and clinical outcome

3 Material and methods

3.1 Patients and tumor samples

All patients included in the thesis in paper I, II and V were diagnosed with tonsillar SCC in the County of Stockholm during 1970–2007 and treated at the Karolinska University Hospital. In the third paper, tumor samples were obtained and collected from Metaxa Cancer Hospital, Piraeus, Greece, from patients diagnosed with oral and oropharyngeal cancer between 1986 and 2007. In the fourth paper, hypopharyngeal cancer samples were from patients diagnosed during 2000-2007 and treated at the Karolinska University Hospital. Tumor samples were assessed by a pathologist.

The diagnosis for all patients was selected according to International Classification of Diseases (ICD) system. The ICD classification is the standard diagnostic tool for epidemiology, health management and clinical purposes including the analysis of the general health situation of population groups. It should be noted that for the studies in paper I and II the patients were selected according to the ICD-7. The more recent ICD-10 classification was not used in Paper I since it was important to use the same classification from 1970-2007.

Patient data was collected from the Swedish Cancer Registry. Swedish Cancer Registry was founded in 1958 and contains data for more than 98% of all Swedish cancer cases. This registry provides information about medical data like site of tumor, diagnosis, histopathology, treatment and follow-up data like date of death or cause of death.

3.2 DNA and RNA extraction

All samples included in the thesis were from formalin-fixed paraffin embedded (FFPE) tissues. DNA extraction in papers I - IV was performed using High Pure RNA paraffin kit, from Roche Diagnostics, with exclusion of the DNase treatment. RNA extraction of the samples in paper II was performed with the same kit with inclusion of the DNase step.

Methodological considerations: The Roche kit is especially designed for preparation of RNA from FFPE samples. However, it works well for both RNA and DNA preparations.

The quality of DNA and RNA prepared from FFPE samples is not as good as DNA or RNA prepared from fresh frozen samples. For most samples it is possible to obtain PCR-products with a length of up to 150 bp, while amplicons with a length of 300-400 bp can be obtained from fewer samples. Thus all PCRs in this thesis were performed with amplicon length of <150 bp, with the exception of CPI/CPIIG with an amplicon of 187 bp. The CPI/CPIIG assay is also somewhat less sensitive than the other included HPV assays when used for the analysis of HPV in DNA obtained from FFPE samples. It should be noted that for the older samples

included in Paper I, (samples from 1970-1990) a larger fraction (up to 24%) had to be discarded due to too low DNA quality.

In order to avoid and check for HPV contamination, sections from an empty paraffin block, blank samples, were included between each tumor sample and treated and included in the PCR analysis similarly to the tumor samples.

3.3 PCR analysis

In Paper I-III, the purified DNA was analyzed for the presence of HPV by “standard” PCR, i.e. in a PCR reaction with one primer couple/reaction and where the presence of a specific amplicon was analyzed by gel electrophoresis. In Paper IV, the HPV analysis was performed by Luminex bead based analysis, where 24 HPV-types were analyzed simultaneously. To assure the quality of the DNA, and that the DNA was possible to amplify in a PCR reaction, analysis of the cellular gene S14 was performed in Paper I-III and of β -globin in Paper IV. In Paper I-III, three different HPV PCRs were performed: 1) HPV16 specific PCR, 2) GP5+/6+ PCR and, if both of these were negative, 3) CPI/IIG PCR. Samples positive with GP5+/6+ or CPI/IIG but negative for HPV16 were sequenced to identify the HPV-type. In all assays a negative (water) sample and a positive control were included. For “standard” PCR 100 ng (rarely up to 200 ng) DNA was analyzed/reaction. PCR products were examined with UV light on a 2.5-3.0% agarose gel stained with ethidium bromide. All primers are presented in Table 1.

3.3.1 S14 PCR

S14 PCR amplifies a 127 bp part of the human ribosomal S14 gene and is used to validate the quality of the DNA preparation.

Protocol for S14 PCR:

PCR-mix (50 μ l/sample)

	Final concentration/amount	<u>PCR</u>
Sample	10 μ l	94°C (1 min)
S14 sense/anti-sense primers	15 pmol/primer	40 cycles with:
10xPCR buffer*	1x	94°C (30 sec)
dNTP	200 μ M/ dNTP	50°C (30 sec)
MgCl ₂	1.5 mM	72°C (45 sec)
BSA	4 μ g/ μ l	72°C (10 min)
Taq-polymerase	1 U/ μ l	

*buffer from Applied Biosystems

3.3.2 General HPV PCR

In Paper I-III, detection of HPV was performed by PCR, using two separate pairs of general primer; GP5+/6+ and CPI/IIIG (Table 1). These primer pairs both target a number of different HPV types, including all HR-HPV. GP5+/6+ targets the L1 ORF and gives an amplicon of 130-150 bp, whereas CPI/IIIG recognize the E1 ORF and gives an amplicon of around 188 bp.

Protocol for GP5+/6+

PCR-mix (50 µl/sample)

	Final concentration/amount	PCR program
Sample	10 µl	94°C (4 min)
GP5+/6+ primers	10 pmol/primer	40 cycles with:
10xPCR buffer*	1x	94°C (1 min)
dNTP	200 µM/ dNTP	44°C (1 min)
MgCl ₂ (25mM)	1.5 mM	71°C (2 min)
BSA	4 µg/µl	71°C (5-10 min)
Taq-polymerase	5 U/µl	

*buffer from Applied Biosystems

Protocol for CPI/IIIG PCR:

PCR-mix (50 µl/sample)

	Final concentration/amount	PCR program
Sample	10 µl	94°C (5 min)
CPI primer	17 pmol	40 cycles with:
CPII primer	26 pmol	95°C (1 min)
10xPCR buffer*	1x	55°C (1 min)
dNTP	1.25 mM/ dNTP	72°C (2 min)
MgCl ₂	2.5 mM	72°C (10 min)
BSA	10 µg/µl	
Taq-polymerase	2.5 U/µl	

*buffer from Applied Biosystems

3.3.3 HPV16 type specific PCR

A HPV16 type specific PCR was also run in order to identify the presence of this HPV type in the tumors. Type specific primers, targeting HPV16 E6 were used for detection (Table 1).

Protocol for HPV16 PCR:

PCR-mix (50 µl/sample)

	Final concentration/amount	PCR program
Sample	10 µl	95°C (4 min)
HPV-16 E6 primers	10 pmol/primer	40 cycles with:
10xPCR buffer*	1x	95°C (30 sec)
dNTP	1.25 mM/dNTP	55°C (30 sec)
MgCl ₂	2.5 mM	72°C (60 sec)
Taq Polymerase Gold	5 U/µl	72°C (10 min)

*buffer from Applied Biosystems

3.4 HPV analysis using the Luminex based Multiplex HPV assay

For the study on hypopharyngeal cancer in Paper IV, the presence of HPV was analyzed with a HPV multiplex assay using a MagPix instrument from Luminex. This assay was developed by the group of Michael Pawlita in Heidelberg [123]. We have set up the assay in our lab in accordance with their protocol. In this assay the presence of 24 different HPV-types is analyzed simultaneously. These include all 15 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), 3 putative HR-HPV types (25, 53 and 66) and 6 LR types (6, 11, 42, 43, 44 and 70) (classification according to Munoz et al [124]). In addition, the β -globin gene is also assayed for, to validate the presence of amplifiable DNA.

This assay is initiated with a GP5+/GP6+ PCR with some important differences. The sensitivity with the standard GP5+/GP6+ differs radically for different HPV types [125]. While the sensitivity for HPV16 is good (approximately 10 copies in a standard PCR assay), the sensitivity for e.g. HPV39, 56, 68, 73 and 82 is very low (10 000-100 000 copies). To obtain a more equal sensitivity for all HR-HPVs, Schmitt et al designed a primer set with broad spectrum GP5+/GP6+ primers (BSGP primers), consisting of 9 different GP5+ and 3 different GP6+ primers, Table 1, [125]. With this primer set a more equal amplification was obtained for all HR-HPV types, as well as all other types included in the Luminex assay, with a sensitivity of 10-100 copies [125]. These primers were used in the Luminex HPV assay in Paper IV where all reverse primers are biotinylated.

In the MagPix instrument, magnetic beads with different colors are analyzed. Up to 50 different types of beads, each with a separate color, can be analyzed simultaneously. To each of the 25 bead types (24 HPV-types and β -globin) included in the assay a specific probe was coupled. Although the BSGP primers amplifies a number of different HPV types, each probe only recognize one specific type. In the Luminex assay, denatured PCR-products are incubated with the bead mixture at a specific hybridization temperature (42°C). During this incubation, the PCR-amplicons for each HPV type present in the reaction, bind to the type specific probes on the beads. PCR-products not bound to beads, as well as surplus primers,

are then washed away. After washing, the beads are incubated with fluorescent streptavidin, which binds to the biotinylated primers. The whole assay is performed in 96-well plates.

In the MagPix analysis, beads from each well are spread and analyzed on a magnetic plate. By the use of two different lasers, each bead is identified by its color and the presence of fluorescent streptavidin, indicating the attachment of amplicons to the bead. The output from the MagPix is, for each well, the Median Fluorescent Intensity (MFI) for the 50-100 beads of each type analyzed/well (sample). In Paper IV the signal was calculated as $MFI - 15 - 1.5 \times \text{background}$ (obtained from a well without a sample).

Methodological considerations HPV analysis

As pointed out the GP5+/GP6+ primers have a low sensitivity for some HPV types. These may thus be missed in the standard HPV assay. The use of three different primer pairs compensate to some extent for this. However, it is still possible that some types were undetected in these assays. Many of these samples have now been reanalyzed in the Multiplex HPV assay and there are very few cases of additional HPV types found. Mostly these are cases where there is a clear signal for HPV16 and a weak signal for another type, indicating the presence of a weak infection by another type not involved in the tumor development.

Since the Multiplex HPV assay has a higher sensitivity than the standard PCR assays we have compared the values obtained from this assay with the result by the standard HPV16 PCR for tonsillar samples. We have found that 5-10 ng tumor DNA is enough to obtain a strong signal for HPV16 positive tumors in the Multiplex assay. In Paper IV we started with the analysis of 10 ng DNA. However, since few samples were positive, the tests were repeated with 50 ng DNA but the result with 10 and 50 ng was virtually the same.

Since only BSGP primers are included in the Luminex assay, only the presence of the L1 region is assayed for. Thus, tumors lacking this region may be assayed as HPV negative. In later studies we have included HPV16 E6 primers and an E6 specific probe in this assay. However, this was not included for the study in Paper IV. We have now analyzed several hundred tonsillar cancer samples with BSGP + HPV16 E6 primers and found that only very rarely do we find tumors that are negative for the L1 region and positive for the E6 region. It is thus unlikely that this has affected the results of the study on HPV in hypopharyngeal cancer.

3.5 Analysis of HPV viral load

In Paper II, we were using a real time quantitative TaqMan PCR (qPCR) to identify and measure the viral load of HPV copies per genome equivalent. HPV16 primers and probe were as described in a previous paper [25]. Each sample was analyzed in triplicates and the number of viral copies in each sample was correlated to the values from a standard curve obtained with a serial dilution of a HPV16 plasmid, included in each run. PCR amplification was performed in an iCycler iQ from BioRad.

A human RNase P gene was used as an internal control of the human genome content in every sample, according to the manufacturer's instructions. (TaqMan RNase P Detection Reagents kit from Applied Biosystems). Viral loads in each sample were expressed as the number of HPV copies/ cell genome [126].

Protocol for estimation of viral load.

PCR-mix (25 µl/sample)

	<u>Final concentration/amount</u>	<u>PCR program</u>
Sample	10 µl	50°C (2 min)
HPV 16 primers	10 pmol/primer	95°C (10 min)
10xPCR buffer*	1x	40 cycles with:
dNTP	200 µM/dNTP	94°C (15 sec)
MgCl ₂	1.5 mM	60°C (1 min)
Probe	5 pmol	60°C (1 min)
Taq-DNA polymerase (AmpliTaq Gold DNA polymerase)	0.5 U/µl	

*buffer from Applied Biosystems

Methodological considerations, viral load.

As noted in Paper II, there was a large variation in viral load between different samples (0.08-130 copies/cell). Although this should mainly be due to actual differences in viral load between different TSCC, other factors may also contribute to these differences. The most important additional factor is likely the fraction of tumor cells in the tissue. TSCC are more or less homogenous with different amount of stroma in the TSCC. In addition, there is a large variation in the number of tumor infiltrating T-cells [127].

3.6 HPV16 E6/E7 mRNA assay

E6 and E7 mRNA was analyzed by quantitative reverse transcriptase PCR (qRT-PCR) in an iCycler iQ and the values compared with a dilution series of HPV16 plasmids as described

above in the analysis of HPV viral load. First RNA extraction was performed as described above, followed by cDNA synthesis using a SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen). An internal control of the human genome content, the human RNase P gene, was analyzed for each sample. After RNA extraction, a HPV16 type specific PCR was run, in order to confirm the absence of remaining HPV DNA. DNA melting curves were evaluated to assess specificity. These started from 40°C and was increased by 0.5°C every 10th second until 120°C was reached.

Protocol for mRNA analysis

PCR mix (25 µl/sample)

	<u>Final concentration/amount</u>	<u>PCR program</u>
cDNA	10 µl	50°C (2 min)
HPV16 primers	10 pmol/primer	95°C (10 min)
iQMN SYBR Green Supermix*	12.5 µl	40 cycles with:
*BioRad Laboratories		95°C (15 sec)
		60°C (30 sec)
		74°C (30 sec)

Methodological considerations, mRNA analysis.

Crucial for this assay is the quality of the RNA preparation and the cDNA synthesis. Both were assayed by the inclusion of RNase P in the analysis. If the sample was not positive for RNase P it was not possible to include this in the evaluation. Another critical question is if all DNA was destroyed in the DNase step. The HPV16 PCR on the prepared DNA, before the cDNA step, was necessary to validate this. These tests confirmed the complete absence of HPV DNA in all samples. Although qRT-PCR is a quantitative assay, we did not evaluate the result with regards to quantity but as positive or negative. For a quantitative estimation of E6 and E7 mRNA, a comparison with the expression of several household genes would have been required, since it is possible that there is some variation in the expression of one gene such as RNase P.

Table 1. Primers used for PCR analysis

Name	sequence	position	gene
GP5+	5'-TTT GTT ACT GTG GTA GAT ACT AC-3'	6624-6646 ¹	L1
GP6+	5'-GAA AAA TAA ACT GTA AAT CAT ATT C-3'	6765-6741 ¹	L1
CPI	5'-TTA TCW TAT GCC CAY TGT ACC AT-3'	1963-1941 ¹	E1
CPIIG	5'-ATG TTA ATW SAG CCW CCA AAA TT-3'	1776-1798 ¹	E1
HPV-16.1	5'-TCA AAA GCC ACT GTG TCC TGA-3'	421-441 ¹	E6
HPV-16.2	5'-CGT GTT CTT GAT GAT CTG CAA-3'	520-540 ¹	E6
HPV16 E6.F	5'-GAG CGA CCC AGA AAG TTA CCA-3'	122-142	E6 (for RNA)
HPV16 E6.R	5'-AAA TCC GCA AAA GCA AAG TCA-3'	252-232	E6 (for RNA)
HPV16 E7.F	5'-ACC GGA CAG AGC CCA TTA CAA-3'	699-719	E7 (for RNA)
HPV16 E7.R	5'-GTG CCC ATT AAC AGG TCT TCC-3'	818-798	E7 (for RNA)
S14 sense	5'-TCG AAA GGG GAA GGA AAA GA-3'	2275-2256 ²	S14
S14 antisense	5'-CAG TGA CAT GGA CAA AAG TG-3'	2148-2167 ²	S14

Primers included in the Luminex assay

Forward

GP5+	5'-TTT GTT ACT GTG GTA GAT ACT AC-3'	L1
BSGP5+-2	5'-TTT GTT ACT GTT GTI GAT ACT AC-3'	L1
BSGP5+-3	5'-TTT GTT ACT GTT GTI GAT ACC AC-3'	L1
BSGP5+-4	5'-TTT GTT ACT TGT GTI GAT ACT AC-3'	L1
BSGP5+-5	5'-TTT TTA ACT GTT GTI GAT ACT AC-3'	L1
BSGP5+-6	5'-TTT GTT ACT GTG GTA GAC ACT AC-3'	L1
BSGP5+-7	5'-TTT GTT ACA GTI GTA GAC ACT AC-3'	L1
BSGP5+-8	5'-TTT GTT ACA GTI GTA GAT ACC AC-3'	L1
BSGP5+-9	5'-TTT GTT ACT GTG GTA GAT ACC AC-3'	L1
MS3.F	5'-AAT ATA TGT GTG CTT ATT TG-3'	β-globin

Reverse 5' Biotinylated

Bio-GP6+	5'-GAA AAA TAA ACT GTA AAT CAT ATT C-3'	L1
Bio-GP6+-b	5'-GAA AAA TAA ATT GTA AAT CAT ACT C-3'	L1
Bio-GP6+-c	5'-GAA AAA TAA ATT GCA ATT CAT ATT C-3'	L1
Bio-MS10.R	5'-AGA TTA GGG AAA GTA TTA GA-3'	β-globin

¹ in NC_001526.1 HPV16 European type reference sequence

²M13934

3.7 Immunohistochemistry

p16

P16 analysis, by immunohistochemistry (IHC), in Paper IV, was performed with the p16INK4a primary monoclonal mouse anti-human p16INK4a antibody (dilution 1:100; clone JC8; Santa Cruz Biotechnology) on 4 µm sections of FFPE tissues. Epitope retrieval was performed by heating and then treating the sections with peroxidase blocking reagent. All sections were incubated with monoclonal antibody p16INK4a, followed by incubation with visualization reagent and developed in DAB. As a negative antibody control, the monoclonal mouse IgA2 was used. This staining was verified and assessed by light microscopy and the evaluation was graded according to a 3-tier scale (0: 0%; 1: 1-25%; 2: 26-74%; 3: 75-100%). In different studies we have considered that samples with of grade 2-3 or only grade 3 as p16 positive. However, it should be noted that virtually all samples were either of grade 0 or grade 3 while hardly any samples were of grade 1 or 2. [67].

EGFR

In Paper V, the presence of EGFR and EGFR phosphorylated at 2 different tyrosines was analyzed by IHC on 4 µm FFPE sections. Antibodies used for staining were; rabbit monoclonal antibody EGFR D38B1 (for EGFR), rabbit polyclonal antibody pEGFR (Tyr1148) and mouse monoclonal antibody pEGFR (Tyr1068) 1H12, all diluted 1:200 and all from Cell Signaling Technology. The incubation of the slides was performed with a biotinylated secondary anti-mouse, or anti rat, antibody (1:200, Vector Laboratories, Burlingame, CA, U.S.A.) followed by incubation with the avidin-biotin-complex-PO using the VECTASTATIN® Elite® ABC kit (Vector Laboratories) and developed in DAB. The evaluation was graded according to a 3-tier scale: (0: 0%, 1: 1-25%, 2:26-75% or 3:76-100%. The intensity of the staining was also evaluated according to the following scale: absent (0), weak (1), moderate (2) or strong (3).

Methodological considerations, IHC:

IHC of p16 is quite straightforward. The staining is mostly strong and the tumor cells either all or nearly all positive or all or nearly all negative. However, for IHC of EGFR the situation is different. As described in Paper V, most TSCC, both in our own study and in other studies, have a moderate or strong EGFR staining regardless of HPV status. The interpretation of the results will thus depend on which cutoff is used for separating positive and negative samples and the interpretation of weak, moderate and strong staining. This is probably the reason for the differences in frequency of HPV positive samples between different studies. As pointed out in Paper V, our results for EGFR were in line with the results in several other studies. In addition, regardless of if the cutoff was between grade 0-2 and 3 or between 0-1 and 2-3, more HPV- than HPV+ samples were EGFR positive. For pEGFR it is also possible that the

phosphorylation is affected by the treatment of the sample, i.e. the time before the sample is formalin fixed [128]. Differences in technique and sample treatment may thus affect the result obtained by different groups.

3.8 Statistical analysis

Pearson Chi-square test was performed to make the analysis of the HPV status and clinical parameters correlation in Paper I and II.

Fisher's exact test was used to compare differences in tumor differentiation in Paper I, and HPV+ TSCC between 1992-1998 and 2000-2007 in Paper III, HPV and p16 overexpression in Paper IV and EGFR immunostaining with TNM classification, stage or histopathological differentiation in Paper V.

An independent, two-sided t-test was performed to compare the mean age for patients with HPV+ and HPV- TSCC in Paper I and with positive and negative immunostaining in Paper V.

In Paper II, IV and V, survival data was presented in Kaplan-Meier curves and the log-rank test was used for comparison of survival curves. In the multivariate analyze, a Cox proportional hazards model was used to adjust for covariates. The log-rank test was performed to compare differences in survival rate.

4 Results and Discussion

4.1 Paper I

Incidence of human papilloma virus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: An epidemic of viral-induced carcinoma?

Aim and background of the study

As described in the background on tonsillar cancer (1.3.2.4), we published a study in 2006, where we demonstrated a parallel increase, from the year 1970 to 2002, in the incidence of tonsillar cancer in Stockholm and also in the prevalence of HPV in these tumors [56]. The aim of the present study was to follow up the earlier study with an analysis of tonsillar tumors from the year 2003-2007. We wanted to explore if the earlier increase in both incidence of tonsillar cancer and HPV prevalence continued during this period. In addition we wanted to compare the incidence of HPV+ and HPV- tonsillar cancer during the period 1970-2007.

Short description of material and methods

In the study by Hammarstedt et al, 203 tumors from patients diagnosed 1970-2002 at the Karolinska University Hospital were analyzed for the presence of HPV [56]. In the present study a further 98 samples from patients diagnosed 2003-2007 were analyzed for the presence of HPV DNA. All tumors were analyzed for HPV DNA and the result was combined with the study from 2006. In addition around 60% of the HPV positive tumors were also analyzed for the presence of HPV E6 and/or E7 RNA.

Main results

- The prevalence of HPV in tonsillar cancer in Stockholm had continued to increase during the years 2003-2007 and 85% of the TSCC were HPV+ during this period.
- HPV16 continued to dominate among the HPV+ TSCC and all with the exception of 6 (7%) were positive for HPV16, while the rest were positive for HPV33, 35 or 59, or, in 3 cases, the type was not identified.
- The incidence of TSCC in Stockholm had continued to increase, from 0.74 per 100 000 person years 1970-1979 to 1.65, 2000-2006.
- By combining the values for HPV prevalence and incidence of TSCC, we made an estimate of the incidence of HPV+ and HPV- TSCC in Stockholm during the period 1970-2006. We found that while the incidence of HPV+ TSCC had increased from 0.18 to 1.25/100 000

person years during the years 1970-2006, the incidence for HPV- TSCC had, during the same period, decreased from 0.56 to 0.39 with a peak at 0.76 during the 1980's. Thus the incidence of HPV+ TSCC had increased 7-fold during this period.

Discussion

With this study we could show that both the HPV prevalence and incidence of TSCC in Stockholm had continued to increase. Thus, HPV induced TSCC continues to be an important and increasing health problem. Of special interest was that we could show both an increase in HPV+ TSCC and a decrease in HPV- TSCC. We speculated that this decrease in HPV- TSCC, after a peak during the 1980's, was due to a decrease in smoking among men, since the trend was similar to trends for other cancer types caused by smoking, e.g. lung cancer. In addition, other types of HNSCC, where HPV is not an important causative factor, also show a trend of decreasing incidence. Noteworthy, this was the first time the trends for HPV+ and HPV- TSCC were analyzed separately, and we were the first to demonstrate a specific increase of HPV+ TSCC in contrast to a decrease of HPV- TSCC. This study, and especially the results presented in Figure 3, with separate trends for HPV+ and HPV- TSCC, has had an important impact internationally. Many other research groups have referred to this study when discussing this subject. Since this study was published, similar trends for HPV+ and HPV- oropharyngeal cancer has also been demonstrated in the US where the proportion of HPV oropharyngeal tumors increased dramatically from 16.3% during the 1980s to 72.7% during the 2000s [59]. This increase was especially prominent among young white men. In the Netherlands, the fraction of HPV+ oropharyngeal SCC increased from 5% in 1990 to 29% 2010 [35].

The HPV prevalence in TSCC was similar to the prevalence in tongue base cancer from patients, diagnosed during the same period, at the Karolinska University Hospital. Thus for patients diagnosed 2006 -2007, 84% of base of tongue cancer cases were HPV-positive [57].

It can be noted that the HPV prevalence in TSCC from our group is higher than in many studies from other countries. There are several possible reasons for this: 1) In Sweden smoking, especially among men, has declined both more and earlier than in most other countries, and as a consequence, the fraction of HPV+ TSCC should be more prominent in Sweden. 2) It is possible that we, 20-30 years ago, had a higher prevalence of HPV infection in Stockholm as compared to other areas, but we do not know if this is the case. 3) There is some variation between different studies in what is regarded as a HPV+ TSCC. In the present study we counted all TSCC that were HPV DNA positive as HPV+. In some studies only HPV DNA and RNA positive or HPV DNA and p16 positive tumors were included, e.g. [35]. However, in the present study, 98% of the HPV DNA positive tumors were positive also for

E6 and/or E7 RNA and the data would thus not have been much different if 1-2 HPV RNA negative tumors would have been excluded. 4) In the present study we have only included TSCC and not oropharyngeal SCC from other subsites. In some studies by other groups all types of oropharyngeal SCC were included. We have demonstrated, in a separate study, that oropharyngeal SCC of non-tonsillar, non-tongue base are mostly HPV- [67]. Thus, if all oropharyngeal SCC were included, the total HPV prevalence would have been lower.

4.2 Paper II

Human papillomavirus is a favourable prognostic factor in tonsillar cancer and its oncogenic role is supported by the expression of E6 and E7

Aim and background of the study

Also this study was a follow up on the study on HPV in TSCC from 2006 [56]. There were three separate aims for the present study was: 1) To analyze the relation between the presence of HPV and patient survival. Earlier studies had indicated that patients with HPV+ oropharyngeal cancer have a better prognosis [49, 68]. However, the number of patients included in these studies was rather limited. Here, we had the opportunity to analyze this for a much larger number of patients. 2) To investigate if there was a relation between the viral load (HPV copies/cell) in HPV+ TSCC and the clinical outcome as found in some earlier studies [25]. 3) To confirm the presence of HPV E6 and/or E7 RNA in these tumors demonstrating that the HPV genome was actively transcribed.

Short description of material and methods

150 patients, of a total of 203 from the 2006 study, were included in an analysis of 5-year survival in correlation to the presence or absence of HPV DNA. Viral load was analyzed by quantitative PCR for 86 HPV+ samples and correlated to clinical outcome. Presence of HPV E6 and E7 mRNA in 53 HPV DNA+ samples was analyzed by RT-PCR.

Main results

- Presence of HPV DNA in TSCC was positively correlated to clinical outcome. Disease specific survival was 81% for HPV+ TSCC in comparison to 36% for patients with HPV- TSCC.
- When HPV+ TSCC were divided into 4 categories depending on the number of HPV copies/cell, we found no correlation between HPV viral load and clinical outcome.

- HPV E6 and/or HPV E7 RNA was detected in 50 of 53 (94%) analyzed HPV DNA+ samples. Thus HPV was expressed in the vast majority of the samples.

Discussion

The finding that the presence of HPV is positively correlated to clinical outcome can potentially be of great clinical importance. Although this result was not new, this was at the time the largest study where this was shown. At this time there was also a controversy since some groups did not find such a correlation. Our result has now been corroborated in a number of studies, from different countries, and is now widely accepted [72, 129-130]. It is essential to note that, in the present study, the majority of these patients only received conventional radiotherapy and/or surgery. Since this study was performed, the treatment of all HNSCC patients, at the Karolinska University Hospital, has been intensified and now often includes accelerated radiotherapy and adjuvant therapy with e.g. EGFR inhibitors. The result is an increase in harmful side effects. Since the majority of patients with HPV+ TSCC fare well already with less intensive therapy, it may be possible to abstain from intensive therapy for the majority of patients with HPV+ TSCC. This possibility is now a major point of discussion among researchers in this field.

The reason for the better prognosis for patients with HPV+ TSCC is still not clearly established. A likely explanation is that the immune defense is more prone to target tumors with foreign viral antigens. In accordance with this, our group have in a separate study demonstrated that HPV+ TSCC have more tumor infiltrating CD8+ T-cells than HPV- TSCC and that the number of these cells is correlated to the clinical outcome of the patient [131].

In contrast to the correlation between the presence of HPV DNA and clinical outcome, the lack of correlation between viral load and clinical outcome found in our study, is not yet clearly established and there are some discrepant results [25, 132]. It is important to note that in our study, the vast majority of HPV DNA positive TSCC also were E6 and or E7 mRNA positive. This indicates that the majority of the TSCC were indeed caused by HPV. HPV DNA + RNA positivity is now often considered a “golden standard” for HPV testing in HNSCC [34]. In some other studies on HPV in oropharyngeal cancer, fewer of the HPV DNA positive samples were also RNA positive e.g. [132]. A possible reason for this discrepancy is differences in the detection limit of the HPV DNA assay. In studies where a very sensitive method was used, more samples with very low amounts of HPV DNA may have been identified. In e.g. the study by Ribeiro et al these studies some samples had a HPV viral load far below 1 copy/tumor cell [66]. HPV DNA in these samples may thus have been incidental.

4.3 Paper III

Human Papillomavirus Frequency in Oral and Oropharyngeal Cancer in Greece

Aims and background of the study

In a previous study, we found an increased presence of HPV in tonsillar and base of tongue cancer in Sweden [56]. In the present study our aim was to investigate the presence of HPV in different head and neck cancer samples from patients diagnosed in Greece.

Short description of material and methods

The study included 115 paraffin-embedded tumor samples from the Metaxa Cancer Hospital, Piraeus, Greece, from patients diagnosed between 1986 and 2007, with oral or oropharyngeal cancer. Thirty-one patients were diagnosed with TSCC, 38 with tongue cancer and 46 with oral cavity cancer. 12 samples were excluded from the study and the remaining 103 samples were analyzed for HPV, both by general and type-specific HPV PCR.

Main results

- 13% of the analyzed tumors were HPV+ and the majority of these were HPV16+.
- Nearly all HPV+ samples were TSCC, where 12/28 (43%) were HPV+.
- There was a tendency to an increase in HPV prevalence with time. Only 1/6 (17%) collected 1992-1998 was HPV+ in contrast to 11/22 (50%) collected 2000-2007.
- Of the tongue cancer samples only 1/38 (3%) were HPV positive, while none of the 41 oral cavity cancer samples was HPV positive.

Discussion

As was expected from our studies on HPV in HNSCC from Swedish patients, HPV was predominantly found in TSCC. Although the HPV prevalence in TSCC (43%) was lower than in Sweden (68%, 2000-2002, [56]) the number of analyzed samples (28) from Greece was too low to clearly establish this. In Sweden, there is a decrease in smoking, while Greece is a country with a very high adult tobacco usage. It was interesting to observe that, in spite of this, there was a rather high HPV prevalence in TSCC also in Greece.

The HPV prevalence in tongue cancer was much lower than the corresponding figures for Stockholm during this period [64, 133]. However, there is an important difference in the analysis of tongue cancer from Stockholm and Greece. In the material from Stockholm, oral

and base of tongue cancer were analyzed separately, while in the samples from Greece these subsites were not separated. Since, usually, only base of tongue cancer is HPV positive, it is thus difficult to compare the result from Greece and Stockholm [68].

The tendency for an increase in HPV prevalence in TSCC with time is in line with our published data from the Stockholm area as well as with a recent report from the US (Paper I and [59]). Due to the limited number of cases from the 1990's, the data should be interpreted with caution. Nevertheless, the data suggest that the incidence of HPV-positive cancer in Greece is increasing and that HPV is gradually becoming a more important factor for TSCC also in Greece.

4.4 Paper IV

Presence of human papillomaviruses and p16 expression in hypopharyngeal cancer

Aim and background of the study

Although, HPV, especially HPV16, is now acknowledged as a risk factor for tonsillar and tongue base SCC, the influence of HPV on tumor development for HNSCC from other subsites is less well studied. Many earlier studies on this topic have included a mixture of HNSCC from different subsites. However, as our studies on HPV in tonsillar and base of tongue cancer has shown, it is important with large studies on HNSCC cancer from specific and well defined subsites (Paper I and [56-57]). Hypopharyngeal cancer is one of the head and neck cancers with the worst prognosis and treatment has not improved over the years. There are very few studies that have focused on HPV in hypopharyngeal cancer specifically. The aim of the present study was thus to analyze the presence of HPV in a large number of hypopharyngeal cancer in order to see if HPV might be a contributory factor in this disease. We also analyzed overexpression of p16 as an indicator of expression of HPV E7.

Short description of material and methods

In this study we investigated the presence of HPV and overexpression of p16 in 119 hypopharyngeal cancer biopsies from patients diagnosed 2000-2007, in the Stockholm area. The presence of HPV DNA was analyzed by PCR and a bead based Multiplex HPV assay with a MagPix instrument. Presence of β -globin as marker for cellular DNA was assayed and 10 samples with low β -globin values were excluded. Analysis of overexpression of p16 was performed by IHC. The result was correlated with overall and disease-free survival by univariate and multivariate analysis.

Main results

- Only 7 hypopharyngeal tumors (6%) were HPV DNA positive.
- HPV 16 was the most frequent type.
- From all tumors 16% overexpressed p16.
- All HPV16 positive tumors overexpressed p16 while the other HPV+ tumors (HPV51, 53 and 56) did not.
- Three out of four patients with HPV16+ tumors were alive and tumor free >5 years after treatment while the fourth of these patients died of unknown cause after 3.5 years.
- There was no significant correlation between the disease free survival of the patients and any of the parameters analyzed with exception of age.

Discussion

All HPV subtypes found in the tumors belong to the high risk or potentially high risk group. However, the presence of HPV in this cohort was very low, 6%, indicating that HPV cannot be considered a major cause for hypopharyngeal cancer in the Stockholm region. p16 has been shown in many studies to be a good marker for the involvement of HPV in the carcinogenesis of oropharyngeal cancer [35, 38], but our study shows that it is not a reliable biomarker for the presence of HPV in hypopharyngeal cancer. The fact that all HPV16+ tumors overexpressed p16, in comparison to only 16% in the whole cohort, indicates that HPV16 is a causative factor for these cancers. The high survival of patients with HPV16+ tumors is in line with the survival of patients with HPV16+ TSCC. However, the number of HPV16+ tumors were much too few to clearly state that patients with HPV16+ hypopharyngeal cancers have a better prognosis. Many more hypopharyngeal tumors have to be analyzed to establish this. The tumors that were positive for HPV51, 53 and 56 were all p16 negative, indicating that HPV was not active in these tumors and were not causative for tumor development although this is not a conclusive proof. Noteworthy, in our cohort, overexpression of p16 in the tumors was not correlated to HPV or to prognosis. This demonstrates the peril of using p16 as a surrogate marker for HPV in HNSCC in general and to assume that HNSCC patients with p16 positive tumors have a better prognosis. As described in Paper V our results differ drastically from the results of the only other large study on HPV in hypopharyngeal cancer that we know of where 82% were HPV positive [94]. We do not know the reason for this difference but it is noteworthy that only 11% in the study by Ernoux-Neufcoeur et al were p16 positive, indicating that at most a few very caused by HPV. Since our study demonstrates few hypopharyngeal cancers to be caused by HPV, other potential biomarkers should be analyzed to find those that are correlated to patient's response to treatment, in order to individualize patient treatment and to improve the quality of life and survival for these patients.

4.5 Paper V

Epidermal growth factor receptor (EGFR) and phosphorylated EGFR in relation to human papilloma virus (HPV) status and clinical outcome in tonsillar squamous cell carcinoma

Aim and background of the study

As shown in Paper II, HPV+ TSCC patients have a better clinical outcome than HPV- patients with a disease specific survival after conventional RT of around 81%. In spite of this, the intensity of the treatment of TSCC patients has increased in recent years, in line with the treatment of HNSCC patients in general, and now often includes accelerated RT and adjuvant therapy. Treatment of many TSCC patients can probably be less intense. However, since a minority of patients with HPV+ TSCC does not respond to conventional therapy, the question is which patients can receive a reduced treatment and still remain tumor free. HPV is a good biomarker for prognosis but is not enough. There is thus a need to combine HPV with other biomarkers. EGFR overexpression in HNSCC has been associated with poor prognosis, increased tumor growth, metastasis and resistance to chemotherapy and radiation therapy and is thus a candidate biomarker for prognosis [134-135]. The aim of the present study was to evaluate if expression of EGFR and/or EGFR phosphorylated on tyrosine residues 1068 or 1148 in TSCC in combination with HPV status could give a better prediction of response to treatment than HPV status alone.

Short description of material and methods

All 83 patients included in the study were treated during 2000-2006 with intention to cure, mainly with conventional RT. The samples were stained by IHC for total EGFR and EGFR phosphorylated on tyrosine 1068 (Tyr1068) or 1148 (Tyr1148). The result was correlated to tumor HPV status, clinical outcome and disease free survival.

Main results

- There was a significant correlation between the presence of phosphorylated EGFR, both for Tyr1068 and Tyr1048, and tumor HPV. There was also a tendency to a correlation between overexpression of EGFR and HPV status, although this was not significant.
- TSCC were more often positive for Tyr1148 than for Tyr1068 and there was no clear correlation in the phosphorylation of these two sites.
- There was no significant correlation between EGFR and clinical outcome when patient groups were stratified by HPV status.

Discussion

As mentioned above, several studies have shown an association between overexpression of EGFR in HNSCC and a worse clinical outcome [99, 102-104]. In addition, some studies have shown a correlation between phosphorylation of EGFR and clinical outcome [110-111]. As further described in Paper V, there are also studies where no such correlation has been found [107-108, 136]. Our results are thus in line with the results of the latter studies. It is important to note that none of the studies on phosphorylated EGFR in HNSCC has focused on oropharyngeal cancer in relation to the HPV status of the tumor. In our study we could not find any correlation between overexpression of EGFR or phosphorylation of EGFR on tyrosine 1068 or 1148 and a worse prognosis. Today, it is accepted that HPV is more common in tonsillar cancer than in other head and neck subsites and is correlated with a better clinical outcome [32]. Since, as we found in this study, there is a correlation between the presence of HPV and phosphorylation of EGFR in TSCC, it should be plausible to find a correlation between EGFR and clinical outcome for TSCC patients, if the HPV status of the tumors is not taken into account. We conclude that EGFR is not an appropriate biomarker to use together with HPV to predict treatment outcome. However, it is important to note that the treatment of patients included in our analysis was conventional, and did not include any EGFR inhibitors like Cetuximab. This might have influenced our results, and it is possible that a correlation between EGFR and clinical outcome would have been found if the patients would have received treatment with EGFR inhibitors. Thus, our results should be treated with caution.

In other studies by our group, we have found other biomarkers that better correlate to clinical outcome, e.g. tumor infiltrating T-cells and HLA class I expression [127, 131]. These markers are thus more likely to be useful to individualize the treatment of patients with TSCC than EGFR.

5 Summary and Conclusions

- The incidence of HPV positive tonsillar cancer in the Stockholm area has increased 7-fold between the years 1970 and 2007 while the incidence of HPV negative tonsillar cancer has decreased during the same period.
- The expression of E6 and E7 mRNA in HPV16 positive tonsillar cancer supports an oncogenic role of HPV16 in this cancer type.
- Patients with HPV positive tonsillar cancer have a better clinical outcome than those with HPV negative tonsillar cancer.
- There is a high HPV prevalence in tonsillar cancer from Greece, indicating that HPV is an important etiological factor for the development of this cancer type in Greece.
- HPV is not an important factor for the development of hypopharyngeal cancer in Stockholm and p16 overexpression is not correlated to the presence of HPV in this cancer type.
- The presence of EGFR Tyr1068 and Tyr1148 in tonsillar cancer is related to the presence of HPV, but is probably not independently correlated to treatment outcome and thus not useful as marker together with HPV for prediction of clinical outcome.

6 Future perspectives

In Paper I we demonstrated a 7-fold increase in the incidence of HPV+ TSCC in Stockholm during the last three decades. We do not know if the incidence will continue to increase or not during the following decade. Since public HPV vaccination among young girls has now, after a delay, been initiated, the incidence of all HPV16 induced cancer, even for TSCC among men, is likely to be reduced. This reduction will probably not be visible within the next two decades, since the women that are vaccinated are young and HPV induced tumors usually take decades in developing. The amount of reduction will depend on how many of the young women will be vaccinated, if also young men will be vaccinated, and how effective HPV vaccination is against oral HPV infection. The incidence of HPV- TSCC will likely continue to decline due to decreased smoking. It will be important to follow the incidence of both HPV+ and HPV- TSCC in the future.

Despite the good clinical outcome for the majority of patients with HPV+ TSCC, as shown in Paper II, the trend has been to intensify treatment, due to the bad prognosis for HNSCC patients in general. It is likely that many with HPV+ TSCC now receive unnecessary intensive treatment, resulting in long term harm for the patient that may have been avoided. To be able to reduce treatment, it is important to find other biomarkers for prognosis that can be used, together with HPV, to predict treatment outcome. As presented in Paper V, we evaluated EGFR and phosphorylated EGFR as potential biomarkers for prognosis. However, we concluded that these were not useful for this purpose. As described in the discussion of Paper V, we have in our group also evaluated other, more promising, markers e.g. tumor infiltrating CD8+ T-cells and tumor HLA class I expression [127, 131]. For the benefit of the patients, it is necessary to pursue the search for such biomarkers and to try them out in clinical trials.

In Paper IV, we investigated the prevalence of HPV in hypopharyngeal cancer. As noted the result was vastly different to the situation in TSCC. Only a few cancers were HPV positive in line with the very low overall survival of patients with hypopharyngeal cancers. Although those with HPV positive hypopharyngeal cancer seemed to fare better they were so few that the overall survival was not much affected. For these patients the question is thus not how to reduce the treatment, but how to optimize the treatment to the specific tumor. New treatments are needed, but it may also be necessary to find biomarkers that distinguish which tumor is sensitive to a specific treatment.

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