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HUMAN PAPILLOMAVIRUS AND OTHER BIOMARKERS IN NECK MASSES OF UNKNOWN ORIGIN AND IN HEAD AND NECK CANCER

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Human papillomavirus and other biomarkers in neck masses of unknown origin and in head and neck cancer

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To my family

ABSTRACT

Background. Human papillomavirus (HPV) is, in addition to smoking and alcohol, a risk factor for developing oropharyngeal squamous cell carcinoma (OPSCC), and more specifically tonsillar- and base of tongue squamous cell carcinoma (TSCC & BOTSCC respectively). Interestingly, HPV-positive (HPV+) TSCC/BOTSCC has a remarkably better clinical outcome than HPV-negative (HPV-) TSCC/BOTSCC and head and neck cancer in general. However, the role of HPV and other biomarkers in cancer of unknown primary in the head and neck region (HNCUP), i.e. where only a lymph node metastasis and no primary tumour is found, is not well understood. Moreover, the genetic landscape of HPV-positive HNCUP and the prognostic implications of certain mutations in TSCC/BOTSCC has not been studied extensively. Furthermore, using fine-needle aspiration cytology (FNAC) from neck masses for HPV-detection and potentially for predicting an HPV-positive TSCC/BOTSCC as the final diagnosis would be highly clinically useful, but has not been studied prospectively in a cohort consisting of both malignant and benign neck masses.

Aims. To investigate HPV DNA and mRNA and other prognostic biomarkers (p16, p53, CD8+ tumour infiltrating lymphocytes (TILs), HLA Class I expression) in HNCUP in relation to clinical outcome. Moreover, to study mutations in HPV+ HNCUP and TSCC/BOTSCC in relation to prognosis and to analyse whether HPV-detection in FNAC from neck masses is reliable and if a finding of HPV could predict an HPV+ TSCC/BOTSCC as the final diagnosis.

Results. In Paper I, we show that HPV is a favourable prognostic factor in HNCUP, and in Paper II, we validate this finding in a separate cohort (3-year overall survival 86% vs. 54% for HPV+ and HPV- HNCUP respectively, in the combined cohorts). In Paper II, we also demonstrate that HPV mRNA is expressed in the vast majority of HPV DNA+ HNCUP. Moreover, in Paper I we find that high p53-expression correlates to a poor prognosis, and in Paper II that a low number of CD8+ TILs are potentially related to a poor outcome, while HLA Class I expression does not appear to be a prognostic factor in HNCUP. In Paper III, we show that TP53, CDKN2A and PIK3CA are the most commonly mutated genes in HPV+ HNCUP, and that having a mutation in FGFR3 correlated to a poor prognosis in HPV+ TSCC/BOTSCC. In Paper IV, we demonstrate that HPV DNA detection in FNAC from neck masses of unknown origin is reliable and could be used prospectively to predict an HPV+ TSCC/BOTSCC as the final diagnosis. HPV DNA was not found in malignant conditions other than HPV+ TSCC/BOTSCC or in any benign conditions, including branchial cleft cysts.

Conclusions. HPV appears to have a similar causative and prognostic role in HNCUP as in TSCC/BOTSCC. Investigation of HPV-status should therefore be part of the diagnostic work-up of an HNCUP. Examination of HPV DNA-status using FNAC is useful in the clinical investigation of patients with neck masses of unknown origins, including patients eventually diagnosed with HNCUP or TSCC/BOTSCC.

LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. **Sivars L**, Näsman A, Tertipis N, Vlastos A, Ramqvist T, Dalianis T, Munck-Wikland E, Nordemar S. Human papillomavirus and p53 expression in cancer of unknown primary in the head and neck region in relation to clinical outcome. *Cancer Med. 2014 Apr;3(2):376-84.*

II. **Sivars L**, Landin D, Grün N, Vlastos A, Marklund L, Nordemar S, Ramqvist T, Munck-Wikland E, Näsman A, Dalianis T. Validation of human papillomavirus as a favourable prognostic marker and analysis of CD8+ tumour-infiltrating lymphocytes and other biomarkers in cancer of unknown primary in the head and neck region. *Anticancer Res. 2017 Feb;37(2):665-673.*

III. Bersani C, **Sivars L**, Haeggblom L, DiLorenzo S, Mints M, Ährlund-Richter A, Tertipis N, Munck-Wikland E, Näsman A, Ramqvist T, Dalianis T. Targeted sequencing of tonsillar and base of tongue cancer and human papillomavirus positive unknown primary of the head and neck reveals prognostic effects of mutated FGFR3. *Oncotarget. 2017 May 23;8(21):35339-35350.*

IV. **Sivars L***, Landin D*, Haeggblom L, Tertipis N, Grün N, Bersani C, Marklund L, Ghaderi M, Näsman A, Ramqvist T, Nordfors C*, Munck-Wikland E*, Tani E*, Dalianis T*. Human papillomavirus DNA detection in fine-needle aspirates as indicator of human papillomavirus-positive oropharyngeal squamous cell carcinoma: A prospective study. *Head Neck. 2017 Mar;39(3):419-426.*

**Contributed equally*

RELATED PUBLICATIONS, NOT INCLUDED IN THE THESIS

I. Nordfors C, Grün N, Tertipis N, Ährlund-Richter A, Haeggblom L, **Sivars L**, Du J, Nyberg T, Marklund L, Munck-Wikland E, Näsman A, Ramqvist T, Dalianis T. CD8+ and CD4+ tumour infiltrating lymphocytes in relation to human papillomavirus status and clinical outcome in tonsillar and base of tongue squamous cell carcinoma. *Eur J Cancer. 2013 Jul;49(11):2522-30.*

II. Nordfors C, Grün N, Haeggblom L, Tertipis N, **Sivars L**, Mattebo M, Larsson M, Häggström-Nordin E, Tydén T, Ramqvist T, Dalianis T. Oral human papillomavirus prevalence in high school students of one municipality in Sweden. *Scand J Infect Dis. 2013 Nov;45(11):878-81.*

III. **Sivars L**, Tertipis N, Näsman A, Ramqvist T, Dalianis T (2014) A simple approach for determining presence of HPV DNA from slides previously stained for P16ink4a. *J Cytol Histol S4:014.*

IV. Tertipis N, Hammar U, Näsman A, Vlastos A, Nordfors C, Grün N, Ährlund-Richter A, **Sivars L**, Haeggblom L, Marklund L, Hammarstedt-Nordenvall L, Chaturvedi AK, Munck-Wikland E, Ramqvist T, Bottai M, Dalianis T. A model for predicting clinical outcome in patients with human papillomavirus-positive tonsillar and base of tongue cancer. *Eur J Cancer. 2015 Aug;51(12):1580-7.*

V. **Sivars L**, Tani E, Näsman A, Ramqvist T, Munck-Wikland E, Dalianis T. Human papillomavirus as a diagnostic and prognostic tool in cancer of unknown primary in the head and Neck region. *Anticancer Res. 2016 Feb;36(2):487-93. (Review)*

VI. **Sivars L**, Bersani C, Grün N, Ramqvist T, Munck-Wikland E, Von Buchwald C, Dalianis T. Human papillomavirus is a favourable prognostic factor in cancer of unknown primary in the head and neck region and in hypopharyngeal cancer. *Mol Clin Oncol. 2016 Dec;5(6):671-674. (Review)*

VII. Bersani C, Mints M, Tertipis N, Haeggblom L, **Sivars L**, Ährlund-Richter A, Vlastos A, Nordfors N, Grün N, Munck-Wikland E, Näsman A, Ramqvist T, Dalianis T. A model using four concomitant biomarkers for predicting outcome in patients with human papillomavirus positive tonsillar and base of tongue squamous cell carcinoma. *Oral Oncol. 2017 May;68:53-59*

VIII. **Sivars L**, Landin D, Rizzo M, Haeggblom L, Bersani C, Munck-Wikland E, Dalianis T, Näsman A, Marklund L. Presence of Human papillomavirus (HPV) in branchial cleft cysts – a way to distinguish branchial cleft cysts from cystic metastases? *(Submitted)*

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LIST OF ABBREVIATIONS

ADC	Adenocarcinomas
BCC	Branchial cleft cyst
BOTSCC	Base of tongue squamous cell carcinoma
BSGP	Broad spectrum general primer
CD	Cluster of differentiation
CUP	Cancer of unknown primary
CT	Computed tomography
DFS	Disease-free survival
E	Early region
EBV	Epstein-Barr virus
EV	Epidermodysplasia verucciformis
E6AP	E6 associated protein
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescent in-situ hybridization
FNAC	Fine-needle aspiration cytology
GP	General primer
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HNC	Head and neck cancer
HNCUP	Head and neck cancer of unknown primary
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR	High risk
hTERT	Human telomerase reverse transcriptase
HTLV-1	Human T-lymphotropic virus-1
IARC	International agency for research on cancer

ICD-10	International statistical classification of diseases and related health problems - tenth revision
IHC	Immunohistochemistry
ISH	In situ hybridization
KSHV	Kaposi's sarcoma herpes virus
L	Late region
LCR	Long control region
LR	Low risk
MCV	Merkel cell polyomavirus
MRI	Magnetic resonance imaging
MFI	Median fluorescence intensity
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
OPSCC	Oropharyngeal squamous cell carcinoma
OS	Overall survival
PAD	Pathological anatomical diagnosis
PCR	Polymerase chain reaction
PET	Positron-emission tomography
pRb	Retinoblastoma protein
RT	Radiotherapy
SCC	Squamous cell carcinoma
TIL	Tumour infiltrating lymphocyte
TSCC	Tonsillar squamous cell carcinoma
TSS	Tumour specific survival

1 INTRODUCTION

1.1 INFECTIONS AND CANCER

Of the 14 million annual new cancer cases, worldwide, approximately 2.2 million or 15.4% are attributable to infectious agents, thus making pathogens one of the most common causes of cancer. This is especially true in the less developed part of the world where the percentage of tumours caused by infections are much greater than in developed countries, ranging from 31.3% in Sub-Saharan Africa to 7% in Europe and 4% in North America (Plummer et al., 2016).

High-risk human papillomavirus (HPV), Hepatitis B- and C-virus (HBV & HCV, respectively), Epstein-Barr virus (EBV) and the bacteria *Helicobacter pylori* are the most frequent pathogens associated with cancer, but additional viruses as well as a few trematodes have been shown to be able to cause cancer in humans. A list of biological agents recognized as carcinogenic by the International Agency for Research on Cancer (IARC) and others; the types of cancer they are linked to; and approximate number of new cases per year and agent are depicted in Table 1 (IARC Monograph, 2012; Plummer et al., 2016; Schadendorf et al., 2017). In addition, human immunodeficiency virus (HIV) is sometimes recognized as a tumour virus, although HIV does not have a direct carcinogenic effect, but rather sets the stage for other infectious agents through its immune suppressive effects (Moore et al., 2010).

That cancer can be caused by a transmissible agent was suspected as early as in the 1840's when the Italian physician Rigoni-Stern investigated death certificates of women in Verona and noticed that cancer of the uterine cervix was more common in married women, widows and prostitutes than in nuns, thus leading to the hypothesis that a sexually transmissible agent caused cervical cancer (Rigoni-Stern, 1842; zur Hausen, 2009). Evidence that small transmissible agents, could indeed cause cancer, was provided by Francis Peyton Rous in his famous experiment with hens in 1909 for which he later was awarded the Nobel Prize in physiology or medicine. In the seminal experiment, Rous resected a sarcomatous chest tumour from a hen, grinded and filtered it thoroughly and injected the resulting extract into another hen, which then developed a similar chest tumour (Rous, 1910; Rous, 1911). In 1964, the first human tumour virus – EBV, was discovered in Burkitt's lymphoma cell lines (Epstein et al., 1964). So far, another six human tumour viruses have been described (see Table 1), in addition to a number of viruses causing cancer in other species. In 1983, high-risk HPV types were discovered in cervical cancer (Durst et al., 1983), finding the transmissible agent the Rigoni-Sterns study had hinted about, and landing Harald zur Hausen the Nobel Prize in 2008 (Moore et al., 2010).

It has been suggested that infectious cancer agents can be broadly categorized into two groups, either causing cancer in a direct way or in an indirect way. In the direct way, the agent, typically a virus is present in each cell and produces oncogenes that directly contribute

to the carcinogenesis and maintains the transformed tumour cell phenotype. HPV, Merkel Cell polyomavirus (MCV), EBV and Kaposi's sarcoma herpes virus (KSHV) induce and maintain neoplasms in this way (Moore et al., 2010). The produced oncogenes often target pRb (thus increasing cell cycle progression allowing for replication of the viral genomes) and p53 (e.g. inhibiting apoptosis) leading to transformation of host cells. Indirect carcinogens, e.g. *H. pylori*, are proposed to cause cancer by inducing persistent infection & inflammation with subsequent increased cell division leading to eventual mutations and transformation of the cells. However, the categorization into direct or indirect carcinogens may be an oversimplification, as several agents, e.g. HBV, HCV & Human T-lymphotropic virus-1 (HTLV-1) cannot be categorized into either of these categories, producing both potentially oncogenic proteins and causing persistent inflammation (Moore et al., 2010).

Table 1. Infectious agents causing cancer in humans, the cancer types they are associated with and the estimated number of new cancer cases each agent is associated with worldwide per year (IARC Monograph 100b. 2012; Plummer et al., 2016; Schadendorf et al., 2017).

Pathogen	**Type**	**Associated cancer types**	**Approx. no. cases/year worldwide**
Clonorchis sinensis	Trematode	Cholangiocarcinoma	1.300*
Epstein-Barr virus (EBV), also known as human herpesvirus 4 (HHV4)	Herpesvirus	Nasopharyngeal carcinoma Lymphomas	120.000
Helicobacter pylori	Bacteria	Gastric carcinoma Gastric lymphomas	770.000
Hepatitis B virus (HBV)	Hepadnavirus	Hepatocellular carcinoma	420.000
Hepatitis C virus (HCV)	Flavivirus	Hepatocellular carcinoma Lymphomas	170.000
High-risk human papillomavirus (HPV)	Papillomavirus	Cervical cancer Anogenital cancer Head & neck cancer	640.000
Human T-lymphotropic virus-1 (HTLV-1)	Retrovirus	Adult T-cell leukemia	3.000
Kaposi's sarcoma herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8)	Herpesvirus	Kaposi's sarcoma Primary effusion lymphoma	44.000
Merkel cell polyomavirus (MCV)	Polyomavirus	Merkel cell carcinoma	1.500**
Opisthorchis viverrini	Trematode	Cholangiocarcinoma	1.300*
Schistosoma haematobium	Trematode	Bladder carcinoma	7.000

*Number denotes cancer attributable to infection by any of the two liver flukes. ** Number denotes cases of Merkel Cell carcinoma in the United States 2008. No worldwide calculations available.

A point worth noting is that cancer development following infection with any of the above-mentioned agents should be regarded as biological accidents. The pathogen does not gain any evolutionary advantages by causing cancer, e.g. it does not increase transmission or viral burden. Carcinogenesis is rather an unwanted side effect of the pathogens strategy to carrying out its life cycle and replicate or to evade the host immune system (Moore et al., 2010).

After this brief introduction to infections & cancer in general, the rest of the thesis will focus on HPV with special attention given to its role in head & neck cancer, oropharyngeal cancer, tonsillar and base of tongue cancer and most specifically cancer of unknown primary of the head and neck region (HNCUP).

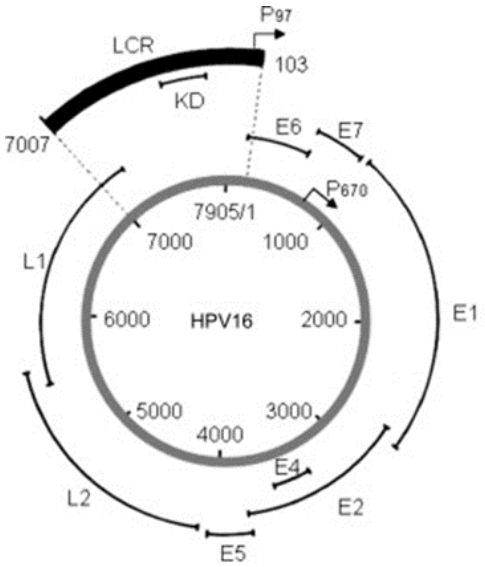
1.2 HUMAN PAPILLOMAVIRUS (HPV)

1.2.1 General background

There are more than 200 different types of HPV, which can be divided into high risk (HR) and low risk (LR) types depending on their ability to induce tumours (Papillomavirus Episteme, NIH, 2017; Tommasino, 2014). High risk types can give rise to e.g. cervical- and anogenital cancers as well as subsets of head and neck squamous cell carcinomas (HNSCC). The International Agency for Research on Cancer (IARC)/World Health Organization (WHO) are currently listing 12 HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) as carcinogenic in humans with another 13 as putative carcinogens (IARC Monograph 100b, 2012). The most frequent HR types causing cervical cancer, accounting for approximately 70% of the cases, are HPV16 and HPV18, while HPV16 alone stands for > 80% of HPV+ HNSCC and > 90% of HPV+ tonsillar squamous cell carcinoma (TSCC) and base of tongue squamous cell carcinoma (BOTSCC) (de San Jose et al., 2010; Ndiaye et al., 2014). The low risk types can give rise to a number of conditions including genital warts (e.g. HPV6 & 11), common skin warts (e.g. HPV1 & 2) and recurrent respiratory papillomatosis (e.g. HPV6 & 11) (Tommasino, 2014). However, under certain circumstances, such as in immunocompromised individuals, or in patients with certain conditions such as epidermodysplasia verucciformis (EV) some cutaneous LR HPV-types (e.g. HPV5 and 8) have been implicated in cancer development as well (Tommasino, 2014). Below, first the molecular and then the clinical aspects of HPV infection will be discussed in greater detail.

1.2.2 Viral particle and the HPV-genome

All HPV types are small (55 nm in diameter) with an icosahedral capsid and a circular double-stranded DNA genome of approximately 8000 base pairs (Tommasino, 2014). The genome consists of an early region (E), a late region (L) and a long control region (LCR), the latter containing regulatory elements (Tommasino, 2014). There are six regulatory proteins (E1, E2, E4-E7) involved in various steps in the viral life cycle, and two structural proteins, the major (L1) and minor (L2) capsid proteins (Tommasino, 2014). See Figure 1 for a schematic representation. The proteins and their involvement in the viral life cycle are described in more detail further down with a special focus on E6 & E7 – the two most important proteins involved in HPV-induced carcinogenesis (Moody & Laimins, 2010).



Viral Protein	Functions and Features
E1	It forms a heterodimer complex with E2 and controls viral replication
E2	It regulates early gene promoter and together with E1 viral DNA replication
E4	It may mediate the viral particle realease by destabilizing cytokeratin network
E5	It stimulates mitogenic signals of growth factors
E6	It inactivates many cellular proteins and is one of the major viral oncoproteins
E7	It inactivates many cellular proteins and is one of the major viral oncoproteins
L1	It is the major capsid protein and is the component of the HPV prophylactic vaccine
L2	It is the minor capsid protein

Figure 1. Schematic representation of the organization of the human papillomavirus 16 genome, and the major functions of the viral proteins. LCR = Long control region. P97 and P670 = the early and late promotors respectively. (Tommasino, 2014; with permission from publisher).

1.2.3 Classification

HPVs are classified according to the homology of the well-conserved L1 gene, as determined by whole gene sequencing. Human papillomaviruses belong to one of five genera (Alpha-, Beta-, Gamma-, Mu- and Nu-papillomaviruses) with types within one genus typically showing less than 60% homology of the L1 region to that of other genera (Bernard et al., 2010). They are further divided into species and then types with more than 200 different types sequenced in full so far (Papillomavirus Episteme, NIH, 2017). Each type is at least 10% different in its L1 nucleotide sequence compared to all other types (Bernard et al., 2010). The different genera and types show differences in behaviour and are associated with different diseases infecting either mucosal or cutaneous epithelia. The high-risk types belong to the Alpha-species, although the Alpha-species include mucosal LR (e.g. HPV6 & 11) and cutaneous HPV-types as well (Tommasino, 2014). The Beta- and Gammatypes are cutaneous with the Betatypes being implicated in squamous cell carcinoma (SCC) of EV- and other immunocomprised individuals, while the Mu types are cutaneous benign (Tommasino, 2014). Below, the focus will be on the mucosal high-risk types, especially HPV16, which is the most well studied HPV-type.

1.2.4 Viral life cycle and carcinogenesis

HR HPVs are sexually transmitted and carry out their life cycle in mucosal epithelia, where they infect the cells in the basal layer. It has been suggested that micro wounds in the epithelium and the subsequent wound healing response facilitates a successful infection. Infection may then be cleared by the host immune response (usually within 6-12 months) or in a small number of cases, result in an asymptomatic carrier state, which then later may result in neoplasia. HPV gains entry to the keratinocytes in a complex and poorly understood

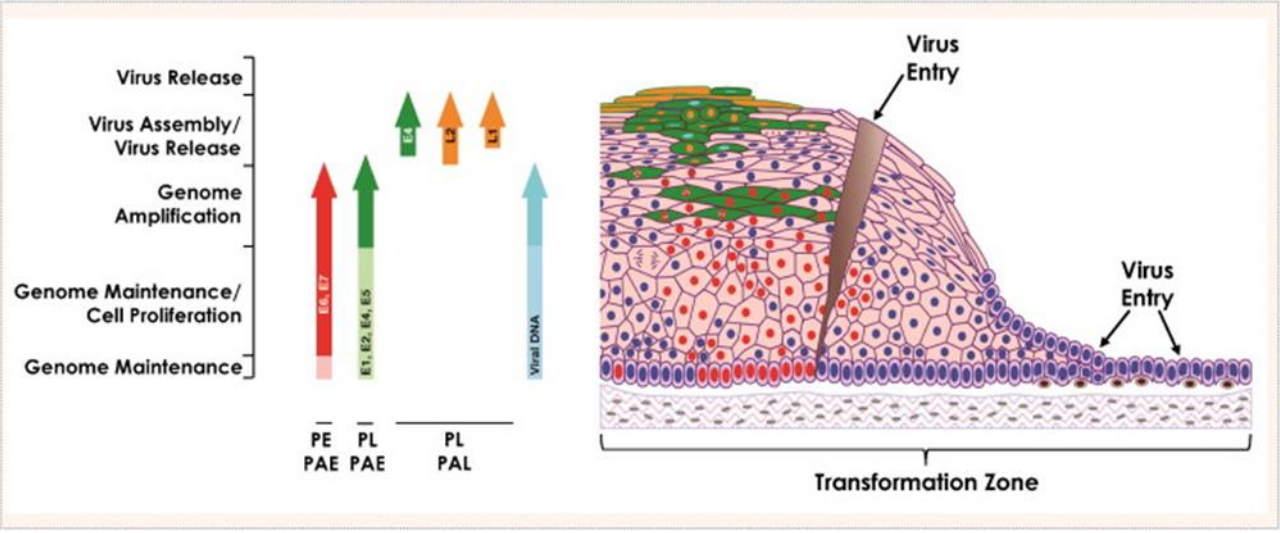


Figure 2. Life cycle of high-risk HPVs and expression patterns of the different viral proteins in cervical epithelium (Doorbar, 2012; with permission from publisher).

fashion involving L1 and L2 and heparan sulfate proteoglycans on the basement membrane and on the basal cells themselves. It then enters the cells by endocytosis and the virions are transported to the nucleus, where the HPV DNA can then either exist in an episomal, non-integrated, form, or become integrated into the host genome. The HPV life cycle is thereafter tightly linked to the maturation of the keratinocytes. See Figure 2 for a schematic picture of the life cycle of HR HPV and expression of the various HPV proteins in cervical epithelium (Doorbar, 2012).

Upon persistent infection, HR HPV can cause tumour development. The viral contribution to the carcinogenesis is complex and below only the most central mechanisms will be presented, see also Figure 3. The two most important HPV-proteins involved in carcinogenesis are E6 and E7 and continuous expression of these proteins is necessary for maintaining a malignant phenotype (Tommasino, 2014).

E6 binds to the E6-associated protein (E6AP). The resulting E6/E6AP-complex then binds to the core domain of p53 leading to rapid ubiquitination of p53, thus targeting it for degradation in the proteasome (Moody & Laimins, 2010). Hence, in HPV-induced cancers, p53 is dysregulated at the protein level rather than mutated as in many other tumour types (O´Sullivan et al., 2015). The transcription factor p53 is one of the most important tumour suppressor genes with many functions, e.g. to halt the cell cycle in case of cellular stress, thus giving the cell time to repair any DNA-damage. If this is not possible, p53 can induce apoptosis (Hanahan & Weinberg, 2011). Cells infected with HR HPV, thus lose important cell cycle control mechanisms and effective DNA-repair leading to cell division despite mutations or DNA damage, and this way eventually also may develop a malignant phenotype. Furthermore, the E6/E6AP-complex is also involved in transcriptional activation of the human telomerase reverse transcriptase (hTERT) gene coding for a subunit of the

telomerase complex, allowing for increased telomerase activity in HR HPV infected cells. This helps the cells to maintain telomere length and proliferate indefinitely without entering a state of replicative senescence (Moody & Laimins. 2010).

E7 binds to the Retinoblastoma protein (pRb), another important tumour suppressor, and its related proteins p107 and p130, all three involved in cell cycle control. pRb controls the entry of cells into the S-phase of the cell cycle. Active, hypophosphorylated, pRb binds to and inhibits members of the E2F family of transcription factors, which activate genes necessary for DNA synthesis. Binding of E7 leads to degradation of pRb through proteasomal pathways. E2F is released when pRb is lost, which leads to the promotion of cell cycle progression and transformational properties in cells infected with HR HPVs. Theoretically, loss of pRb would lead to p53 mediated cell growth inhibition and apoptosis if the cells would still have functional p53. This explains why HR HPVs have developed strategies targeting both p53 (with E6) and pRb (with E7) to promote proliferation. LR HPV E7 also binds to pRb, but with less affinity (Moody & Laimins, 2010). Down regulation of pRb also leads to up regulation of the p16 protein, and p16 overexpression is therefore often used as a surrogate marker for HPV-induced HNSCC (Venuti & Paolini, 2012).

E5 is also considered to contribute to tumour development, e.g. by stimulating the mitogenic signals of growth factors (Tommasino, 2014). E5 has furthermore been reported to down-regulate human leukocyte antigen (HLA)-Class I expression on the surface of cells, and thus help HPV-infected cells to avoid detection by the host immune system (Campo et al., 2010).

The other HPV-proteins have lesser roles in tumour development. E1 and E2 are e.g. involved in controlling viral replication (Tommasino, 2014). Loss of E2 upon viral integration into the host genome, leads to increased transcription of E6 and E7 (Moody & Laimins, 2012). E4 has been suggested to be involved in viral particle release. L1 is the major capsid protein, can self-assemble into virus like particles and is the major component of the prophylactic HPV-vaccines, while L2 is the minor capsid protein (Tommasino, 2014), see also Figure 1.

After this introduction to the more molecular aspects of HPVs involvement in cancer, we will in the next section turn our attention towards more clinical and epidemiological features.

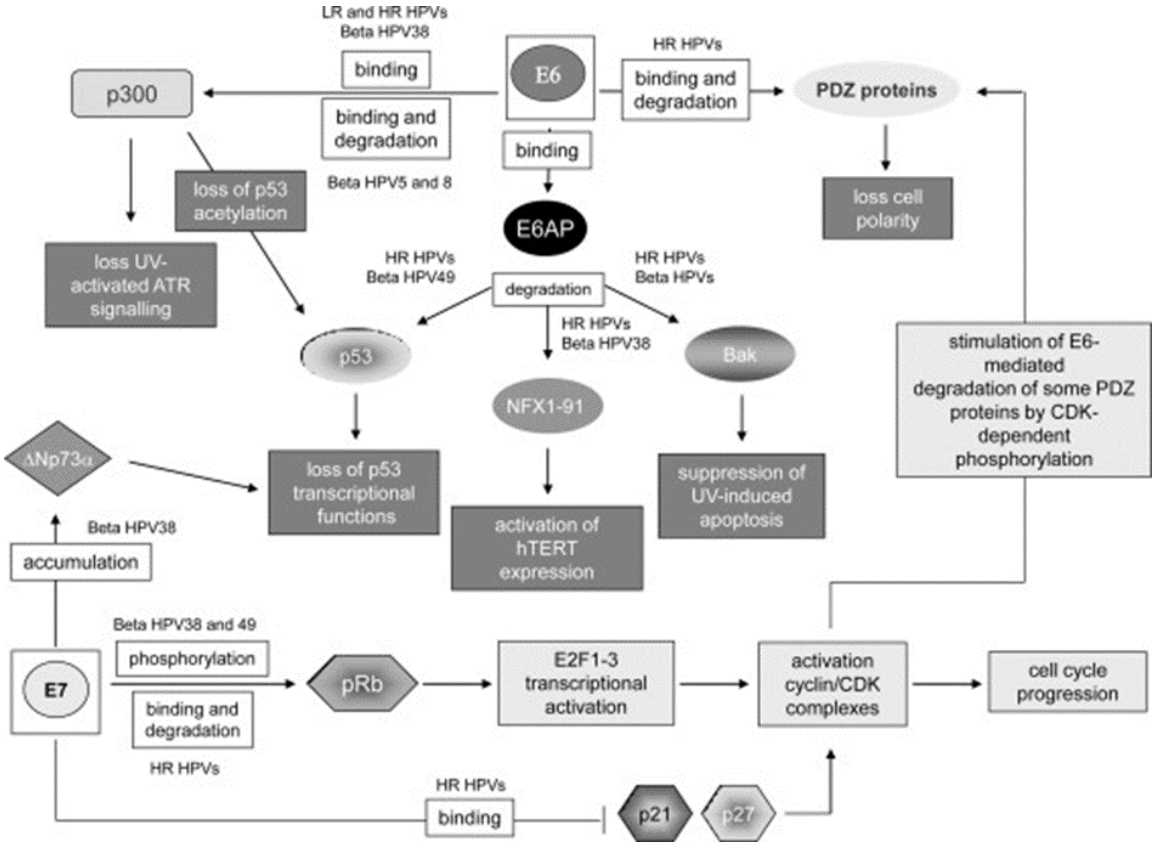


Figure 3. HPV E6 and E7 and their contributions to carcinogenesis (Tommasino, 2014; with permission from publisher).

1.2.5 HPV in cancer and preventive measures

HR HPVs have been estimated to cause of almost all of the 530 000 cases of cancer of the uterine cervix worldwide per year (Plummer et al., 2016). HPV16 and 18 dominate and stand for approximately 70% of the cases of invasive cervical cancer, while HPV16, 18 or 45 together are found in 94% of the cervical adenocarcinomas (de San Jose et al., 2010). In addition, HR HPVs are indicated to be the cause of ~51% of penile carcinomas, 88% of anal carcinomas, 78% of vaginal carcinomas and 15-48% of vulvar carcinomas (depending on the age of the patient, with higher prevalence among younger women) worldwide (Plummer et al., 2016). HR HPVs are also the cause of certain subsets of HNSCC and these will be described below. In total, HR HPVs are estimated to cause approximately 640 000 cases of cancer per year, adding up to 4.6% of all cancer cases (Plummer et al., 2016). HR HPVs are thus important carcinogens and significant resources have been allocated to prevent HPV-related cancers. Preventive measures consisted in the past decades of screening for cervical cancer when available, and more recently of preventive HPV vaccination.

For cervical cancer, in many countries, there are screening programs for precursor lesions in cytology samples and/or for HR HPVs. For TSCC or BOTSCC however, there are no screening programs available, since there are no established precursor lesions for TSCC or BOTSCC, as is the case with cervical intraepithelial neoplasia (CIN) in cervical cancer.

Today, prophylactic HPV-vaccines are based on self-assembled L1 proteins, which form virus like particles. Gardasil (Merck, USA), approved by the US Food and Drug Administration (FDA) in 2007 covers HR HPV types 16 and 18 as well as LR types 6 and 11, responsible for causing genital warts, while Cervarix (Glaxo-SmithKline, UK), FDA approved shortly thereafter, only covers HPV 16 and 18 (O'Sullivan et al., 2015). Recently, a nona-valent vaccine – Gardasil 9 (Merck, USA), covering HPV-types 6, 11, 16, 18, 31, 33, 45, 52 and 58 has been introduced (Drolet et al., 2014). The studies behind the introduction of these vaccines, showing very good efficacy and few side effects, have been performed in cervical cancer (Villa et al., 2005; Villa et al., 2006; FUTURE II Study Group, 2007). The vaccines have however also been suggested to be effective for preventing other HR HPV-induced tumours, e.g. HPV-positive TSCC and BOTSCC. Programs for vaccinating girls have been started in many countries and a few countries, e.g. Australia, have also started to vaccinate boys, something currently being discussed in Sweden (Ali et al., 2017; Folkhälsomyndigheten, 2017).

1.3 HEAD AND NECK CANCER, OROPHARYNGEAL CANCER AND OTHER CONDITIONS CAUSING NECK MASSES

1.3.1 General background on head and neck cancer

The term head and neck cancer (HNC) includes cancer of e.g. the larynx, the hypopharynx, the oropharynx, the nasopharynx, the oral- and nasal cavities and salivary glands as well as cancers of unknown primary in the head and neck region (O´Sullivan et al., 2015). It is the 6th most common form of cancer in the world with ~600.000 cases per year and a mortality rate of ~50% (Ferlay et al., 2008). However, it is less frequent in the developed world with e.g. approximately 1300 cases per year in Sweden (Nationellt vårdprogram, Huvud- och Halscancer, 2015). The great majority of HNC cases (80-90%) are squamous cell carcinomas (HNSCC) (Nationellt vårdprogram, Huvud- och Halscancer, 2015), of which oropharyngeal squamous cell carcinoma (OPSCC) and cancer of unknown primary (HNCUP) will be discussed more, while e.g. adenocarcinomas, salivary gland tumours or lymphomas will not be presented in detail.

It has long been established that smoking and alcohol are common risk factors for HNSCC (Wenig, 2016). Additionally, in 2007, HR type HPV16 was added to the list as a causative agent for OPSCC and more specifically TSCC, by the International Agency for Research on Cancer (IARC) (IARC-Monographs, 2007). Other causes of HNSCC include e.g. bethel nut chewing, poor mouth hygiene, and certain smokeless tobaccos, although Swedish "snus" has not been linked to HNSCC (Luo et al., 2008; O´Sullivan et al., 2015).

There are some studies characterizing the genomic landscape of HNSCC. The Cancer Genome Atlas (TCGA) includes e.g. whole exome sequencing by next generation sequencing (NGS) of 279 flash-frozen HNSCC - 172 from the oral cavity, 72 from laryngeal sites, 2 from the hypopharynx and 33 OPSCC (Cancer Genome Atlas Network, 2015). The 36 HPV-

positive tumours had frequent activating mutations of PIK3CA, an oncogene implicated in e.g. cervical cancer. Loss of TRAF3, encoding a protein involved in antiviral immune response, and where the loss leads to abnormal NF-kB signalling was also found in HPV positive tumours. Furthermore, amplification of E2F1, which encodes a transcription factor important for the cell cycle was also frequently seen (Cancer Genome Atlas Network, 2015).

Almost all smoking related tumours showed loss of function mutations in TP53 and inactivation of CDKN2A, which encodes p16, while these alterations were very rare in HPV-positive tumours (Cancer Genome Atlas Network, 2015). Patients with HPV-positive tumours and, interestingly, HPV-negative tumours with wild type TP53 had better survival than HPV-negative, TP53-mutated tumours (Cancer Genome Atlas Network, 2015).

In another study, Chung et al. (2015), showed that using FFPE tumours was comparable to using fresh frozen tissue when performing NGS in HNSCC, allowing comprehensive genome analysis in a standard clinical setting (Chung et al., 2015). Furthermore, Chung et al. (2015), analysed 236 cancer-related genes in 252 formalin-fixed paraffin-embedded (FFPE) HNSCC from a variety of locations including regional and distant metastasis. In concordance with the previously mentioned study, PIK3CA was the most frequently mutated gene in the 84 HPV-positive tumours. Other frequently mutated genes in HPV-positive tumours included PTEN, SOX2 and MLL2. In the HPV-negative tumours again TP53 and CDKN2A were frequently mutated. In both HPV-positive and HPV-negative tumours mutations in tumour suppressor genes were more common than in oncogenes, although HPV-positive tumours had a higher likelihood of having only oncogene expression compared to HPV-negative tumours (Chung et al., 2015). Similar mutational profiles were also found in smaller studies, specifically examining genes commonly mutated in cancer (Lechner et al., 2013; Seiwert et al., 2015; Tinhofer et al., 2016), as well as by whole genome sequencing (Stransky et al., 2011).

In addition to in OPSCC, HPV has also been implicated as a causative agent in a small proportion of hypopharyngeal squamous cell carcinoma cases (Dalianis et al., 2015; Wendt et al., 2014) and HPV is also sometimes found in other types of HNSCC, e.g. in approximately 4-5% of oral- and laryngeal SCC (Plummer et al., 2016). However, the IARC does not consider HPV as a causative agent in these cancer types (IARC-Monographs, 2007).

In the next section, OPSCC, where HPVs causal role is well established, is presented (IARC-Monographs, 2007).

1.3.2 Oropharyngeal squamous cell carcinoma (OPSCC)

Anatomically, the oropharynx includes the tonsils, the base of tongue, the soft palate, the uvula and the back wall of the pharynx posterior to the oral cavity (Wenig, 2016). HPV causes cancer of the tonsils (TSCC) and the base of tongue (BOTSCC), the two subsites of the oropharynx containing lymphoid tissue, but is typically not involved in the carcinogenesis of the other oropharyngeal locales (Haeggblom et al., 2017; Marklund et al., 2012).

HPV-positive OPSCC, especially TSCC and BOTSCC, appear to be different clinical and biological diseases as compared to their corresponding HPV-negative counterparts, with HPV-positive tumours more often affecting younger people and non-smokers than HPV-negative tumours (O´Sullivan et al., 2015). Notably, the metastases from HPV-positive TSCC and BOTSCC are frequently (30-50%) cystic, while the metastases from HPV-negative HNSCC most often are solid (O´Sullivan et al., 2015). See also above section on the genetic differences between HPV-positive and HPV-negative HNSCC.

A sharp rise in the incidence of OPSCC, and especially TSCC and BOTSCC has been observed since 1970 in many countries including Sweden (Chaturvedi et al., 2011; Näsman et al., 2009; Rietbergen et al., 2013). Today, approximately 325 persons are diagnosed with OPSCC per year in Sweden, of which ~70% are men and ~75% of the tumours are HPV-positive (Nationellt vårdprogram Huvud- och Halscancer 2015; Näsman et al., 2015). The proportion of HPV-positive OPSCC varies considerably between countries with a higher proportion in e.g. Sweden (Näsman et al., 2015) and the USA (Chaturvedi et al., 2011) as compared to e.g. in Germany (Tahtali et al., 2013) and the Netherlands (Rietbergen et al., 2013). Furthermore, the proportions of HPV-positive TSCC and BOTSCC have increased the last decades (Chaturvedi et al., 2011; Näsman et al., 2009), analogous to the increase in TSCC and BOTSCC incidence, leading to the hypothesis that an HPV-epidemic is causing the rise in TSCC and BOTSCC incidence (Chaturvedi et al., 2011; Näsman et al., 2009).

The incidence of HPV-negative TSCC and BOTSCC in contrast, are decreasing in some countries, e.g. in Sweden and the USA, likely due decreased smoking in these countries (Chaturvedi et al., 2011; Näsman et al., 2009).

Notably, patients with HPV-positive TSCC or BOTSCC have significantly much better clinical outcome than patients with HPV-negative TSCC or BOTSCC (Ang et al., 2010; Chaturvedi et al., 2011; Lindquist et al., 2007), with approximately 80% versus 40% 5-year disease-free survival in Sweden, respectively, in the study by Lindquist et al. (2007). HPV-status, however, does not clearly appear to have a prognostic effect at other oropharyngeal sites than the tonsils or base of tongue (Marklund et al., 2012).

1.3.3 Cancer of unknown primary in the head and neck region (HNCUP)

In 3-5% of all head and neck cancer cases, a primary tumour is not found despite a thorough clinical workup - so called cancer of unknown primary (HNCUP) (Pavlidis & Pentheroudakis, 2012). Between 2008 and 2012, in Sweden, there were 233 cases of HNCUP, and this calculates to an incidence rate of 0.49 cases per 100 000 inhabitants (Nationellt vårdprogram Huvud- och Halscancer, 2015). As other head and neck cancers, HNCUP is more common in men than in women. Squamous cell carcinoma is the most common form, accounting for 75-85% of the cases and will be the focus of the rest of the thesis (Cervezo et al., 2011; Nationellt vårdprogram Huvud- och Halscancer, 2015; O´Sullivan et al., 2015).

Previously it has been thought that CUP presenting at different parts of the body share common biological traits leading to rapid progression and early dissemination (Varadhachary & Raber, 2014). However, today increasing evidence points to CUP as different site-specific metastasis retaining the properties of the primary tumour, and that they just happen to share the attribute of having a primary tumour that escapes recognition (Pavlidis & Pentheroudakis, 2012). Reasons for not finding the primary tumour includes that the primary tumour is very small and therefore missed during examination or due to spontaneous regression of the primary tumour (Cerezo et al., 2011).

HNCUP has a much better clinical outcome than CUP in general with a 5-year survival of 35-50% (Economopoulou et al., 2015), compared to CUP in general, which has a dismal prognosis with a median survival of only 6 to 10 months (Tothill et al., 2013). HNCUP thus appear to have more in common with head and neck cancer than with CUP at other parts of the body.

It is likely that the more favourable clinical outcome in HNCUP could in part be due to that some of these tumours are in fact HPV-positive OPSCCs, or that they are regional metastases as opposed to distant metastases from a non-HNSCC tumour. In this thesis the first hypothesis has been examined in Paper I.

Recently, a few studies have investigated genetic alterations in general CUP, however not taking into account specific subsites and including very few HNCUP. Ross et al. (2015), analysed 3769 exons from 236 cancer-related genes in 200 CUP and found at least 1 genetic alteration in 96% of the samples – most frequently TP53 (55%), KRAS (20%) and CDKN2A (19%) (Ross et al., 2015). They also subdivided the cohort into adenocarcinomas (ADC) (n=125) and non-ADC (n=75, including 8 SCC) with the same three genes as the most commonly mutated genes in both subgroups, although alterations in the receptor tyrosine kinase/RAS pathway were more often mutated in ADCs (Ross et al., 2015). Gatalica et al. (2014), analysed 1806 CUP with a variety of techniques including sequencing, immunohistochemistry (IHC) and in situ hybridization (ISH) in search of potential drug targets, finding mutations in 96% of the samples (Gatalica et al., 2014). TP53 and KRAS were the most frequently mutated genes with EGFR and HER2 the most commonly amplified genes (Gatalica et al., 2014). Tothill et al. (2013), examined 701 genes in 16 CUP patients including 3 HNSCC, two of them p16-positive by IHC, finding therapeutic gene targets or pathways in 12/16 (Tothill et al., 2013).

Clearly, little is known about genetic changes in HNCUP, and in Paper II we therefore examined hotspot mutations in oncogenes and tumour suppressor genes in HPV-positive HNCUP.

1.3.4 Benign conditions causing neck masses

Cancer must always be excluded when a patient present with a neck mass. There are, however, a number of benign conditions causing neck masses and especially conditions

causing cystic lesions on the neck can pose a challenge to distinguish from a cystic metastasis from a HNSCC (Katabi & Lewis, 2017)

In Table 2, a few benign conditions causing cystic lesions on the neck are mentioned and throughout the thesis branchial cleft cysts (BCC) will be discussed in more detail since this condition is well known to be particularly difficult to distinguish from cystic metastases (Gourin & Johnson, 2000). Notably, HPV-positive OPSCC neck metastases have been shown to frequently be cystic and thus pose a particular challenge to distinguish from BCCs, while HPV-negative OPSCC metastases rarely are cystic (O´Sullivan et al., 2015; Wenig, 2016). However, e.g. lymphomas, colloid nodular goiters and lymph nodes on the neck enlarged due to infection or sarcoidosis will not be discussed since they rarely are mistaken for a HNSCC.

BCCs are congenital embryological remnants suggested to originate from the branchial apparatus in the neck (Wenig, 2016). They often present as a painless mass located on the lateral neck near the mandibular angle, often debuting between 20 and 40 years of age (Katabi & Lewis, 2017). They are quite common, representing 20% of all neck cysts and 90% of cysts located on the lateral neck (Katabi & Lewis, 2017).

The cysts are often lined with non-stratified squamous cell epithelium, but ciliated respiratory epithelium may also occur. The cyst walls contain lymphoid tissue giving them the alternative name *lymphoepithelial cysts*. The cysts can be associated with fistulas with openings in the pharyngeal wall or tonsillar region (Wenig, 2016). However, whether BCCs or other benign neck masses can harbour HPV has not been studied extensively and this will be discussed later in the thesis.

Table 2. Examples of benign conditions that can cause cystic lesions on the neck (Katabi & Lewis, 2017).

Branchial cleft cysts
Thyroglossal duct cysts
Ranula, also called renention cysts or mucocele
Dermoid cysts
Teratoid cysts
Warthin’s tumours
Pleomorphic adenomas
Lymphangiomas

1.4 DIAGNOSTIC PROCEDURES OF HEAD AND NECK MASSES

1.4.1 Head and neck cancer including oropharyngeal squamous cell carcinoma

In head and neck cancer the first sign of the disease, which brings the patient to the doctor, is often a lymph node metastasis on the neck presenting as a neck mass (Nationellt vårdprogram

Huvud- och Halscancer, 2015). It can however also be due to symptoms such as pain, hoarseness, one-sided nasal congestion, difficulty swallowing or a mucosal wound that does not heal (O´Sullivan et al., 2015). The subsequent diagnostic workup includes e.g. fine-needle aspiration cytology (FNAC) of the neck mass, computed tomography (CT) of the head, neck & thorax, sometimes magnetic resonance imaging (MRI) of the head and neck, and tumour biopsies for a pathological anatomical diagnosis (PAD) if a primary tumour is evident. Together, these procedures reveal the site of the primary tumour in most cases and this is e.g. how OPSCC, including TSCC and BOTSCC are mainly diagnosed (Nationellt vårdprogram Huvud- och Halscancer, 2015). For OPSCC, HPV-analysis is often performed on FFPE biopsies, either using PCR for HPV DNA or analysing expression of the surrogate marker p16 using immunohistochemistry (O´Sullivan et al., 2015). Today however, some centres require the presence of both for defining positive HPV status (Näsman et al., 2013; Smeets et al., 2007).

1.4.2 Cancer of unknown primary in the head and neck region

In Sweden, the clinical investigation of HNCUP starts with the above-mentioned FNAC, CT and MRI. If no primary tumour is found the investigation continues. If the FNAC shows non-SCC, e.g. adenocarcinoma, a PET-CT is recommended, since the primary tumour is then likely not a head and neck cancer, and today a full-body PET-scan is sometimes performed. Furthermore, a panendoscopy of the upper aerodigestive tract, including blind biopsies from the nasopharynx and base of tongue is performed, as well as bilateral tonsillectomy (Nationellt vårdprogram Huvud- och Halscancer, 2015). Location of a SCC metastasis in level I or II of the neck gives rise to suspicion of OPSCC, while a metastasis located in the lower part of the neck more likely has a non-head and neck cancer as a primary (Nationellt vårdprogram Huvud- och Halscancer, 2015). Since HNCUP is a diagnosis made after all other diagnoses have been excluded, the HNCUP diagnosis is dependent of the clinical work-up that has been performed. The clinical investigation, e.g. if bilateral tonsillectomy (where TSCC may be detected) is performed, may vary between different centres, as well as different studies, and this influences the incidence of HNCUP, the proportion HPV-positive HNCUP as well as the clinical outcome in the studies (Boscolo-Rizzo et al., 2015).

1.4.3 Branchial cleft cysts

The clinical investigation of a suspected branchial cleft cyst is dependent on the age of the patient as the risk for head and neck cancer, which needs to be ruled out, increases with age. At the Karolinska University Hospital all patients receive a thorough clinical examination including palpation and fine-needle aspiration cytology (FNAC) on two occasions. For patients over 29 years of age the FNAC-analysis includes DNA-analysis, where aneuploidy indicates malignancy. If a tumour is suspected or apparent, panendoscopy including palpation and biopsies are performed. For patients over 40 years of age, a CT with intravenous contrast of the neck, thorax and base of skull is done, and in addition, if no tumour is evident during panendoscopy, bilateral tonsillectomy is performed and multiple biopsies are taken from the

base of tongue and epipharynx (Vårdprogram, Halscysta – Lateral., 2016). HPV-testing is infrequently performed on FNAC or surgical specimens (see Paper IV).

1.4.4 HPV-detection in fine-needle aspirates from neck masses

As mentioned above, fine-needle aspiration cytology (FNAC) of neck masses is an important step in the diagnostic work-up of a suspected HNSCC. The aspirate is smeared on a glass and examined using a light microscope. The sensitivity and specificity of this method depends on the quality of the aspirate and the smear and are improved if the procedure is guided by ultra sound (Nationellt vårdprogram Huvud- och Halscancer, 2015). Most often the method can distinguish between benign and malignant conditions, and between carcinomas, lymphomas and sarcomas, although the interpretation can be difficult at times and require specially trained personnel. The material obtained can also be used for molecular methods (Nationellt vårdprogram Huvud- och Halscancer, 2015). There are also liquid cytology methods available and detection of HPV using these methods can be employed in cervical cancer screening (Whitlock et al., 2011). However, FNAC is not used for the collection of material, since the uterine cervix is accessible for brushing and scraping.

Being able to reliably detect HPV in FNAC from neck masses would be desirable and has not been studied extensively (Faquin, 2014). This would facilitate improved diagnosis and prognosis making in a number of malignant and benign conditions in the head and neck region and may lessen the need for more invasive procedures (Faquin, 2014). This would be especially true for HNCUP since a neck dissection sometimes is performed to validate the result from cytology with histopathology. Also, as mentioned above, if the metastasis is HPV-positive, then the primary tumour is probably an HPV-positive TSCC or BOTSCC (Begum et al., 2007) and the patients likely have a good prognosis (Ang et al., 2010; Lindquist et al., 2007). It is possible that these patients may not need as intensive treatment as patients with HPV-negative HNCUP, in analogy to the de-escalation of treatment that has been proposed for HPV-positive TSCC/BOTSCC (Mirghani et al., 2015). If HPV-diagnosis in FNAC is shown to be reliable enough, these patients could potentially be spared some treatment and subsequent adverse effects.

There have been several studies investigating HPV-detection in FNAC from neck masses and they will be briefly mentioned below, for a more detailed review see Sivars et al. (2016). Unfortunately, most of the studies have been rather small and of retrospective character, using a variety of techniques (often ISH in the older studies), and a variety of starting material (smears or cell blocks, fresh frozen or FFPE) (Baldassarri et al., 2015; Barwad et al., 2012; Begum et al., 2007; Bishop et al., 2012; Guo et al., 2014; Jannapureddy et al., 2010; Kerr et al., 2014; Lastra et al., 2013; Smith et al., 2014; Solomides et al., 2012; Umudum et al., 2005; Zhang et al., 2008). Typically, the studies find that HPV-detection is feasible, but not perfect and that a finding of HPV can predict an OPSCC with a very good specificity of 90-100%, but only with an average sensitivity of 50-60% (Begum et al., 2007; Jannapureddy et al., 2010; Zhang et al., 2008;). There are also a few studies using commercially available methods for HPV-detection in gynaecological cytology samples, showing promise for the

analysis of FNAC samples as well, although these methods were not perfect either (Bishop et al., 2012; Guo et al., 2014; Lastra et al., 2013; Smith et al., 2014). Recently, in addition, two prospective studies have been performed using the Roche cobas 4800 system (Roche Molecular System, Pleasanton, CA) for HPV detection, showing >90% correlation of HPV-status between cytology and FFPE material (Baldassarri et al., 2015; Kerr et al., 2014), as well as being highly indicative of an OPSCC (Baldassarri et al., 2015).

To summarize, HPV detection in FNAC shows great promise, but there is at the moment no consensus of which method that is the best, neither concerning how to best prepare and store the sample until testing, nor how to best detect DNA (Faquin, 2014). Furthermore, in the above studies mostly patients with malignancies where examined. It would be interesting to test for HPV DNA in FNAC, as well as HPV mRNA, in a broader unselected cohort of patients with unknown neck masses. In this thesis we have attempted to do just this in Paper IV.

1.5 TREATMENT OF HEAD AND NECK MASSES

1.5.1 Head and neck cancer

Treatment of HNSCC varies depending on subsite, but can consist of surgery, radiotherapy or chemotherapy, alone or in combinations (O´Sullivan et al., 2015). In addition, the EGFR-inhibitor cetuximab is approved for use in HNSCC (O´Sullivan et al., 2015) and, very recently, immune checkpoint inhibitors have been approved in recurrent HNSCC (Ferris et al., 2017). Below, the treatment for OPSCC will described briefly, and the treatment for HNCUP will be described in greater detail.

1.5.2 Oropharyngeal squamous cell carcinomas

Radiotherapy (RT) in a curative dose is the main treatment option in Sweden today. Chemotherapy (cisplatin) or targeted therapy (cetuximab) is often added in advanced cases. Surgery can be applied in selected cases, or if there is tumour left on the neck after RT (Nationellt vårdprogram Huvud- och Halscancer, 2015). However, since HPV-positive TSCC and BOTSCC have much better clinical outcome than corresponding HPV-negative cancer, it has been suggested that treatment could be de-escalated for HPV-positive cancer (Mirghani et al., 2015). For this purpose, several studies have focused on finding additional predictive markers to use in conjunction with HPV, and in some studies, it has been shown e.g. that high CD8+ lymphocyte counts, and low or absent HLA class I expression was correlated to better prognosis in HPV-positive cancer (Nordfors et al., 2014; Näsman et al., 2013;).

1.5.3 Cancer of unknown primary in the head and neck region

Treatment of HNCUP varies between different centres and there is a lack of evidence for what treatment is optimal (Balaker et al., 2012). The main treatment options today consist of either neck dissection with post-operative radiotherapy or radiotherapy with or without chemo-/targeted therapy (Nationellt vårdprogram Huvud- och Halscancer, 2015). Neck dissection allows confirmation of the cytological diagnosis with histopathology. In a radical

neck dissection, all lymph nodes in levels I to V are removed as well as the sternocleidomastoid muscle, the internal jugular vein and the accessory nerve. Today, a modified radical neck dissection is often performed instead, which spares as many structures as possible depending on the anatomy of the individual patient. The radiotherapy consists of up to 68 Gy, in advanced cases with concomitant chemotherapy, most often cisplatin based. However, sometimes the EGFR-inhibitor cetuximab is used as an adjuvant instead. The radiotherapy often leads to severe side effects, e.g. mucositis with subsequent pain, difficulty swallowing and nutritional difficulties, as well as skin reactions, dry mouth and osteoradionecrosis. Surgery could lead to adverse effects such as pain, lymph oedema, nerve damage as well as cosmetic issues (Nationellt vårdprogram Huvud- och Halscancer, 2015).

If in fact an HPV-positive HNCUP originates from an HPV-positive OPSCC, then treatment could potentially be tapered for this group of tumours. Therefore, in Papers I and II, in addition to HPV status, CD8+ tumour infiltrating lymphocytes (TILs), HLA class I expression and p53 status were investigated in HNCUP in correlation to clinical outcome in order to find additional biomarkers.

1.5.4 Branchial cleft cysts

If nothing malignant is found during investigation (see above) and a branchial cleft cyst is the final diagnosis and the patient is below 40 years of age, the cyst is carefully extirpated avoiding it's braking. If the patient is 40 years old or above, a more extensive procedure is performed, where lymph nodes in the same neck region as the cyst are removed in addition to the cyst (Vårdprogram, Halscysta – Lateral, 2016). Patients with branchial cleft cysts thus clearly undergo extensive invasive procedures as part of the diagnostic procedure and treatment, with subsequent side effects, including risk for damage to the hypoglossal-, superior laryngeal-, vagal-, glossopharyngeal- and accessory nerves as well as to the jugular vein and carotid artery, if surgery is applied, despite having a benign condition (Vårdprogram, Halscysta – Lateral, 2016).

1.6 REFLECTIONS ON PERSONALIZED TREATMENT IN HNCUP, OPSCC AND BRANCHIAL CLEFT CYSTS

Cancer is not one disease, but many, and this applies also to HNC, including OPSCC and HNCUP. Therefore, all patients with a certain condition should not necessarily be treated the same way. For branchial cleft cysts, the scope of the diagnostic procedures and the optimal treatment for different age groups is still a matter of debate.

Notably, patients with OPSCC receive the same treatment, regardless of that patients with HPV-positive TSCC and BOTSCC have, as mentioned previously, a significantly better 5-year survival than patients with HPV-negative TSCC or BOTSCC. Despite this, they are treated similarly and the treatment for all TSCC and BOTSCC has furthermore been intensified in recent years. It is plausible that all patients with HPV-positive TSCC/BOTSCC

are not helped by this intensified approach and de-escalation of treatment for this patient group has therefore been suggested (Mirghani et al., 2015). There are currently several de-escalation trials ongoing (Mirghani et al., 2015). On the other hand, for TSCC/BOTSCC-patients with a poor prognosis there is need for new treatments as well as biomarkers to identify these patients.

For HNCUP there is, as already mentioned, a controversy as to if a neck dissection should be performed or not (Balaker et al., 2012) and biomarkers selecting patients that could benefit from having (or not having) the extensive surgical procedure would be welcome. If an HPV-positive HNCUP has a much better prognosis than an HPV-negative HNCUP, diagnosing HPV-positive status of the HNCUP could potentially be used to select a less intensive treatment, in analogy to the de-escalation of treatment proposed for HPV-positive TSCC/BOTSCC. However, one complicating matter for HNCUP is that it is such a rare entity making randomized controlled trials between different treatments difficult to perform.

In addition, biomarkers that could help find the likely site of the primary tumour could help steer further diagnostic procedures and treatment (e.g. RT) towards the suspected location, potentially sparing other locations. If HPV-positive HNCUP indeed originates from HPV-positive TSCC/BOTSCC, as has been suggested, HPV-positive status of an HNCUP could potentially steer investigation and therapy towards the oropharynx.

For patients with branchial cleft cysts there are other challenges. These patients undergo extensive invasive procedures as part of diagnostics and treatment in order to rule out an HNC. The major challenge here is that it is sometimes difficult to distinguish BCCs from cystic metastases. Today, if HPV is found in a cervical cyst it is assumed to be a metastasis from an HPV-positive TSCC/BOTSCC. However, whether HPV is found or not in other cervical cysts, e.g. BCCs, have not been studied extensively. If it can be ruled out that BCCs harbour HPV DNA, investigation of HPV-status would be of value in the clinical work-up of BCCs, potentially helping to discriminate between BCCs and cystic TSCC/BOTSCC metastases. Further studies would then be needed to evaluate if HPV-negative status (together with a clear benign cytology) in a BCC could potentially influence treatment.

To summarize, some treatments work well for some patients, but not as well for others. Personalized treatment would therefore be very welcome to avoid overtreatment leading to unnecessary side effects for some patients and under treatment of others. Still, to facilitate the realisation of personalised treatment in HNSCC, more knowledge is needed. In the next section, questions unanswered at the time of the initiation of this thesis project are presented. Since then, we have tried to answer some of these questions and these are presented in the papers of this thesis.

1.7 UNANSWERED QUESTIONS AT THE TIME WHEN THIS THESIS PROJECT WAS INITIATED

In 2013, when I as a medical student started working on the projects described in this thesis, the role of HPV in HNCUP had not been studied extensively. Although, HPV DNA had been found in HNCUP in a few studies (Compton et al., 2011; Park et al., 2012; Tribius et al., 2012), the prognostic implications of positive HPV-status were inconclusive: two studies had failed to show any prognostic effect of positive HPV-status (Compton et al., 2011; Tribius et al., 2012), while one did show a survival benefit (Park et al., 2012). The study by Park et al. (2012), however, included patients who had not undergone bilateral tonsillectomy, a procedure nowadays part of the standard clinical work-up of HNCUP, and which often leads to that a primary tumour is found – frequently an HPV-positive TSCC with a good prognosis. These patients would therefore not be classified as HNCUP today. Furthermore, the presence of HPV mRNA and the prognostic significance of additional biomarkers had not been studied extensively in HNCUP. For this purpose, we investigated these issues in Papers I and II.

Moreover, as mentioned above, the mutational landscape of HPV-positive HNCUP was largely unexplored and the prognostic significance of different mutations in OPSCC not fully elucidated. We analysed these two topics in Paper III.

Additionally, if presence of HPV DNA in FNAC from a cervical lymph node could accurately predict an HPV-positive TSCC or BOTSCC as final diagnosis in a broad and unselected cohort of patients with benign and malignant neck masses had not been studied prospectively. Such a study was therefore conducted and described in Paper IV.

Finally, if HPV DNA is present in other neck masses than metastases from HPV-positive TSCC/BOTSCC, e.g. in branchial cleft cysts, had not been investigated thoroughly, something we also examined in Paper IV.

2 AIMS

- To examine the prevalence of HPV DNA in HNCUP, and whether HPV DNA and/or p16-status, as well as p53-expression were correlated to clinical outcome. (Paper I)

- To validate our findings regarding HPV DNA, p16 and p53 from Paper I in a separate HNCUP cohort and also to analyse additional biomarkers: HPV16 mRNA, CD8+ tumour infiltrating lymphocytes (TILs), and HLA class I expression in relation to clinical outcome in the combined cohorts. (Paper II)

- To find prognostic markers in TSCC/BOTSCC, as well as to compare the presence of hot spot mutations in cancer related genes in HPV-positive TSCC/BOTSCC, HPV-negative TSCC/BOTSCC and HPV-positive HNCUP using targeted next generation sequencing. (Paper III)

- To investigate whether the detection of HPV DNA and HPV16 mRNA in FNAC was reliable, whether HPV detection in FNAC from a neck mass could prospectively predict an HPV-positive TSCC/BOTSCC as the final diagnosis or whether HPV DNA was present in benign neck masses, e.g. branchial cleft cysts, as well. (Paper IV)

3 STUDY SUBJECTS, MATERIAL AND METHODS

3.1 GENERAL INTRODUCTION TO METHODS OF HPV-DETECTION IN HNSCC

Detection of HPV in patient material can be done in numerous ways including detection of HPV DNA or RNA, viral proteins in the material, or detection of proteins that are upregulated by HPV. Detection of antibodies against HPV in serum can also be investigated. Which method that is the best is under debate and varies according to what material you have access to and whether you are interested on HPV infection on the individual or the population level (Näsman A, 2013; Venuti & Paolini, 2012).

Presently, detection of E6 and E7 mRNA in fresh-frozen tumour tissue is generally regarded as the golden standard (Smeets et al., 2007, Venuti & Paolini, 2012). Fresh-frozen tissue is however often not easily available, since it is impractical in routine clinical practise and therefore formalin-fixed paraffin-embedded (FFPE) material is often used instead. When working with FFPE material, HPV DNA can be analysed by several techniques including fluorescent in situ hybridization (FISH), a variety of different PCR-based techniques, or by sequencing, with the first two being the most common (Venuti & Paolini, 2012). However, just finding HPV DNA in the tumour does not automatically prove that the neoplasm is HPV-driven – it could be due to a transient infection or a false positive due to the high sensitivity of e.g. PCR (Smeets et al., 2007).

Overexpression of the p16-protein is often used a surrogate marker of HPV-driven carcinogenesis, since p16 is usually upregulated upon loss of pRb during HPV infection (Venuti & Paolini, 2012). Furthermore, p16 is often lost in other forms of cancer, including HPV-negative HNSCC, making p16 overexpression useful as a surrogate marker for HPV (Cancer Genome Atlas Network, 2015; Venuti & Paolini, 2012) Nevertheless, upregulation of p16 can still occur and around 14% of HPV DNA-negative OPSCC is also p16-positive (Lewis et al., 2010). It has therefore been proposed that initial immunohistochemistry (IHC) staining for p16 followed by HPV DNA analysis of the p16 positive tumours would be the best way of determining clinically relevant HPV infections (Smeets et al., 2007). Notably, combining p16 overexpression with presence of HPV DNA, showed an impressive 100% sensitivity and specificity when applying this algorithm compared to HPV E6 expression (Smeets et al., 2007) and the algorithm was later validated showing an accuracy of 98% (Rietbergen et al., 2013). However, HNSCC expressing HPV DNA and E6*1 mRNA, but lacking p16 overexpression, have also been described and would have been missed using this approach (Hoffman et al., 2012).

In the clinic, and in research studies, HPV DNA (by PCR or ISH) or p16 (by IHC) status are sometimes used alone when determining HPV-status, something that is not optimal, considering that there also are HPV-/p16+ and HPV+/p16- cases. Applying a conservative definition of HPV-positivity, i.e. requiring positivity for both HPV DNA and p16, is

especially important when selecting patients for de-escalating trials in order not to lessen the treatment for patients with poor prognosis. This routine could miss some patients according to Hoffman et al (2012), but it is still a safer approach than using HPV DNA or p16 expression alone. The definition of p16 overexpression, i.e. p16-positive, has varied, but for an indication of active HPV infection today a cut-off of >70% of the tumour cells presenting intense staining is mainly used (Ang et al., 2010; Lassen et al., 2012). It is worth noting that this cut-off has been set rather arbitrarily, but that this rarely poses a problem in OPSCC (according to the experience of others and us), since p16 almost always presents with either 100% or 0% of tumours cells being stained, making interpretations straight-forward (Lassen et al., 2012).

In this thesis, FFPE material was used for HPV DNA detection and p16 analysis in Papers I, II and III as well as HPV mRNA detection in Paper II and next generation sequencing (NGS) in Paper III, while fresh-frozen FNACs was used for HPV DNA and RNA detection in Paper IV. See below for further details.

3.2 STUDY SUBJECTS AND PATIENT MATERIAL

Below, study subjects and patient material will be briefly presented for each paper and then some considerations worth noting will be touched upon. For more details please see respective paper.

Paper I. Patients with HNCUP as defined by ICD-10-code C.77.0 diagnosed between 2000 and 2007 at the Karolinska University Hospital, Stockholm, Sweden with available FFPE-material for analysis were identified from hospital records and included in the study. Patients only given palliative treatment, or with histology other than SCC, or patients declining post-operative radiotherapy or lacking sufficient FFPE-material were excluded, leaving 50 patients in the study. Patients' data were obtained from medical records.

Paper II. All patients with HNCUP (with the same definition as in paper I, above) diagnosed at the Karolinska University Hospital, Stockholm, Sweden between 2008 and April 30th 2013 were identified through the Swedish Cancer Registry. Using the same inclusion- and exclusion criteria as mentioned for Paper I, this resulted in another 19 patients included in Paper II. These 19 patients were used for the validation of the effect of HPV DNA and p16 expression on survival, while the combined 2000-2013 cohorts of 69 patients were used for HPV mRNA analysis and the additional biomarker analysis performed.

Paper III. In a study on hotspot mutations of 50 cancer-related genes, all 20 HPV DNA-positive HNCUP samples from Paper I were included together with a total of 348 TSCC (ICD-10-code C09.0-9) and BOTSCC (ICD-10-code C01.9) diagnosed 2000-2011 at the Karolinska University Hospital. HPV DNA-, p16- and patient data for the included TSCC/BOTSCC were derived from previous studies, where FFPE-material had been used (Nordfors et al., 2014; Näsman et al., 2013; Ramqvist et al., 2015).

Paper IV. Patients undergoing FNAC for a suspected HNSCC/neck mass of unknown origin at the Karolinska University Hospital between 2013 and 2016 were prospectively included in the study. Sixty-six patients had enough material left after routine cytological diagnostic procedures had been performed. The left-over aspirates were fresh-frozen at -20°C and analysed as soon as possible.

Study subjects and patient material considerations: As noted previously, HNCUP is a difficult diagnosis to define. One example of this is that in four patients, included in Papers I and II, a suspected primary tumour was found during follow-up, years after the diagnosis was set. Would these classify as a true HNCUP? Some would argue not (Jensen et al., 2014). We however decided to include these patients in the studies, since we were interested in patients defined as having HNCUP at the time of diagnosis, when the physicians decide on the treatment. After all, physicians cannot, unfortunately, see into the future. The diagnoses of these four indicated patients have been commented on in the discussion parts of the papers.

In Paper IV HPV analysis on FNACs was performed. Over the course of two and half year a total of 66 samples were collected. Samples were collected prospectively when suitable for the collecting clinician and when enough material remained after routine clinical procedures. Therefore, obviously not all the patients that had a neck mass of unknown origin analysed by FNAC at the Karolinska University Hospital during the time period could be included, and a selection bias could have been introduced into the study.

3.3 METHODS

3.3.1 HPV DNA and RNA extraction

In Paper I DNA, and in Paper II DNA and RNA were extracted from FFPE cervical lymph nodes resected during neck dissection using the Roche High Pure RNA Paraffin Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions, but omitting the DNAse treatment. In Paper III DNA had been extracted previously from FFPE biopsies from TSCC and BOTSCC using the same method. In Paper IV DNA was extracted from fresh-frozen aspirates from neck masses using the QIAmp DNA micro kit (Qiagen, Hilden, Germany) and if the aspirate was HPV DNA-positive, RNA was extracted using the RNeasy Micro kit (Qiagen, Hilden, Germany).

Methodological considerations: The Roche High Pure RNA Paraffin Kit is optimized for RNA retrieval; however, it also works fine for extracting DNA and has been in use in our group for a long time (Nordfors et al., 2014; Näsman et al., 2013). As mentioned above, DNA quality in FFPE material is inferior to the DNA quality of fresh-frozen material but is more easily available. Nevertheless, HPV DNA detection from FFPE material is standard procedure at pathology laboratories. In Paper IV we had access to fresh-frozen aspirates. However, due to practical reasons at the clinic the samples were frozen and stored at -20°C and not -70°C. This may have affected our ability to detect HPV RNA. To check for cross

contamination between samples, in all studies, blanks were added and treated in same way as the samples.

3.3.2 HPV DNA and RNA detection

The method for HPV DNA-detection used in this thesis has been developed in Heidelberg, Germany by the Michael Pawlita group and consists of two parts, which will be briefly described below: first a multiplex PCR and then a Luminex bead-based assay for 27 HPV types (Schmitt et al., 2006; Schmitt et al., 2008). A similar method is applied for HPV RNA detection (Ramqvist et al., 2015).

For the HPV DNA PCR, the broad-spectrum general primers (BSGP) 5+/6+ primers that bind to the well-conserved L1 region of the HPV-genome were used. In addition, specific primers directed against HPV16 E6 and HPV33 E6, were added to detect the rare cases, where L1 had been deleted in tumours associated with these HPV-types. As an HPV-positive control, samples with DNA from SiHa cells, corresponding to around 1,10 and 100 genomes of HPV16 per 5µl respectively, were used, while specific primers of the housekeeping gene β-globin were used to control for presence of amplifiable cellular DNA. Finally, RNAse free water was used as negative control. 10 ng of extracted DNA was added for each reaction. See Schmitt et al., (2008), for the primers used. A 15 minutes denaturation step at 94°C was followed by 40 cycles of amplification. Each cycle consisted of a 20 second denaturation step at 94°C followed by a 90 second annealing step at 38°C and an 80 second elongation step at 71°C. The final elongation step was prolonged for 4 minutes (Nordfors et al., 2014).

For RNA detection in Papers II and IV, the samples were DNAse treated using the RNeasy MiniElute Cleanup kit (Qiagen, Hilden, Germany) if needed, and then cDNA was synthesized using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) with random hexamer primers used. A PCR was then run to amplify HPV16 E2, E5, E6*1, E6*2 and E7 mRNA. The housekeeping gene U1A was used to check for presence of amplifiable RNA and absence of β-globin was used to control that no DNA was still present in the sample. See Ramqvist et al. (2015) and Papers II and IV for more details and the specific primers used (Ramqvist et al., 2015).

The amplified DNA and RNA was then analysed using a bead-based assay on a Magpix instrument (Luminex Corp). The HPV DNA assay included 27 HPV types: HPV6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42-45, 51-53, 56, 58, 59, 66-70, 73 and 82, thus including all HR and putative HR HPV types and a number of LR types. The assay also included HPV16 and HPV33 E6 and β-globin. The RNA assay was more limited and included HPV16 E2, E5, E6*1, E6*2 and E7 mRNA, U1A and β-globin (Ramqvist et al., 2015).

The Magpix assay allows for simultaneous detection of up 50 different molecules in a single sample. Briefly, magnetic beads with different colours are coupled to probes specific for the molecules to be measured. Here, e.g. DNA of 27 different HPV-types was coupled to beads with 27 different colours. The previously generated PCR-products were denatured and hybridized to the bead-probe complex. Unhybridized DNA was then washed away and the

PCR-products stained with a flourescent reporter dye. After another washing step, the beads were analysed by two lasers in the Luminex reader. More specifically, one laser evaluates the colour of the beads determining which HPV-types are present, while the other laser detects the reporter dye and semi-quantifies the amount of HPV DNA (or mRNA). The result is given as median fluorescence intensity (MFI) (Schmitt et al., 2006).

In this thesis, to exclude false positive signals and cases where the HPV DNA concentration was clearly too low to be causative of HNSCC, samples were considered positive if the MFI – (1.5xbackground +15) was above 100.

Methodological considerations: The BSGP 5+/6+ primers are a development from the GP 5+/6+ primers (Schmitt et al., 2008). The original GP5+/6+ primers have good sensitivity for e.g. HPV16 (approximately 10 copies needed), but considerably weaker sensitivity for other HPV types, e.g. 10 000 – 10 0000 genome copies would be needed for detection of certain HPV-types (Schmitt et al., 2006). By using the developed broad-spectrum GP 5+/6+ primers the sensitivity is more equal among the HPV types included in the assay, although still somewhat variable (Schmitt et al., 2008). Nevertheless, since HPV16, for which the sensitivity is extremely good, is the clearly dominating type in HNSCC, this did likely not pose a problem for the studies included in this thesis. Furthermore, the BSGP 5+/6+ primers are also a good choice when working with FFPE material, which often contain fragmented DNA, since they result in a shorter product (around 150 bp) than e.g. the PGMY9/PGMY11 primers, which give a product of around 450bp (Venuti & Paolini, 2012). Finally, the Magpix assay allows for simultaneous detection of many HPV-types (or mRNAs) in a single sample, making it a time saving and rather affordable method when working with large sets of samples (Schmitt et al., 2006).

3.3.3 Immunohistochemistry (IHC)

The standard IHC procedure applied in this thesis consisted of 4µm FFPE sections initially de-paraffinized and re-hydrated followed by antigen-retrieval in citrate buffer in a microwave oven. Thereafter were endogenous peroxidase activity quenched using hydrogen peroxide and slides were treated with horse serum to block unspecific binding sites. Thereafter, the slides were ready to be incubated with the primary antibody (see below). After incubation with the first antibody, a biotinylated secondary anti-mouse antibody was applied (dilution 1:200, Vector Laboratories, Burlingame, USA) followed by incubation with an avidin-biotin-complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, USA). The slides were then developed in DAB (Vector Laboratories, Burlingame, USA) and counterstained using haematoxylin. All evaluations were performed using light microscopy with researchers blinded to clinical outcome.

p16

In Paper I the primary antibody p16INK4a (clone: JC8, dilution 1:100, Santa Cruz Biotech, Dallas, USA) was applied overnight at 8°C to study p16 expression. This antibody had previously been used for the p16 data included in Paper III. However, when the study in

Paper II was initiated this antibody was no longer available, and instead the antibody from the Ventana Cintec p16 Histology kit (Roche AB, Stockholm, Sweden) was incubated with the sample undiluted (as it was ready to use) for 30 minutes at room temperature. In Paper IV, p16 data was collected from patient records if available. Slides were considered p16-positive if >70% of tumour cells exhibited strong staining in all papers (Ang et al., 2010; Lassen et al., 2012).

p53

In Papers I and II the mAb p53 (clone: DO-1, dilution 1:100, Santa Cruz Biotech, Dallas, USA) that recognises both wild-type and mutant p53 was applied overnight at 8°C to study p53 expression. The percentage of tumour cells stained was then estimated (Yemelyanova et al., 2011).

CD8+ tumour infiltrating lymphocytes (TILs)

In Paper II the mAb CD8 (clone: 4B11, dilution 1:40, Leica Biosystems, Newcastle, UK) was used overnight at 8°C to study the number of CD8+ TILs. Two researchers independently counted the number of stained lymphocytes in five randomly selected representative 40x high power magnification fields of tumour tissue. The average number of the 10 observations was then used to divide the patients into quartiles (Nordfors et al., 2014).

HLA Class I expression

To study HLA Class I expression in the tumours in Paper II the mAb HC10/HLA Class I (dilution 1:40, a kind gift from Dr Soldano Ferrone at the University of Pittsburgh, PA, USA) was applied overnight at 8°C. The tumour tissue in the samples was then scored to have absent staining, weak staining intensity (compared to surrounding normal tissue) or high staining intensity (the same intensity as surrounding normal tissue) (Näsman et al., 2013).

Methodological considerations. The difficulty with IHC does not lie so much in the method itself, but in the interpretation of the staining achieved. Inter- and intra-observer variability can be considerable.

For p16 however, this is rarely a problem since, as mentioned above, typically all or none of the tumour cells show strong staining (Lassen et al., 2012). Furthermore, notably between Paper I and Paper II, the antibody used to analyse p16 expression differed, since the first antibody no longer was commercially available. This however did not likely affect the data in a major way, since only modest differences were observed between the two antibodies for p16 detection in a recent study (Shelton et al., 2017).

The counting of CD8+ TILs is tedious, but in general straightforward. However, a few tumours show marked intra-tumour heterogeneity regarding the number of CD8+ TILs in different areas of the tumour and can be more difficult to evaluate.

P53 staining and HLA Class I expression are somewhat more difficult to interpret, especially given intra-tumour heterogeneity. To minimize errors, all IHC analysis were performed independently by two researchers who had to agree to a unanimous decision, otherwise the sample was analysed again.

It is also worth noting that p53 analysis by IHC is tricky, since the functioning antibodies used cannot distinguish between mutant and wild-type p53. An overexpression could thus consist of either mutant, non-functional p53 (which would presumably be bad for the outcome) or of functional p53 (which would presumably be good), perhaps creating a mixed population among the group showing high p53 expression. Moreover, tumours with p53 mutations have in some cases been shown to display a complete absence of p53 staining upon IHC examination, further complicating p53 analysis by IHC (Yemelyanova., 2011).

3.3.4 Sequencing

In Paper III, targeted next generation sequencing was performed on OPSCC and HPV-positive HNCUP. The method is better described elsewhere, e.g. in Paper III and will only be summarily described here. Briefly, the Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific, Waltham, USA) was used to amplify by PCR mutation hotspots in 50 genes commonly mutated in cancer, covering around 2800 hotspots described in the Cosmic database. See Table 3 for the genes included. Sequencing was performed on an Ion Proton benchtop sequencing platform (Thermo Fisher Scientific, Waltham, USA) and the Torrent Suite Software (Thermo Fisher Scientific, Waltham, USA) was used for variant calling. Samples with poor DNA quality, germline mutations, and variants with projected low impact were excluded, see Paper III for details.

Methodological considerations: Performing NGS on FFPE material is not ideal since formalin fixing may lead to fragmentation of DNA and thus poor DNA quality. For HPV DNA detection this does not pose a problem, but for sequencing it can, especially when performing whole genome sequencing with large amplicons. Furthermore, deamination artefacts leading to false positives can occur with FFPE-material (Moorcraft et al., 2015). In Paper III, however targeted sequencing was performed, which works better with fragmented DNA, since the amplicons are shorter. Internal controls were also used to check the DNA quality. Furthermore, the method used has previously been documented to work well with FFPE material (de Leng et al., 2016). Nonetheless, a few mutations that might have been detected if using fresh-frozen material could have been missed.

Table 3. Genes included in the Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific, Waltham, USA).

ABL1	EGFR	GNAS	KRAS	PTPN11
AKT1	ERBB2	GNAQ	MET	RB1
ALK	ERBB4	HNF1A	MLH1	RET
APC	EZH2	HRAS	MPL	SMAD4
ATM	FBXW7	IDH1	NOTCH1	SMARCB1
BRAF	FGFR1	JAK2	NPM1	SMO
CDH1	FGFR2	JAK3	NRAS	SRC
CDKN2A	FGFR3	IDH2	PDGFRA	STK11
CSF1R	FLT3	KDR	PIK3CA	TP53
CTNNB1	GNA11	KIT	PTEN	VHL

3.3.5 Statistical methods

In this thesis, the Kaplan-Meier method was used to generate survival curves and to calculate overall survival (OS) and disease-free survival (DFS) with the significance of the differences in survival between groups calculated using the Log-rank test. Hazard Ratios were calculated using uni- and multivariate Cox regression. Chi-square or Fisher's exact test was used for calculating the significance of differences between categorical variables, while an independent t-test was applied for age differences between two groups. Calculations were performed using SPSS software (IBM, Armonk, NY, USA). Two-sided p-values were reported and p-values below 0.05 were considered as statistically significant.

4 RESULTS AND DISCUSSION

4.1 HUMAN PAPILLOMAVIRUS AND P53 EXPRESSION IN CANCER OF UNKNOWN PRIMARY IN THE HEAD AND NECK REGION IN RELATION TO CLINICAL OUTCOME. (PAPER I)

Aim

In Paper I we aimed to examine the prevalence of HPV DNA in HNCUP, and whether HPV DNA and/or p16-status as well as p53-expression correlated to clinical outcome.

Material and methods in brief

Fifty lymph node metastases from patients diagnosed with HNCUP at the Karolinska University Hospital between 2000 and 2007 were analysed for HPV DNA, using a bead-based assay and for p16- and p53-expression using immunohistochemistry, see Paper I for details.

Main results

HPV DNA was detected in 40% of the cases. Patients with HPV DNA-positive HNCUP had significantly better 5-year overall survival (OS) compared to patients with HPV DNA-negative HNCUP (80.0% vs. 36.7%, $p = 0.004$). Similarly, p16-positive HNCUP had significantly better 5-year OS than p16-negative HNCUP (76.2% vs. 37.9%, $p = 0.007$) and HPV DNA+/p16+ HNCUP had significantly better outcome compared to HNCUP that were either HPV DNA or p16-negative (77.8% vs. 40.6% 5-year OS, $p = 0.017$). Having absent/intermediary-low p53-expression in the tumour tissue correlated to a better 5-year OS as compared to having a tumour with high p53-expression (69% vs. 14%, $p < 0.001$).

Discussion

That HPV DNA was found in HNCUP was expected since some HNCUP likely originates from HPV-positive TSCC/BOTSCC, however the proportion of HPV-positive cases had not been examined in this region before. In a recent systematic review on HPV-prevalence in HNCUP, our study had the highest proportion HPV+/p16+ cases among "true definite" HNCUP (Boscolo-Rizzo et al., 2015). This could be explained by that Sweden has a larger proportion HPV-positive TSCC/BOTSCC than many other countries (Näsman et al., 2015; Rietbergen et al., 2013; Tahtali et al., 2013). In addition, HPV DNA and overexpression of p16 correlated to a high degree, as has been shown for TSCC/BOTSCC (Ndiaye et al., 2014).

Furthermore, to our knowledge, we showed for the first time that HPV DNA and overexpression of p16 are favourable prognostic factors in a "true definite" HNCUP setting. Previous studies on the subject either failed to reach significance (Compton et al., 2011; Tribius et al., 2012) or included patients were a primary tumour was found during examination or patients that would not be considered fully investigated today (e.g. not having

had tonsillectomy) (Park et al., 2012; Vent et al., 2013). Subsequently, others and we have confirmed that HPV DNA and/or p16 overexpression are favourable prognostic factors in HNCUP (Axelsson et al., 2017; Dixon et al., 2016; Jensen et al., 2014; Keller et al., 2014; Schroeder et al., 2017; Paper II). Thus, accumulating evidence suggests that HPV status is of prognostic importance not in only in TSCC/BOTSCC, but in HNCUP as well, which further strengthens the thesis that HPV-positive HNCUP originates from HPV-positive TSCC/BOTSCC.

In this study, p53 expression was analysed by IHC, and as mentioned above, p53 is often degraded and rarely mutated in HPV-positive cancers, while it is often mutated in many other types of cancer, and then often correlated to poor prognosis (Yemelyanova et al., 2011). It is therefore not surprising that HNCUP with low p53-expression in this study had better prognosis than HNCUP with high p53-expression. However, unexpectedly, only a trend and not a statistically significant correlation between HPV-status and p53-expression was observed ($p = 0.118$). Furthermore, p53 was an even better prognostic marker than HPV in this study indicating that it may have an independent prognostic effect in HNCUP, regardless of HPV-status and that these markers could potentially be used together. HPV-positive HNCUP with low p53 expression showed a tendency towards having the best prognosis and HPV-negative HNCUP with high p53 expression having the worst, but the number of patients was limited and further studies would be of importance. One such study has since been published (Yildirim et al., 2017) showing statistically significant worse 2-year tumour specific survival (TSS) in multivariate analysis for p16 negative/p53 positive HNCUP compared to all other p16/p53 combinations grouped together (defining p53-positive as >10% of tumour cells stained). Yildirim et al. (2017) did however not find any significant differences on TSS between p16-negative and p16-positive HNCUP, or between p53-negative and p53-positive HNCUP alone. However, this is another small, retrospective study and it would be most interesting to see results from a larger, prospective study on this subject.

Conclusion

Our data suggest that HPV-status should be investigated during the diagnostic work-up of HNCUP, since it provides valuable prognostic information and since a finding of HPV could steer further diagnostic procedures and potentially treatment towards the oropharynx. Moreover, patients with HPV-positive HNCUP could possibly receive less treatment than today, e.g. by omitting neck dissection, in analogy to the de-escalation of treatment proposed for HPV-positive TSCC/BOTSCC. Today, the work by us and others have led e.g. to that HPV and p16-status are now listed as “essential biomarkers” for the investigation of HNCUP in the UICC Manual of Clinical Oncology (O’Sullivan et al., 2015) and that according to the guidelines from the College of American Pathologists, HPV-testing should be performed in a SCC of unknown primary, if it is located in a cervical upper or mid jugular chain lymph node (Lewis et al., 2017).

4.2 VALIDATION OF HUMAN PAPILLOMAVIRUS AS A FAVOURABLE PROGNOSTIC MARKER AND ANALYSIS OF CD8+ TUMOUR-INFILTRATING LYMPHOCYTES AND OTHER BIOMARKERS IN CANCER OF UNKNOWN PRIMARY IN THE HEAD AND NECK REGION. (PAPER II)

Aim

In Paper II we aimed to validate our findings regarding HPV DNA, p16 and p53 from Paper I in a separate HNCUP cohort, and also to analyse additional biomarkers: HPV16 mRNA, CD8+ tumour infiltrating lymphocytes (TILs) and HLA class I expression in relation to clinical outcome in the combined cohorts.

Materials and methods in brief

Nineteen HNCUP patients diagnosed at the Karolinska University Hospital between 2008 and 2013 were used to study HPV DNA, p16 and p53. In addition, these 19 HNCUP together with the 50 HNCUP from Paper I were used for analysis of HPV16 mRNA, CD8+ TILs and HLA Class I expression. A bead-based multiplex assay was used for HPV DNA and mRNA analysis and IHC for the other markers (for more details see paper II).

Main results

Of the 19 HNCUP diagnosed 2008-2013, 63% were HPV DNA-positive. Patients with HPV DNA-positive HNCUP had significantly better 3-year OS and DFS compared to those with HPV-negative HNCUP (91.7% vs. 42.9%, $p = 0.028$ for OS and 100% vs. 66.7%, $p = 0.045$ for DFS). Similar trends were observed when comparing HPV DNA+/p16+ HNCUP with HPV DNA-/p16- HNCUP and when comparing p16+ HNCUP with p16- HNCUP, however reaching significance only for OS in the former comparison. In the entire 2000-2013 cohort, HPV mRNA evaluation was possible in 86% of the HPV16 DNA-positive cases, of which 92% were positive for HPV16 E6 and E7 mRNA. HNCUP in the three quartiles with the highest numbers of CD8+ TILs had significantly better 3-year OS and DFS compared to HNCUP in the quartile with the lowest number of CD8+ TILs. However, when dividing the cohort according to HPV-status comparing quartiles in the same manner, significance was reached only for 3-year DFS among patients with HPV-negative HNCUP. No significant differences in OS or DFS were observed when comparing absent/weak vs. high HLA Class I expression.

Discussion

Despite only 19 patients in the 2008-2013 cohort, we managed to validate HPV DNA as a favourable prognostic factor in HNCUP. This shows the profound effect HPV-positive status has on clinical outcome. Remarkably, 3-year DFS was 100% among patients with HPV DNA-positive HNCUP, although one must of course note that this group consisted of only 11 patients. Moreover, considering the entire 2000-2013 cohort, resulting in one of the largest HNCUP cohorts to date, 3-year DFS was 90.6% and 3-year OS 84.8% among HPV-positive HNCUP, which is similar, possibly slightly better, than that observed for HPV-positive

TSCC/BOTSCC in Sweden (Attner et al., 2011; Lindqvist et al., 2007). Furthermore, our data are in the same range as in other recent large studies on survival in HNCUP (Jensen et al., 2014; Schroeder et al., 2017). This further highlights the importance of testing for HPV in HNCUP.

HPV16 E6 and/or E7 RNA have to our knowledge only been investigated in HNCUP in two small previous studies (Bishop et al., 2012; Bussu et al., 2014), finding HPV mRNA in 2 and 10 cases respectively. In the 2000-2013 cohort, these data were confirmed this for the first time in a larger study, by the detection of HPV E6 and E7 mRNA in the great majority (92%) of the HPV16 DNA positive HNCUP. This is of importance, since a finding of HPV DNA in a tumour does not necessarily imply that it is caused by HPV, since it could also be due to a transient infection. The finding of HPV E6 and E7 mRNA, indicates transcriptionally active HPV, and supports the carcinogenic role of HPV in HNCUP. Subsequently, yet another study, has confirmed that HPV E6*I mRNA is indeed found in a proportion of HNCUP, further cementing HPVs role in the carcinogenesis of this disease (Schroeder at al., 2017).

When not taking HPV-status into consideration, we found that HNCUP with very few CD8+ TILs, indicating an insufficient immune response to the tumour, had poor prognosis, which is in line with what has been shown before in a variety of cancer types, including ovarian-, and colorectal cancer as well as TSCC/BOTSCC (Galon et al., 2006; Nordfors et al., 2014; Sato et al., 2005). However, having a high number of CD8+ TILs correlated to being HPV DNA+/p16+, not surprising giving the viral component of the disease, making it difficult to draw any conclusion regarding the separate effect of the lack of CD8+ TILs. Notably, when we divided the cohort according to HPV-status we did not find a prognostic impact of the number of TILs in the HPV-positive group, as previously seen in HPV+ TSCC/BOTSCC (Nordfors et al., 2014). This could partly be due to the excellent prognosis among HPV+ HNCUP patients, with very few events and thus requiring a large cohort to show a statistically significant difference. We conclude that in order to investigate the possibility to use CD8+ TIL counts as a prognostic factor in conjunction to HPV-status in HNCUP, larger, preferably multicentre, studies need to be performed.

The Dalianis group have previously showed that in HPV-positive TSCC/BOTSCC, having absent/weak HLA class I expression, somewhat counter intuitive, conferred a survival benefit as compared to having a high degree of HLA class I expression (Näsman et al., 2013). In this study however, absent/weak HLA class I expression was not correlated to clinical outcome in any group. This could be due the small cohort and few events, but it could of course also be due to that HNCUP differs from TSCC/BOTSCC in this aspect. It would not be surprising if HPV-positive HNCUP differs from HPV-positive TSCC/BOTSCC in some aspects, since we are comparing metastases to primary tumours. This is also emphasized by some data in Paper III.

In the 2008-2013 cohort, having absent/low p53 expression correlated to HPV DNA-positive status. Given this and the fact that the cohort consisted of only 19 patients with very few events, a separate role of p53 expression on survival was not pursued. Likewise, the effect on

prognosis of a lack of HPV E2 mRNA, previously showed to be a negative prognostic factor in HPV-positive TSCC/BOTSCC (Ramqvist et al., 2015), could not be investigated, since only three HPV16 DNA positive HNCUP lacked HPV16 E2 mRNA.

Conclusion

The obtained data further strengthen the role of HPV as an important prognostic factor in HNCUP and our emphasis on that investigation of HPV-status should be part of the diagnostic work-up in HNCUP (see also the discussion for Paper I). Moreover, our data further reinforce the thesis that HPV-positive HNCUP arises from HPV-positive TSCC/BOTSCC, since both display transcriptionally active HPV (as determined by HPV E6 and E7 expression) and exhibit an excellent clinical outcome. This further highlights the potential of HPV-positive status to influence treatment. However, to disclose other prognostic biomarkers possible to use together with HPV-positive status to further improve identification of patients with an excellent outcome, larger cohorts will have to be investigated. Due to the rareness of HPV-positive HNCUP and its excellent prognosis, multicentre studies would be preferable.

4.3 TARGETED SEQUENCING OF TONSILLAR AND BASE OF TONGUE CANCER AND HUMAN PAPILLOMAVIRUS POSITIVE UNKNOWN PRIMARY OF THE HEAD AND NECK REVEALS PROGNOSTIC EFFECTS OF MUTATED FGFR3. (PAPER III)

Aim

In Paper III we aimed to find prognostic markers in TSCC/BOTSCC, as well as to compare the presence of hot spot mutations in cancer related genes in HPV-positive TSCC/BOTSCC, HPV-negative TSCC/BOTSCC and HPV-positive HNCUP using targeted next generation sequencing (NGS).

Material and Methods in brief

Targeted NGS was performed on DNA from 348 TSCC/BOTSCC diagnosed 2000-2011 and the 20 HPV DNA-positive HNCUP from Paper I. The Ion AmpliSeq Cancer Hotspot Panel v2, covering mutations in frequently altered regions in 50 genes commonly mutated in cancer, was used on the Ion Proton sequencing platform (see Paper III for details).

Main results

HPV-positive TSCC/BOTSCC contained significantly fewer mutations/tumour than HPV-negative TSCC/BOTSCC (0.92 vs. 1.68), with HPV-positive HNCUP presenting an intermediate of 1.32 mutations/tumour. The most commonly mutated genes were in HPV-positive TSCC/BOTSCC: PIK3CA (20.1%), TP53 (9.3%) and FGFR3 (7.2%) and in HPV-negative TSCC/BOTSCC: TP53 (63.8%), PIK3CA (6.4%) and IDH2 (6.4%). In HPV-positive HNCUP, TP53 (26.3%), CDKN2A (15.8%) and PIK3CA (15.8%) were the most

frequently mutated genes. TP53, IDH2 and NOTCH1 were significantly more often mutated in HPV-negative TSCC/BOTSCC compared to in HPV-positive TSCC/BOTSCC, while PIK3CA was more often mutated in HPV-positive TSCC/BOTSCC than its negative counterpart. For HPV-positive HNCUP, TP53 was significantly more often mutated than in HPV-positive TSCC/BOTSCC, but significantly less often mutated than in HPV-negative TSCC/BOTSCC.

Patients with HPV-positive TSCC/BOTSCC had significantly worse 3-year DFS if they had a mutation in FGFR3 compared to if they had wild type FGFR3 ($p = 0.002$). The most common FGFR3 variant was S249C (11 of 19 cases). When comparing this variant to patients with other FGFR3 variants or wild type FGFR3, patients with the S249C variant had a significantly worse 3-year DFS ($p = 0.009$). One FGFR3 mutation was found for HPV-negative TSCC/BOTSCC, while no FGFR3 mutations were found for HPV-positive HNCUP.

Discussion

That PIK3CA was the most frequently mutated gene in HPV-positive TSCC/BOTSCC, and that TP53 was the most commonly mutated gene in HPV-negative TSCC/BOTSCC and more often mutated there than in HPV-positive TSCC/BOTSCC are in line with previous studies (Cancer Genome Atlas Network, 2015; Chung et al., 2015; Lechner et al., 2013; Seiwert et al., 2015; Tinhofer et al., 2016).

Notably, FGFR3 mutations, including the S249C variant, have also been shown in HNSCC, but to our knowledge this is the first time they have been correlated to a worse prognosis in HPV-positive TSCC/BOTSCC (Chung et al., 2015; Lechner et al., 2013; Seiwert et al., 2015; Tinhofer et al., 2016). Interestingly, FGFR3 are potentially targetable by drugs, making FGFR3 an intriguing subject for further studies (Gust et al., 2013; Miyake et al., 2010).

To our knowledge, this is the very first study investigating mutations in HPV-positive HNCUP. Since the cohort consisted of only 19 patients, the results need, of course, to be interpreted with some caution, but at least this study offers a first glimpse into the mutational landscape of HPV-positive HNCUP. Interestingly, when analysing our data, HPV-positive HNCUP appears to be an intermediate group compared to HPV-positive and HPV-negative TSCC/BOTSCC in some features.

The mutation rate per tumour (1.32), was as mentioned above, in between HPV-positive TSCC/BOTSCC (0.92) and HPV-negative TSCC/BOTSCC (1.68). Likewise, the mutation rate of TP53 was higher in HPV-positive HNCUP (26.3%) than in HPV-positive TSCC/BOTSCC (9.3%), but much lower than in HPV- negative TSCC/BOTSCC (63.8%). However, HPV-positive HNCUP was more similar to HPV-positive TSCC/BOTSCC than to HPV-negative TSCC/BOTSCC with regard to some other characteristics. More specifically, 47.4% of the HPV-positive HNCUP contained variants, similar to 48.7% in HPV-positive TSCC/BOTSCC, both much less than the 74.5% variants observed in HPV-negative

TSCC/BOTSCC. Furthermore, HPV-positive HNCUP exhibited similar mutation rate of PIK3CA as HPV-positive TSCC/BOTSCC (15.8% vs. 20.1% respectively), which is more than in HPV-negative TSCC/BOTSCC (6.4%). However, HPV-positive HNCUP exhibited a higher mutational rate of CDKN2A than both HPV-positive and HPV-negative TSCC/BOTSCC (15.8% vs. 4.3% vs. 4.3% respectively).

There are limitations to this study, and a major one is that the number of variants noted were very few, e.g. only three HPV-positive HNCUP had PIK3CA or CDKN2A variants respectively, and it is therefore difficult to draw any conclusion of these findings as they need to be verified in larger cohorts. Furthermore, in this study, it is worth pointing out that only 50 genes were examined, and in these 50 genes, only the most commonly mutated regions were covered, i.e. hot spots. Thus, certain mutations might have been missed. However, carrying out whole genome (or whole exome) sequencing on such a large cohort would have required significantly more resources (e.g. financial and bioinformatical) than available.

Our data show some similarities between HPV-positive HNCUP and HPV-positive TSCC/BOTSCC and strengthen the thesis that HPV-positive HNCUP originates from HPV-positive TSCC/BOTSCC. In other aspects however, there are differences, which is to be expected, since we in this study compare metastases with primary tumours (see also Paper II). Another potential reason for the differences observed between HPV-positive HNCUP and HPV-positive TSCC/BOTSCC could be the higher frequency of smokers in the HPV-positive HNCUP cohort than in the HPV-positive TSCC/BOTSCC cohort, since smoking is known to cause mutations in e.g. TP53 (Brennan et al., 1995). It could therefore potentially be of interest to compare HPV-positive HNCUP to metastases from HPV-positive TSCC/BOTSCC, with a similar proportion of smokers between the two groups to disclose possible similarities and differences. Sequencing HPV-negative HNCUP and metastases from HPV-negative TSCC/BOTSCC could add further value to such a study, exploring the biology of HNCUP.

Conclusion

Our study largely confirms the genetic landscape described before for HPV-positive and HPV-negative TSCC/BOTSCC. Notably, we found that FGFR3 mutations (especially the S249C variant) as promising prognostic factors in HPV-positive TSCC/BOTSCC, predicting worse outcome. Since FGFR3 is a potential drug target, this could be of importance for implementing personalised medicine for HPV-positive TSCC/BOTSCC in the future and thus warrants further studies. Moreover, this study offers a first glimpse into the genetics of HPV-positive HNCUP with TP53, PIK3CA and CDKN2A as the most frequently mutated genes. HPV-positive HNCUP exhibited several genetic similarities to HPV-positive TSCC/BOTSCC, but also a few differences. Whether HPV-positive HNCUP should be considered as metastases from HPV-positive TSCC/BOTSCC with additional modifications needs to be studied further in larger cohorts, although data from Papers I-III suggest that this is the case.

4.4 HUMAN PAPILLOMAVIRUS DNA DETECTION IN FINE-NEEDLE ASPIRATES AS INDICATOR OF HUMAN PAPILLOMAVIRUS-POSITIVE OROPHARYNGEAL SQUAMOUS CELL CARCINOMA: A PROSPECTIVE STUDY. (PAPER IV)

Aim

In Paper IV we aimed to investigate whether HPV DNA and HPV16 mRNA detection in fine-needle aspirate cytology (FNAC) was reliable and whether HPV detection in FNAC from a neck mass could prospectively predict an HPV-positive TSCC/BOTSCC as the final diagnosis.

Material and methods in brief

FNACs from 66 patients with enlarged neck masses were prospectively analysed for HPV DNA and HPV16 mRNA (if HPV16 positive) using a bead-based multiplex assay. Results were correlated to final diagnosis and HPV-status from histopathological specimens (if available).

Main results

All 66 FNACs contained enough material for DNA analysis and 17/66 FNACs contained HPV16 DNA. No other HPV types were detected. For all 17 patients with an HPV-positive FNAC, the final diagnosis was an HPV-positive TSCC or BOTSCC. Three FNACs from OPSCC were HPV DNA-negative – two TSCC and one OPSCC originating from the back wall of the oropharynx. All 17 malignant non-OPSCC cases were HPV DNA negative and this was true also for the 29 cases of benign conditions, of which 18 were branchial cleft cysts. When available, HPV status of corresponding histopathological specimens showed perfect concordance to the HPV status from the FNACs. HPV16 E7 and E6*I mRNA were detected in all 7 HPV16 DNA-positive samples that were possible to evaluate for mRNA.

Discussion

In this study, we demonstrate that a finding of HPV DNA in FNACs prospectively collected from enlarged neck masses could predict an HPV-positive TSCC/BOTSCC as the final diagnosis. Notably, the specificity and sensitivity for predicting an HPV+ OPSCC/TSCC/BOTSCC was 100% and the presence of HPV DNA was not observed in any other malignant or benign conditions. These data are to our knowledge, the first of their kind in a prospective setup including an unselected cohort of patients with both malignant and benign conditions. In a larger study, it is possible that the specificity and the sensitivity would not have been 100% since a small proportion of HNSCC other than OPSCC are also HPV-positive (Plummer et al., 2016). Furthermore, it has been shown when evaluating DNA from FNACs on glass slides with low cellularity that it is not always possible to evaluate the presence of HPV DNA optimally (Channir et al., 2016). Nevertheless, our data are in line with previous studies, generally showing that HPV-detection in FNAC is feasible and that a finding of HPV DNA indicates an OPSCC with a very good specificity of 90-100%

(Baldassarri et al., 2015; Barwad et al., 2012; Begum et al., 2007; Bishop et al., 2012; Guo et al., 2014; Jannapureddy et al., 2010; Kerr et al., 2014; Lastra et al., 2013; Smith et al., 2014; Solomides et al., 2012; Umudum et al., 2005; Zhang et al., 2008).

Importantly though, our study included not only verified HNSCC, but benign conditions as well. As mentioned above, if HPV is found in a cervical lymph node metastasis it is assumed to be an HPV-positive OPSCC. However, whether HPV can be found in other, benign, conditions causing neck masses has not been extensively studied. Should this be the case then this could potentially lead to overtreatment of these benign conditions if they indeed harbour HPV. In this study, all 29 patients with benign conditions had HPV-negative FNACs, including the 18 branchial cleft cysts (BCC). The latter is a condition of special interest since BCCs are sometimes very difficult to distinguish from cystic metastases from HPV-positive OPSCC (see introduction). BCCs are furthermore sometimes associated with fistulas originating in the tonsils – a possible point of entry to the cyst for viruses. However, in this study, all BCCs were HPV-negative, although, of course, 18 cases are too few to conclude that BCCs can never be HPV-positive and this should be, and has in fact, been investigated further by us.

For optimal diagnosis of an HPV-positive TSCC/BOTSCC, we and others, have suggested that the presence of an HPV DNA-positive result should be in association with p16 overexpression (Näsman et al., 2009; Smeets et al., 2007). This combination is close to the golden standard, which is the possibility to assess for HPV mRNA. The assessment of p16 expression is problematic in FNACs since the material and cellularity of the samples is such that it is suboptimal for such an investigation and the result difficult to interpret. Instead, we here analysed for the presence of HPV16 mRNA. It was possible to obtain mRNA from 7 of the 16 tested HPV16 DNA-positive samples and all were HPV mRNA positive indicating an active HPV infection.

It is possible that the optimal assessment of HPV status in FNACs would be to assess for HPV mRNA, but presently, we do not know how efficiently this can be done and whether it is practical in clinical use. As mentioned previously, RNA is much less stable than DNA and requires optimal handling of the samples, e.g. ideally fresh frozen specimens or storage in RNA-conserving solutions. In this study we did not have a method optimized for RNA detection, but still managed to detect HPV mRNA in all 7/16 tested HPV DNA-positive samples, where RNA detection was possible, indicating that HPV mRNA detection in FNAC is indeed conceivable. This should be investigated further in studies with protocols optimized for RNA-detection.

Conclusion

HPV DNA detection in FNAC from neck masses was feasible and in this study the presence of HPV DNA in FNAC indicated an HPV-positive TSCC/BOTSCC as the final diagnosis with a specificity and sensitivity of 100%. Furthermore, HPV DNA was not found in any other malignant conditions than HPV-positive TSCC/BOTSCC or in benign conditions,

including BCCs. The data therefore indicates that the presence of HPV DNA should be investigated using FNAC in patients with neck masses of unknown origins. This could be helpful for an early diagnosis of an HPV-positive TSCC/BOTSCC, as well as potentially for distinguishing between cystic metastases and benign cysts, e.g. BCCs. HPV mRNA detection in FNAC should be studied further using protocols optimized for mRNA detection.

5 CONCLUSIONS

- In Sweden, a large proportion of HNCUP is HPV DNA-positive and most HPV16 DNA-positive HNCUP samples express HPV16 mRNA (Papers I and II)

- Patients with HPV-positive HNCUP have a better clinical outcome than patients with HPV-negative HNCUP (Paper I and II)

- CD8+ TILs and p53-expression are interesting biomarkers to use in combination with HPV-status, but need to be studied further in larger cohorts (Papers I and II)

- Investigation of HPV-status should be part of the diagnostic work-up of an HNCUP (Papers I and II)

- Patients with HPV-positive TSCC/BOTSCC with a mutation in the FGFR3 gene have a worse clinical outcome compared to HPV-positive TSCC/BOTSCC with wild type FGFR3 (Paper III)

- In HPV-positive HNCUP, TP53, PIK3CA and CDKN2A were the most frequently mutated genes (Paper III)

- HPV-positive HNCUP share many similarities with regard to prognostic biomarkers with HPV-positive TSCC/BOTSCC, but there also differences (Papers I – III)

- HPV DNA-detection in FNAC is feasible and detection of HPV DNA in a FNAC from a neck mass of unknown origin is highly indicative of an HPV-positive TSCC/BOTSCC (Paper IV)

- HPV DNA was not found in FNAC from other malignancies than HPV-positive TSCC/BOTSCC or in benign conditions, including BCCs (Paper IV)

- HPV mRNA detection in FNAC is promising but needs to be studied further (Paper IV)

- Investigation of HPV DNA-status using FNAC is useful in the diagnostic work up of patients with neck masses of unknown origins (Paper IV).

6 FUTURE PERSPECTIVES

When discussing future perspectives related to HPV, first of all the prophylactic HPV-vaccines must be mentioned. HPV-vaccines have been shown to be effective, and safe, in preventing cancer of the uterine cervix (FUTURE II Study Group, 2007; Villa et al., 2005; Villa et al., 2006). Presently, no direct evidence exists that the vaccine is able to prevent HPV-positive TSCC/BOTSCC/HNCUP, and there are no pre-stages either one can monitor as in cervical cancer. Therefore, it will take some decades before one can observe the effects on HPV-positive TSCC/BOTSCC/HNCUP, since it takes a very long time to develop.

Nonetheless, since HPV16 is the cause of >80% of HPV-positive TSCC/BOTSCC/HNCUP and HPV16 is covered in all available HPV-vaccines it is highly plausible that the vaccine will work in preventing HPV-positive TSCC/BOTSCC/HNCUP as well. Furthermore, studies have already showed a decline in the prevalence of HPV16 in the oral cavity of vaccinated individuals, giving circumstantial evidence for an effect on HPV-positive TSCC/BOTSCC/HNCUP (Chaturvedi et al., 2018; Grün et al., 2015). It is thus conceivable that we in the future (in 20-30+ years) will see a decline in the incidence of HPV-positive TSCC/BOTSCC/HNCUP. However, to achieve this, it is of utmost importance that the coverage of the vaccine is as high as possible. In Sweden today, HPV vaccine coverage is fairly good, at around 80% among young girls (Folkhälsomyndigheten, 2016). It is important to keep it this way and not like in Denmark, lose participants in the vaccination program due to e.g. unwarranted fear of side effects (Brinth et al., 2015; European Medicines Agency, 2015). Here, as health care professionals and researchers, we have an important task to inform patients and the public about the benefits and safety of the vaccine. Furthermore, as mentioned previously, HPV causes cancer not only in women, but also in men, e.g. ~80% of HPV-positive TSCC/BOTSCC as well as anal- and penile cancers (Plummer et al., 2016). It is thus most unfortunate that we in Sweden do not vaccinate boys and it is imperative that we in the future include boys in the vaccination program. On a global scale, HPV related cancers are more prevalent in the developing world than in the developed world (Plummer et al., 2016). It is thus vital that the vaccines are made available, and affordable, to the public also in these parts of the world.

Clearly, the advent of the HPV-vaccine has the potential to decrease the incidence of HPV-positive TSCC/BOTSCC/HNCUP substantially. However, vaccination coverage will likely never be 100%, even if we start to vaccinate boys. Moreover, it likely takes 20-30 years to develop TSCC/BOTSCC. For this reason, HPV-positive TSCC/BOTSCC/ HNCUP will not be eradicated in the near future and there is still a need for improved therapy for e.g. HPV-positive HNCUP for a long period of time. As mentioned, others and we and have shown that HPV-positive HNCUP has a better clinical outcome than HPV-negative HNCUP. The question for the future however, is if this should influence treatment or not. For HPV-positive OPSCC there are trials investigating de-escalation of treatment on going (Mirghani et al., 2015). The problem with HNCUP is that it is such a rare entity that randomized controlled

trials (RCT) will be very difficult to perform. Indeed, very few RCT's have yet been performed comparing treatments for HNCUP, while retrospective studies have shown very little difference in clinical outcome when comparing different treatments, e.g. RT alone vs. surgery alone vs. combination treatment (Balaker et al., 2012). However, these studies have not taken HPV-status into account, and such studies are very much needed and are possibly best performed in collaboration with other centres in order to achieve larger patient numbers for adequate evaluation. Today, it is still very much up to each centre, or even each physician, to decide which treatment is the best for each patient. While we await further studies, HPV-status could be another tool when selecting treatment. Indeed, at the Karolinska University Hospital more recently, HPV-status has been implemented as a parameter for selecting treatment for HNCUP, e.g. now some patients with HPV-positive HNCUP do not undergo neck dissection and receive RT only (Vårdprogram – Okänd primär, 2015). Follow-up studies on the outcome of these patients would be very interesting.

Testing HPV-status in neck masses of unknown origin using FNAC is a very promising diagnostic tool for the future. As FNAC is already part of the diagnostic work-up of these patients, adding an HPV-test of the aspirate could be easily implemented and is something that is infrequently done today at the Karolinska University Hospital (See Paper IV). This would give the clinician early information and, if HPV-positive, could steer further diagnostic procedures towards the oropharynx. Analysing HPV-status in FNAC may be particularly useful for HNCUP, where no primary tumour can be analysed and especially valuable if patients are treated with RT only, where sparse histopathological specimens may be available. Future studies are needed to examine if HPV mRNA detection in FNAC could be useful and whether HPV testing is valuable for the diagnostic work up of BCCs.

To conclude, with the advent of the HPV-vaccines, with improved diagnostic tools for HPV-related HNSCC and also with recent, encouraging results for immunotherapy in HNSCC (Ferris et al., 2016), it is indeed an exciting time for HPV-related TSCC/BOTSCC/HNCUP research! My hope is that the research carried out by us, in this thesis and elsewhere, will be beneficial for people with TSCC/BOTSCC/HNCUP in the future. By contributing in a small way, together with research done by others, I hope the diagnostics and therapy will be improved, leading to better survival, less side effects and a better quality of life.

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*lika viktiga, bara en kunde stå först

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